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Research Performance in Reproductive Biomedicine: A National Scientometrics Study

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Abstract

Recent achievements in reproductive biomedicine have led to a revolution in infertility treatment. A comprehensive understanding of the current status of reproductive medicine is necessary for the development of a forward-looking plan by health policymakers, based on fundamental requirements. This study is a systematic review of the Scopus database to assess reproductive biomedicine publications within Iran and compared to the rest of the world from 1990 to 2020. The data were categorized by geographical distribution across five continents. National data were assessed in comparison with the world and with neighboring countries. Finally, prominent national research institutes in the field of reproductive biomedicine in Iran were identified, and their contributions to the field highlighted. Of the five continents, the highest number of publications and citations is from Europe (36% publications and 41.5% citations). Corresponding numbers for the other continents are 32 and 33% for America, and 26 and 18.4% for Asia respectively. The remaining publications and citations were from Australia (3.8 and 4.1%) and Africa (2.6 and 3.1%). In a national analysis, the highest-ranking institutes in reproductive biomedicine are in Tehran province (50.9% of all Iranian publications), Shiraz (8.8%), Yazd (7.8%), Isfahan (7.1%), and Tabriz (6.9%). More specifically, Tehran University of Medical Science (15.9%), the Royan Institute (12.2%), Shahid Beheshti Medical University (10.1%), Shahid Sadoughi University of Medical Sciences (6.9%), and Tarbiat Modares University (6.7%) account for more than 50% of all Iranian scientific publications. In recent decades, reproductive biomedical research has grown significantly in Iran. Reviewing publications in this field helps health policy decision makers to monitor the direction of research and adjust investment in the treatment of infertility. In addition, it is necessary to expand and organize inter-organizational and international collaborations to improve the research, gain the benefits of different experiences, and engage in international multicenter studies.

Keywords: Bibliometrics, Infertility, Iran, Reproductive, Scientometric

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Introduction

According to reports from the International Committee for the Monitoring of Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO), infertility is a disease of the male or female reproductive system and defined as the failure to achieve a pregnancy after 12 months or more of regular unprotected sexual intercourse. According to global statistics, each year 60 to 80 million couples suffer from infertility complications. The prevalence of infertility is not the same in different societies; for example, its prevalence has risen from 3.5 to 16.7% in more developed nations and from 6.9 to 9.3% in less-developed countries (1, 2). Primary causes of infertility include genetic factors, hormonal disorders, birth defects, or diseases of the reproductive system. Secondary factors are those related to lifestyle, such as obesity, diet, smoking, alcohol consumption, polluted environments, and stressful situations. To overcome this problem and

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increase quality of life, biomedical researchers and medical professionals specializing in various reproductive fields, such as reproductive endocrinology, *in vitro* fertilization (IVF), embryology, andrology, reproductive genetics, reproductive immunology, and more, have been actively engaged in establishing clinical settings dedicated to infertility treatment. The advent of ART has played a pivotal role in this endeavor, giving rise to specialized infertility treatment centers across the globe (3-6). Thanks to the successful implementation of ART, an astonishing 10 million babies worldwide have been born since the birth of the first IVF baby in 1978. With an estimated four million ART cycles conducted annually, approximately one million babies are born via these procedures each year. These numbers reflect the increasing reliance on ART as a reliable and effective method for achieving successful pregnancies around the world (7-9).

According to recent reports, approximately one out of every six couples globally encounter infertility issues at least once during their reproductive lifespan. The current prevalence of infertility, persisting for a minimum of 12 months, is estimated to be around 8-12% in women aged 20-44 worldwide (10-12), while prevalence rates for primary and secondary infertility are slowly increasing. Along with the rest of the world, infertility research in Iran gained much from the work of pioneer researchers, like Saremi and Aflatoonian in 1987. Later the same year, the first infertility treatment department was established at Aban Hospital in Tehran. After that, in 1989, an infertility research center and a clinical center were established at Shahid Sadoughi University of Medical Sciences in Yazd (13). The first IVF clinic was opened at Afshar Hospital in Yazd. The first successful IVF procedure was performed there and the first IVF baby was born in 1990. The Royan Institute was the first IVF clinic in Tehran. It was established in 1991, and the first IVF baby born there was in 1993. Following these achievements, the first embryos resulting from intracytoplasmic sperm injection (ICSI) and *in vitro* maturation (IVM) were born in Yazd, and the first child was born from a frozen embryo. Building on these achievements, the preimplantation genetic diagnosis (PGD) method was developed for the first time in 2004 at the Royan institute (13, 14). Success stories from these centers boosted interest in research in reproductive science and gradually, scientists and experts in the field of reproduction produced further significant accomplishments. Currently, there are more than 60 infertility centers in service in Iran (8-15).

Given the importance of infertility treatment and the remarkable advances of Iranian scientists, there has emerged the need for a scientometric study that comprehensively examines past achievements. The objective of such a study would be to provide valuable insights that can guide future research endeavors in this field. Consequently, we conducted a systematic evaluation of research on this subject, encompassing an analysis of published documents, top institutions, author productivity, networking, and an impact assessment of publications across various regions (16, 17). This type of study is a prerequisite for the future planning and basic management of financial and human resources (18, 19). We mapped studies conducted in this field across different geographical regions of the country and compared them to global trends from 1990 to 2020. The outcomes of this study will serve as a valuable resource for researchers, aiding their understanding of the prospects for diverse research projects within the realm of reproductive biomedicine in Iran. In addition, this research can provide more information for health policy makers concerned about the current low fertility rate in the country and the potential threat of an aging society.

Materials and Methods

Source of data

This aim of systematic review was to undertake a retrospective analysis of publications in reproductive biomedicine originating from Iran over two decades to the end of 2020. First, available databases such as Scopus, Web of Science, and Google Scholar were evaluated. Scopus (http://www.scopus.com) was chosen as the most comprehensive bibliographic database from which to retrieve data as it has the greatest coverage of journals and publications and is updated regularly. The database also enabled analyses by different criteria, such as number of publications per year, author's name, subject area, document type, publication stage, source title, keyword, affiliation, funding sponsor, journal, country, or territory, source type, and language. This type of information is very useful and necessary to bibliometric and scientometric studies. The study included Iranian and non-Iranian publications on reproductive biomedicine and spanned from 1990 until the end of 2020 (http://www.scopus.com) (20, 21).

Data retrieval

The Scopus database was searched for publications with any of the following keywords: "Reproductive science", "Fertility", "Infertility", or "Assisted reproductive technologies" separately. These keywords were selected to ensure that the main subject areas in reproductive biomedicine would be completely covered. These keywords were searched for in the titles, abstracts, and keywords of the reviewed publications. Only publications in English published between 1990 and the end of 2020 were included. Finally, the data from all countries which had more than 500 publications were extracted in Comma-Separated Value (CSV) format. All the CSV files were converted to Microsoft Excel files for statistical analysis (22).

Scientometrics analysis

Microsoft Excel files for 52 countries were combined into one file and all the statistical analyses were performed using Microsoft Excel. The citation rate was calculated using the following formula in Microsoft Excel 2018 (23). Citation rate= (number of citations)/(2021-year of publications)

Results

Overview of the global output

A total of 254394 publications were extracted from 52 countries that had more than 500 English publications in the field of reproductive biomedicine from 1990 to the end of 2020. Next, the contribution of each continent to reproductive biomedical studies was determined; Europe, America, and Asia were found to have produced a remarkably large share of all reproductive biomedicine publications. European countries (including the UK, Germany, Italy, France, Netherlands, Spain, Belgium, Denmark, Switzerland, Sweden, Finland, Norway, Austria, Greece, Poland, Portugal, Ireland, Czech Republic, Romania, and Hungary) accounted for 92,192 publications, almost 36% of the overall total. American countries (including the US, Canada, Brazil, Mexico, Argentina, Colombia, and Chile) had 80,845 publications, equal to 32% of all the total. Asian countries (including Israel, Iran, Turkey, Egypt, Saudi Arabia, Iraq, India, Pakistan, Russian Federation, China, Japan, South Korea, Taiwan, Singapore, Hong Kong, Malaysia, Indonesia, and Thailand) had 65,106 publications, equal to 26% of all the total. Australia had 9564 publications, equal to 3.8% of the total, while African countries (including South Africa, Kenya, Nigeria, and Ethiopia) published 6,687 papers, equal to 2.6% of the total (Fig.1). Countries in brackets were ordered by number of publications.

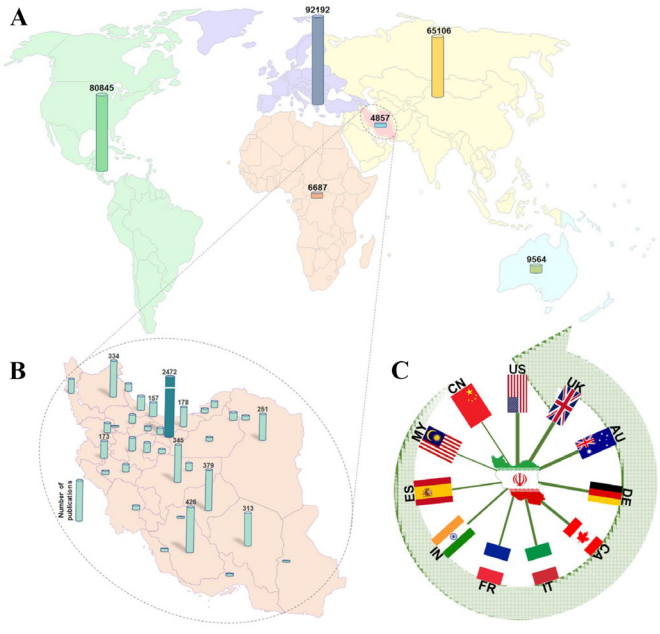

Fig.1: Geographical distribution of all publications in reproductive biomedicine 1990 to the end of 2020. **A.** Global performance in different continents, **B.** National performance in different provinces of Iran, and **C.** The network structure of Iran's scientific collaborations in reproductive biomedicine. The weight of the lines represents the number of collaborative international publications. US; United States, UK; United Kingdom, AU; Australia, DE; Germany, CA; Canada, IT; Italy, FR; France, IN; India, ES; Spain, MY; Malaysia, and CN; China.

In the next step of the study, the quality of the publications was evaluated based on total citations per country or area, and citations per paper in general. In this analysis, publications from Asia were classified into South-East, Middle-East, North, and Central. Further, publications from the Middle East were divided into Israel, Iran, Turkey, Egypt, Saudi Arabia, and Iraq (Fig.2). The publications were classified into 28 different subjects, the top five of which were medicine, biochemistry, genetics, molecular biology, agricultural and biological sciences, environmental sciences, and social sciences.

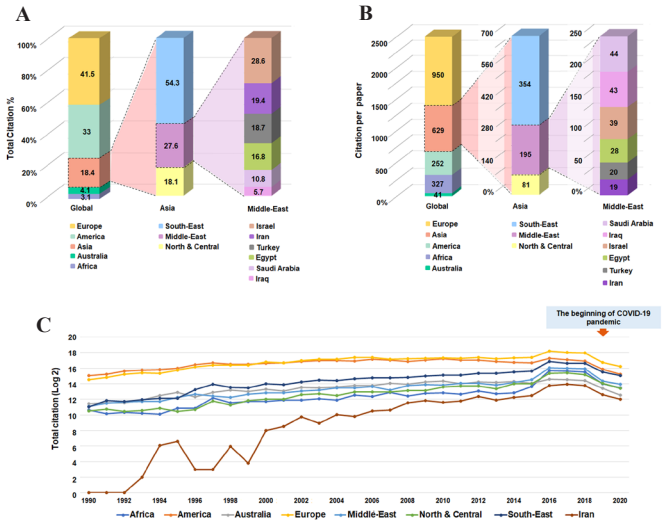

Fig.2: Quantification of reproductive biomedical publications from Iran compared to other countries based on total citations and citations per paper. **A.** The contribution of Iran to the geographical distribution of total citations in reproductive biomedicine publications across five continents (left), Asia (center), Middle East (right), **B.** The contribution of Iran to the geographical distribution of citations per publication in reproductive biomedicine across five continents (left), Asia (center), Middle East (right), **C.** Comparative timeline showing total citations from each continent and Iran. America; US, Canada, Brazil, Mexico, Argentina, Colombia, Chile. Asia; Israel, Iran, Turkey, Egypt, Saudi Arabia, Iraq, India, Pakistan, Russian Federation, China, Japan, South Korea, Taiwan, Singapore, Hong Kong, Malaysia, Indonesia, Thailand. Europe; UK, Germany, Italy, France, Netherlands, Spain, Belgium, Denmark, Switzerland, Sweden, Finland, New Zealand, Norway, Austria, Greece, Poland, Portugal, Ireland, Czech Republic, Romania, Hungary. Africa; South Africa, Kenya, Nigeria, Ethiopia, Bangladesh. Middle East (ME); Israel, Iran, Turkey, Egypt, Saudi Arabia, Iraq.

Further analysis showed that, worldwide, the following scientists had the highest number of publications in reproductive biomedicine; A. Agarwal, C. Niederberger, P. Devroey, A. Pellicer, H. Tournaye, JH. Check, N. Gleicher, Z. Rosenwaks, K. Diedrich, and E. Nieschlag. Presented in order, they are considered the top 10 scientists in this field in the world.

Iran in global and regional reproductive biomedicine research

In terms of quantity, Iranian scientists have contributed to 4,857 publications: 1.1% of publications worldwide, or 4.4% of Asian publications in reproductive biomedicine (Fig.1A). In terms of quality, however, Iran, with an average of 19 citations per paper, accounts for 19.4% of total citations from Asia, while the Middle- East overall accounts for 27.6% of total citations worldwide (Fig.2A, B).

An assessment of international collaborations between Iran and other countries in the field of reproductive biomedicine showed the top five countries were the United States (231 publications), the United Kingdom (147 publications), Australia (111 publications), Germany (106 publications), and Canada (105 publications) (Fig.1).

At the regional level Israel, Iran, Turkey, and Saudi Arabia are the pioneers in reproductive biomedical research, with Iran the second most important country with 25% of total citations from 1990 to 2020. Also, over the years, Iran has led the region in terms of the total number of publications and the total number of citations. It should be noted here that there has been little fluctuation in the number of citations to Iranian publications.

In this analysis, it was found that the outbreak of the COVID-19 pandemic resulted in a decrease in publications in the field of reproductive biomedicine. This reduction could be due to the shift of research focus to different aspects of this pandemic (Fig.3) (21), although it should be mentioned that Iranian researchers have published many articles on the COVID-19 pandemic and reproductive biomedicine complications (24, 25).

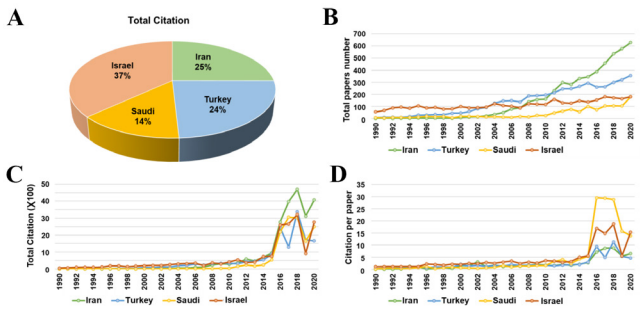

Fig.3: Comparison of the reproductive biomedicine publications from Iran and its regional neighbors based on **A.** Total citations, **B.** Total publications, **C.** Total citations, in the form of a timeline, and **D.** Citation per publication in the form of a timeline.

Reproductive biomedicine research in Iran

The data analysis showed a total of 5298 publications on reproductive biomedicine research in Iran. Of these papers, 4857 were written in English and published between 1990 to 2020. They accounted for 91.68% of the 5298 publications indexed in Scopus, and covered 28 different subjects related to reproductive biomedicine. The top five subjects were medicine (with 3150 publications equal to 65% of the total), biochemistry, genetics and molecular biology (1128, 23%), agricultural and biological sciences (874, 18%), pharmacology, toxicology and pharmaceutics (293, 6%) and veterinary (255, 5%). The most common types of publication in peer-reviewed journals were original articles (4311 equal to 88.8% of the total), review articles (376, 7.7%), letters (66, 1.4%), conference papers (38, 0.7%) and book chapters (22, 0.5%).

Geographical distribution and top Iranian institutes in reproductive biomedicine research

According to the data analysis, the top five cities active in reproductive biomedicine research are Tehran (with 2472 publications equal to 50.9% of the total), Shiraz (426, 8.8%), Yazd (379, 7.8%), Isfahan (345, 7.1%), and Tabriz (334, 6.9%). Tehran, as the capital, has the largest number of publications due to the location of many research centers there. In this regard, it was found that the top 10 research centers in reproduction biomedicine are Tehran University of Medical Science (TUMS), the Royan Institute (RI), Shahid Beheshti Medical University (SBMU), Shahid Sadoughi University of Medical Sciences (SSUMS), Tarbiat Modares University (TMU), University of Tehran (UT), Iran University of Medical Sciences (IUMS), Tabriz University of Medical Sciences (TBUMS), Shiraz University of Medical Sciences (SUMS), and Islamic Azad University (IAU). A more detailed analysis revealed that TUMS (with 771 publications equal to 15.9% of the total), RI (594, 12.2%), SBMU (491, 10.1%), SSUMS (334, 6.9%), and TMU (325, 6.7%) accounted for more than 50% of the publications from 1990 to 2020 (Fig.4). According to a recent study by A. Agarwal, an international scientist in reproductive biomedicine, the Royan Institute was recognized as number 9 out of the top ten institutions globally for expertise in male infertility and ART procedures between 2000 and 2019 (12, 15, 26).

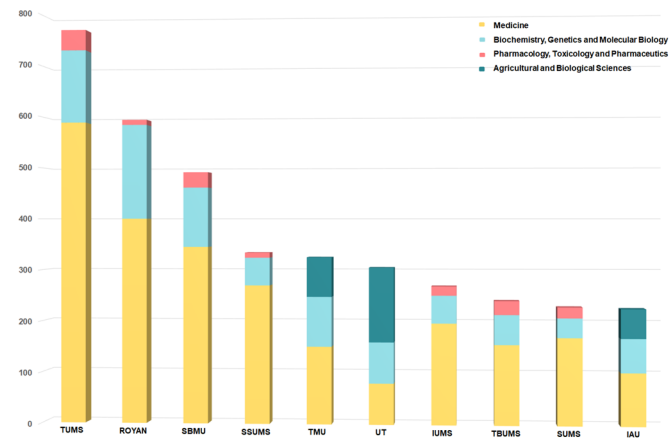

Fig.4: Comparison of the top 10 research centers in reproductive biomedicine in Iran based on the total number publications. TUMS; Tehran University of Medical Science, RI; ROYAN Institute, SBMU; Shahid Beheshti Medical University, SSUMS; Shahid Sadoughi University of Medical Sciences (Yazd), TMU; Tarbiat Modares University, UT; University of Tehran, IUMS; Iran University of Medical Sciences, TBUMS; Tabriz University of Medical Sciences, SUMS; Shiraz University of Medical Sciences, and IAU; Islamic Azad University.

The top Iranian scientists in this field are MH. Nasr Esfahani (101 publications), MA. Khalili (85 publications), M. Tavalaee (70 publications), H. Gourabi (55 publications), MM. Akhondi (52 publications), MR. Sadeghi (51 publications), A. Aflatoonian (49 publications), and A. Moini (49 publications).

MH. Nasr-Esfahani, M. Tavalaee, H. Gourabi, and A. Moini are affiliated with Royan Institute. MA. Khalili and A. Aflatoonian are affiliated with SSUMS, and MM. Akhondi and MR. Sadeghi are affiliated with Avicenna Research Institute.

Discussion

This analysis of research published over the past three decades has revealed that reproductive biomedical research in Iran has been advancing in parallel with global trends. This progress is particularly evident in the field of male infertility and ART, and is reflected in studies renowned worldwide. The increasing number of these studies has been particularly notable since 2005, resulting in significant achievements in the field of reproductive biomedicine at both national and international levels (12).

In support of this claim, it is important to mention that there are currently 61 registered infertility clinics in Tehran, comprising 24 public and 37 private clinics. This signifies the substantial infrastructure development dedicated to address infertility issues and provide related treatments. Furthermore, it is important to highlight the extensive provision of infertility clinics in other provinces across Iran, emphasizing the widespread effort and commitment to reproductive biomedicine in the country (13). The establishment and expansion of these clinics and research-treatment centers are indicative of increasing attention and investment in this field in Iran. The collaborative efforts of Iranian scientists, clinicians, and researchers have contributed to remarkable progress over recent decades. This growth has not only benefitted Iranians but has also made significant contributions to the global body of knowledge in reproductive biomedicine (14, 15, 21).

However, it is essential not only to highlight the strengths but also the weaknesses that need to be addressed for further improvement. Despite the notable number of and citations to Iranian publications, it is crucial to acknowledge that citations per paper compared to neighboring countries in the region remain relatively low. This signifies the need for improving the quality of articles.

One contributing factor to this discrepancy could be the concentration of research centers primarily in Tehran. To overcome this limitation, there is a pressing need to disseminate knowledge and resources throughout the country. By doing so, the accessibility and availability of expertise and research opportunities will increase, promoting more balanced and comprehensive approaches to reproductive biomedicine across Iran.

Additionally, it is worth noting that the COVID-19 pandemic has had a significant impact on reproductive biomedical studies, leading to a decline in research output. Since SARS-CoV-2 has many unknown pathogenic features, it may have some long-term complications in patients (27, 28). To mitigate these potential effects, it is essential for the Ministry of Health and related associations to establish short- and medium-term plans for the post-COVID period. These plans should aim to create an enabling environment that supports the growth of studies in the reproductive biomedicine field, restoring the conditions observed before the pandemic.

By addressing these weaknesses and implementing the necessary measures, the quality and impact of Iranian research in reproductive biomedicine can be significantly enhanced. Collaboration among researchers, the expansion of research centers beyond Tehran, and the development of strategic plans will help to address the problems of the current low fertility rate in the country and the potential threat of an aging society.

Conclusion

This article has discussed and highlighted national publications in reproductive biomedicine in relation to Iran's position in the region and in the world, as well as in international collaborations in the field. We hope that the information provided can significantly help the authorities to make the right financial and human resource decisions based on the needs of the society.

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Authors' Contributions

Z.H., M.L.; Contributed to data collection and curation. P.A., M.V.; Contributed to conception and design. Z.H.; Contributed to formal statistical analysis, interpretation of data, and drafted the manuscript, which was revised by P.A., M.V., A.V.D. M.V.; Was in charge of overall supervision. All authors read and approved the final manuscript.

References

1. Katole A, Saoji AV. Prevalence of primary infertility and its associated risk factors in urban population of central india: a community-based cross-sectional study. Indian J Community Med. 2019; 44(4): 337-341.
2. Sun H, Gong TT, Jiang YT, Zhang S, Zhao YH, Wu QJ. Global, regional, and national prevalence and disability-adjusted life-years for infertility in 195 countries and territories, 1990-2017: results from a global burden of disease study, 2017. Aging (Albany NY). 2019; 11(23): 10952-10991.
3. Skakkebæk NE, Lindahl-Jacobsen R, Levine H, Andersson AM, Jørgensen N, Main KM, et al. Environmental factors in declining human fertility. Nat Rev Endocrinol. 2022; 18(3): 139-157.
4. Deyhoul N, Mohamaddoost T, Hosseini M. Infertility-related risk factors: a systematic review. Int J Womens Health Reprod Sci. 2017; 5(1): 24-29.
5. Aimagambetova G, Issanov A, Terzic S, Bapayeva G, Ukybassova T, Baikoshkarova S, et al. The effect of psychological distress on IVF outcomes: Reality or speculations? PLoS One. 2020; 15(12): e0242024.
6. Borumandnia N, Majd HA, Khadembashi N, Alaii H. Assessing the trend of infertility rate in 198 countries and territories in last decades. Iran J Public Health. 2021; 50(8): 1735-1737.
7. Paskar SS, Kalugina AS, Tkachuk AG. Trends in safe infertility treatment with assisted reproductive technologies. J Obstet Gynaecol Res. 2020; 69(4): 83-88.
8. De Geyter C. Assisted reproductive technology: impact on society and need for surveillance. Best Pract Res Clin Endocrinol Metab. 2019; 33(1): 3-8.

9. Matorras R, Chaudhari VS, Roeder C, Schwarze JE, Bühler K, Hwang K, et al. Evaluation of costs associated with fertility treatment leading to a live birth after one fresh transfer: a global perspective. Best Pract Res Clin Obstet Gynaecol. 2023; 89: 102349.
10. Murgia F, Corda V, Serrenti M, Usai V, Santoru ML, Hurt KJ, et al. Seminal fluid metabolomic markers of oligozoospermic infertility in humans. Metabolites. 2020; 10(2): 64.
11. Cox CM, Thoma ME, Tchangalova N, Mburu G, Bornstein MJ, Johnson CL, et al. Infertility prevalence and the methods of estimation from 1990 to 2021: a systematic review and meta-analysis. Hum Reprod Open. 2022; 2022(4): hoac051.
12. Borumandnia N, Majd HA, Khadembashi N, Alaii H. Worldwide trend analysis of primary and secondary infertility rates over past decades: a cross-sectional study. Int J Reprod Biomed. 2022; 20(1): 37-46.
13. Abedini M, Ghaheri A, Omani Samani R. Assisted reproductive technology in iran: the first national report on centers, 2011. Int J Fertil Steril. 2016; 10(3): 283-289.
14. Aflatoonian A. History of in vitro fertilization and related techniques in Iran: the view of professor Safaa Al-Hasani. Int J Reprod Biomed. 2021; 19(1): 1-4.
15. Moridi A, Roozbeh N, Yaghoobi H, Soltani S, Dashti S, Shahrahmani N, et al. Etiology and risk factors associated with infertility. Int J Women's Health Reprod Sci. 2019; 7(3): 346-353.
16. Baskaran S, Agarwal A, Panner Selvam MK, Finelli R, Robert KA, Iovine C, et al. Tracking research trends and hotspots in sperm DNA fragmentation testing for the evaluation of male infertility: a scientometric analysis. Reprod Biol Endocrinol. 2019; 17(1): 110.
17. Agarwal A, Baskaran S, Panner Selvam MK, Finelli R, Barbarosie C, Robert KA, et al. Scientific landscape of oxidative stress in male reproductive research: a scientometric study. Free Radic Biol Med. 2020; 156: 36-44.
18. Saberi S, Karamzadeh R, Moghadam P, Kadivari M, Behbahani BE, Heydari Z, et al. Research performance in stem cell science and regenerative medicine in Iran: a national comprehensive observation. Arch Iran Med. 2019; 22(6): 318-327.
19. Agarwal A, Panner Selvam MK, Baskaran S, Finelli R, Leisegang K, Barbăroșie C, et al. A scientometric analysis of research publications on male infertility and assisted reproductive technology. Andrologia. 2021; 53(1): e13842.
20. Akhondzadeh S, Ebadifar A, Baradaran Eftekhari M, Falahat K. Medical science and research in Iran. Arch Iran Med. 2017; 20(11): 665-672.
21. Baskaran S, Agarwal A, Leisegang K, Pushparaj PN, Panner Selvam MK, Henkel R. An in-depth bibliometric analysis and current perspective on male infertility research. World J Mens Health. 2021; 39(2): 302-314.
22. Agarwal A, Selvam MKP, Baskaran S, Finelli R, Leisegang K, Barbăroșie C, et al. Highly cited articles in the field of male infertility and antioxidants: a scientometric analysis. World J Mens Health. 2021; 39(4): 760-775.
23. Morshed-Behbahani B, Lamyian M, Joulaei H, Montazeri A. Analysis and exploration of infertility policies in Iran: a study protocol. Health Res Policy Syst. 2020; 18(1): 5.
24. Hezavehei M, Shokoohian B, Nasr-Esfahani MH, Shpichka A, Timashev P, Shahverdi AH, et al. Possible male reproduction complications after coronavirus pandemic. Cell J. 2021; 23(4): 382-388.
25. Nateghi R, Ghashghaei SH, Shokoohian B, Hezavehei M, Ebrahimi B, Shahverdi AH, et al. Female reproductive health in SARS-CoV-2 pandemic era. Int J Fertil Steril. 2021; 15(4): 241-245.
26. Agarwal A, Finelli R, Durairajanayagam D, Leisegang K, Henkel R, Salvio G, et al. Comprehensive analysis of global research on human varicocele: a scientometric approach. World J Mens Health. 2022; 40(4): 636-652.
27. Shahriari Felordi M, Memarnejadian A, Najimi M, Vosough M. Is There any alternative receptor for SARS-CoV-2? Cell J. 2021; 23(2): 247-250.
28. Khoshdel-Rad N, Zahmatkesh E, Shpichka A, Timashev P, Vosough M. Outbreak of chronic renal failure: will this be a delayed heritage of COVID-19? J Nephrol. 2021; 34(1): 3-5.

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Association of *Interleukin-17A* rs2275913 Polymorphism with Recurrent Miscarriage: A Systematic Review and Meta-Analysis Study

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Abstract

Recurrent miscarriage (RM) is a condition defined as having three or more consecutive pregnancy losses before the 20 weeks of pregnancy. The present study was undertaken to investigate association of *Interleukin-17A (IL-17A)* rs2275913 polymorphism with RM. To this end, we searched the international databases (Web of Science, PubMed, Embase, and Scopus) and extracted studies investigating the association of *IL-17A* rs2275913 polymorphism with RM using the appropriate keywords. The collected data were analyzed with the random-effects model and STATA (version 14). A total of five studies met the eligibility criteria, and total sample size was 998 subjects. Mean age of the cases and controls were 31.41 ± 4.16 and 30.56 ± 3.5 years, respectively. Our results disclosed a significant relationship of the *IL-17A* rs2275913 AA genotype [odds ratio (OR)=1.68; 95% confidence interval (CI)=1.16-2.43; I^2=19; P=0.294) with RM. There was no statistically significant correlation between *IL-17A* rs2275913 GG genotype (OR=1.04; 95% CI=0.64-1.7; I^2=59.5; P=0.042) and GA genotype (OR=0.85; 95% CI=0.65-1.1^2; I^2=19.1; P=0.293) with RM. Our findings revealed that the *IL-17A* rs2275913 polymorphism is associated with RM, and the AA genotype of this polymorphism increased possibility of being involved in RM.

Keywords: *IL-17A* rs2275913, Polymorphism, Recurrent Miscarriage

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Introduction

Recurrent or repeated miscarriage (RM) is defined as having three or more consecutive pregnancy losses before the 20 weeks of pregnancy (1). The exact pathophysiology of RM is still unknown; however, several underlying factors, such as chromosomal abnormalities, anatomical defects, hormonal problems, thrombophilic disorders, infections, and immune system factors have been attributed to this condition (2-4). Studies showed that Th1/Th2 immune balance played a prominent role in reproductive phenomena (5), in which the dominant Th2-type response was associated with normal pregnancy, while the Th1-type response correlated with pregnancy failure (6). Cytokine production can be affected by genetic polymorphisms, particularly in the promoter regions, giving rise to high, medium, or low levels of cytokines (7). Association of the such polymorphisms and production of various cytokines has been reported as an important factor for pregnancy (8).

Interleukin (IL)-17 is a well-known proinflammatory cytokine mainly produced by a subset of T-helper cells, i.e. Th17 cells (9, 10). IL-17 family member comprises of six closely related cytokines, from *IL-17A* to *IL-17F* (11). Two of the most studied cytokines, *IL-17A* and *IL-17F*, are located in the adjacent positions on chromosome 6 with almost 50% sequence identity, targeting the same receptor, and exhibiting similar biological properties (12). IL17 is considered an important factor in inflammation and autoimmunity, and it can influence the pathogenesis of RM (6). The rs2275913 SNP, produced via replacement of the guanine (G) by adenine (A) nucleotide base in the *IL-17A* gene promoter,

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was significantly associated with a large number of diseases (13-15). Allelic variants of rs2275913 single-nucleotide polymorphism (SNP) have been indicated to differently bind to the nuclear factor of activated T cells, leading to variations in IL-17A secretion (16).

The aim of the study was to investigate the association of IL-17A rs2275913 polymorphism with RM.

Material and Methods

The present study was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist (17).

Search strategies

A comprehensive search was performed to extract the published studies reporting association of IL-17 polymorphisms with recurrent spontaneous abortion. The used keywords included "rs2275913", "polymorphism", "*IL17A*", "*interleukin-17*", "recurrent miscarriage", "fetal loss", "pregnancy loss", "abortion", "RM", "frequency", "mutation", "variation", and "genotype". These keywords were also combined using Boolean operators ("OR" and "AND") to search international databases, including ISI, PubMed, Embase, and Scopus. Google Scholar was searched for the studies not included in the mentioned databases. Thereafter, references of the extracted studies were checked to find potentially relevant studies. All records were then imported into the EndNote, and duplicate findings were deleted.

Study selection

After eliminating the duplicate studies, title and abstract of the remaining articles were checked to find eligible studies based on the following inclusion and exclusion criteria. Inclusion criteria included original case-control studies on association of the rs2275913 polymorphism and RM with extractable data, studies with similar objectives and statistical methods, sufficient published details to estimate odds ratio (OR) and 95% confidence interval (CI), as well as selection of patients based on the standard and reliable diagnostic parameters. The exclusion criteria included review articles, meta-analyses, congressional abstracts, studies in languages other than English, and withdrawn articles. Eligible studies were selected by two authors rechecked and confirmed by all authors.

Data extraction and quality assessment

Data were extracted from the selected studies by two different authors. The data included location, publication date, genotyping method, age and the number of case and control participants, as well as *IL-17A* rs2275913 polymorphism. All data were reviewed for potential bias by the other authors and then confirmed by all authors. The Newcastle-Ottawa scale was used to assess methodology and quality of the studies. Articles with scores 0-3, 4-6, and 7-9 were considered as low, medium, and high quality respectively; none of the studies scored <4.

Data synthesis and analysis

Mean of the sample size and standard deviations of the predicted data were combined. Studies were then weighted using the inverse-variance method. The Q-test and I^2 index, with an α-level of significance (<10%), were used to verify heterogeneity of the included studies. The heterogeneous data were then analyzed by random-effects model. All data were finally entered into STATA version 14.

Risk of bias between studies

Begg's funnel plots and Egger's test were selected to evaluate publication bias of the data, and $P<0.05$ were considered statistically significant.

Results

The present meta-analysis included five original published studies investigating the association of *IL-17A* rs2275913 polymorphisms with RM (Table 1). The study selection process is illustrated in Figure 1. The total sample size was 998 subjects (440 cases and 459 controls, with mean ages of 31.41 ± 4.16 and 30.56 ± 3.5 years, respectively). For statistical calculations, the genotypes of the subjects were classified into wild type (AA), homozygotes, heterozygotes (GA), and mutant homozygotes (GG) (Table 2).

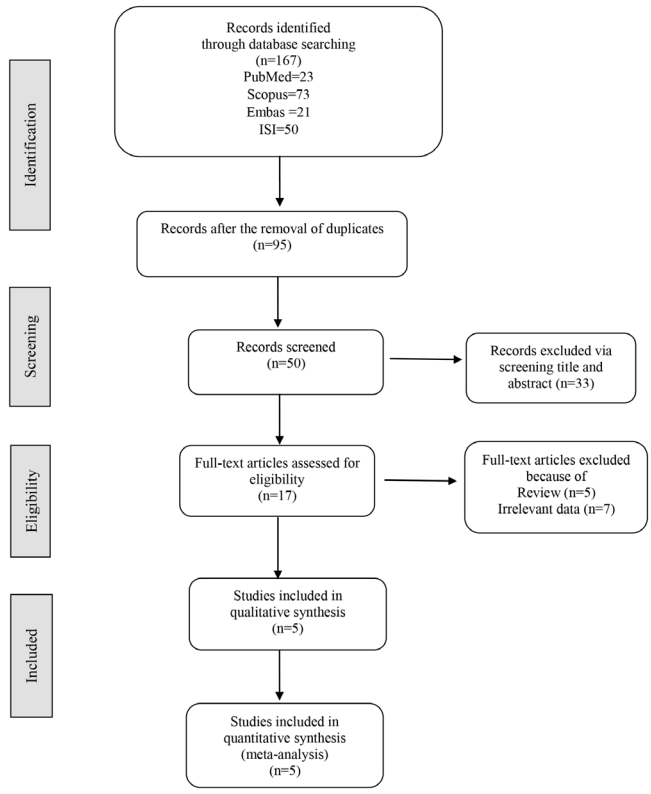

Fig.1: PRISMA flow diagram illustrating the selection of the articles.

Table 1: Characterizations of articles reviewed in the present study

First author (reference)	Publication year	Location	Genotyping method	Sample size		Mean age ± SD		Quality assessment score*
				Case	Control	Case	Control	
Baqer et al. (8)	2021	Iraq	Real-time PCR	50	50			5
Najafi et al. (18)	2014	Iran	PCR-RFLP	85	85	30.84 ± 5.2	29 ± 4.4	5
Alkhuriji (19)	2017	Saudi Arabia	Real-time PCR	100	100	33.2 ± 0. 62	33.1 ± 0.73	5
Bahadori et al. (20)	2014	Iran	PCR-RFLP	85	104			5
Zidan et al. (21)	2015	Egypt	PCR-RFLP	120	120	31.6 ± 6 28	30.6 ± 7.8	6

*; The Newcastle-Ottawa (NOS) scale. PCR-RFLP; Polymerase chain reaction-restriction fragment length polymorphism.

Table 2: The frequency of *IL-17A* rs2275913 genotypes and alleles in recurrent miscarriage (RM) and control group

First author (reference)	Cases				Controls				Cases		Controls	
	Total	AA	GG	GA	Total	AA	GG	GA	A allele	G allele	A allele	G allele
Baqer et al. (8)	50	0	33	17	50	2	28	20	17	83	24	76
Najafi et al. (18)	85	52	7	26	85	46	3	36	130	40	128	42
Alkhuriji (19)	100	11	56	33	100	3	67	30	36	164	55	145
Bahadori et al. (20)	85	7	41	37	104	7	39	58	70	100	79	129
Zidan et al. (21)	120	36	34	50	120	22	49	49	122	118	93	147

According to the random-effects model, there was no statistically significant relationship between IL-17 rs2275913 A allele (OR=0.98; 95% CI=0.66-1.45; I^2=70.9; P=0.008) and G allele (OR=1.02; 95% CI=0.69-1.51; I^2=70.9; P=0.008) with recurrent spontaneous abortion. In line with the fixed-effects model, a statistically significant association was detected between *IL-17A* rs2275913 AA (OR=1.68; 95% CI=1.16-2.43; I^2=19; P=0.294) genotype and RM. The presence of the *IL-17A* rs2275913 AA genotype in women showed an increased risk of RM by 1.68-fold (Fig.2). Furthermore, based on the random-effects model, there was no statistically significant relationship between the *IL-17A* rs2275913 GG genotype (OR=1.04; 95% CI=0.64-1.7; I^2=59.5; P=0.042) and GA genotype (OR=0.85; 95% CI=0.65-1.12; I^2=19.1; P=0.293) with RM (Figs.3, 4).

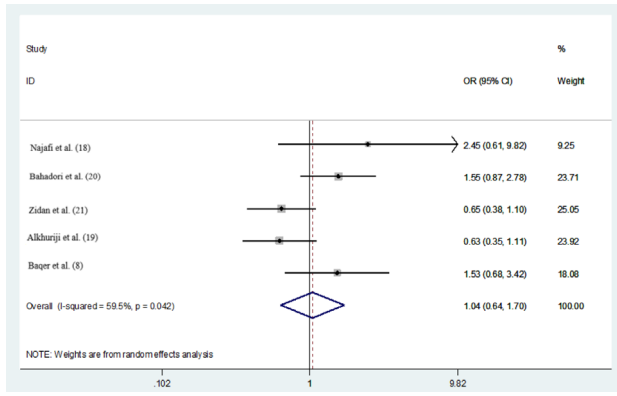

Fig.3: Forest plots for GG genotype showing the relationship of IL-17 rs2275913 polymorphism with RM. Studies are ordered by the date of publication and authors' name based on a fixed-effects model. Square represents the effect estimate of individual studies with more than 95% confidence intervals with the size of squares proportional to the weight assigned to the study in the meta-analysis. Diamond denotes the overall estimation. OR; Odds ratio and CI; Confidence interval.

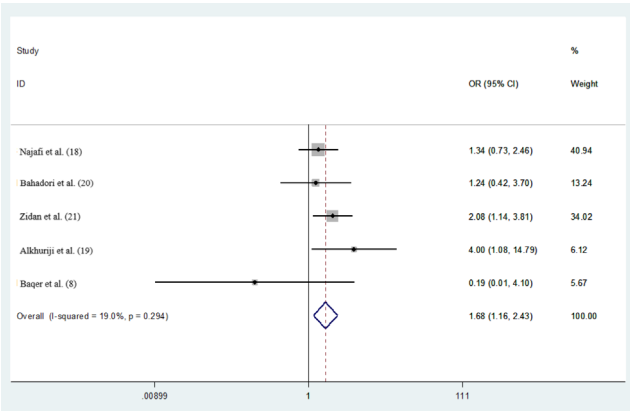

Fig.2: Forest plots for AA genotype showing the relationship of IL-17 rs2275913 polymorphism with RM. Studies are ordered by the date of publication and authors' name based on a fixed-effects model. Square represents the effect estimate of individual studies with more than 95% confidence intervals with the size of squares proportional to the weight assigned to the study in the meta-analysis. Diamond denotes the overall estimation. OR; Odds ratio and CI; Confidence interval.

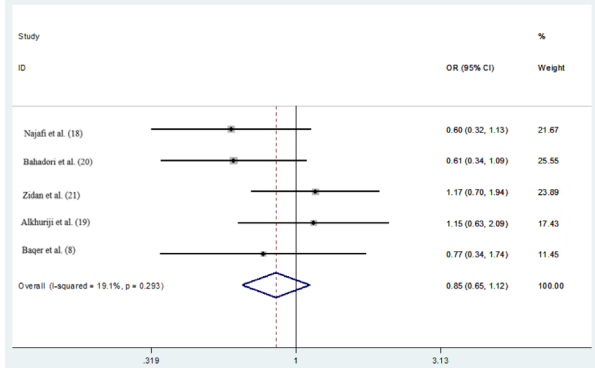

Fig.4: Forest plots for GA genotype showing the relationship of IL-17 rs2275913 polymorphism with RM. Studies are ordered by the date of publication and authors' name based on a fixed-effects model. Square represents the effect estimate of individual studies with more than 95% confidence intervals with the size of squares proportional to the weight assigned to the study in the meta-analysis. Diamond denotes the overall estimation. OR; Odds ratio and CI; Confidence interval.

Risk of bias between studies

No significant publication bias was observed for either outcome using the Begg's (P=0.052) and Egger's (P=0.166) tests. Figure 5 represents the risk of publication bias among studies based on the two above-mentioned tests.

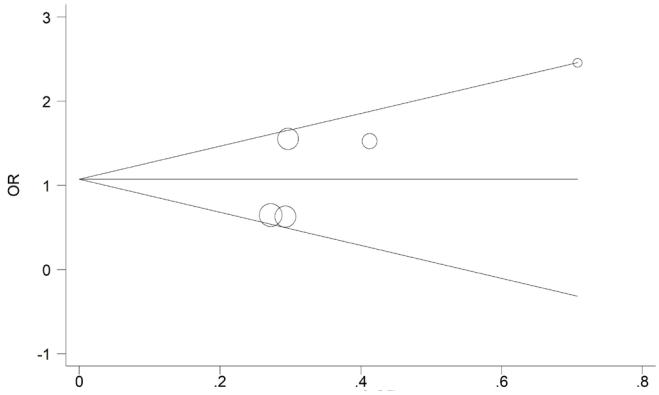

Fig.5: Beggs funnel plot for publication bias, representing a pseudo 95% confidence limit. OR; Odds ratio.

Discussion

Recurrent pregnancy loss or miscarriage of pregnancy is the most serious pregnancy-related disorder. Several immune-related and anatomic causes have been introduced as potential etiological reasons for RM (22, 23). However, further efforts are needed to find a detailed explanation for the pathophysiology of this condition (24). Consequently, new studies are focusing on the polymorphisms of a gene and its possible associations with RM (25). In this regard, the current meta-analysis was conducted to investigate whether the rs2275913 polymorphism is associated with RM or not (26).

As mentioned in the result section, this study included five published papers regarding the association of *IL-17A* rs2275913 polymorphism with RM. Our meta-analysis revealed the relationship of AA genotype with a higher risk of RM, but no significant association was found between single A or G alleles, as well as GA and GG genotypes with RM. According to our search, there was not any previous meta-analysis investigating the association of *IL-17A* rs2275913 polymorphism with RM or any other pregnancy-related condition. However, meta-analyses on cancer and inflammatory diseases such as rheumatoid arthritis have suggested strong associations between the *IL-17A* rs2275913 polymorphism and the mentioned diseases (27, 28).

Studies included in our meta-analysis showed controversial results regarding the association of IL-17A rs2275913 polymorphism with RM. Two studies conducted by Alkhuriji (19) and Zidan et al. (21) reported a significantly higher frequency of the AA genotype among patients than controls, while Baqer et al.'s (8) study stated a protective role for the AA genotype. Baqer's study also showed a significant association between the GG genotype and lower serum *IL-17A* concentration. However, two other studies from Iran did not suggest any significant association with any of the mentioned genotypes (18, 20).

The rs2275913 polymorphism has been studied in other pregnancy-related conditions such as preeclampsia. An original study in China displayed the association of the AG genotype and A allele with preeclampsia, a pregnancy-related inflammatory disease, with a significant role for IL-17 (29). The significant role of Th17 and IL-17 in pregnancy-related conditions, especially RM, has also been thoroughly investigated. Most of the studies in this regard have shown that the higher frequency of IL-17-producing cells and the higher serum concentration of IL-17 have a role and are associated with RM (6, 30). Disturbed Treg/Th17 balance has been exhibited to be an important feature in cases that have experienced RM. The study of Nakashima et al. (31) investigated the proportion of Th17 cells in peripheral blood at all stages of pregnancy and showed that the number of Th17 cells remained unchanged during pregnancy. According to the results of the study by Lee et al. (12). An increase in the number of Th17 cells during pregnancy as well as an increase in the ratio of pro-immune T cells versus regulatory immune T cells may lead to an inflammatory response that may contribute to the development of RM, and this results in consistent with our results which showed the association between the AA genotype of the *IL17A* rs2275913 polymorphism with RM (6).

Our study encountered some limitations that should be considered. First, insufficient sample size may compromise our ability to reveal a statistical relationship, Second, all the studies included in this meta-analysis were from middle east countries, and more worldwide studies are needed. Third, in terms of the experimental process, two studies used real-time PCR, and three studies used the PCR-RFLP method. As these two methods may have different detection accuracy, this difference could result in contradictory results.

Conclusion

The present meta-analysis study indicated that there is a consistent and significant association between the *IL-17A* rs2275913 polymorphism and RM, implying that this polymorphism is a potential marker for RM. Also, the results showed that the presence of AA genotype is associated with a higher risk of developing RM.

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Authors' Contributions

A.K.M, N.Z.; Designed the conception of the study. A.K.M., S.Z., Z.Z., S.I.; Focused on the statical analysis. N.Z., F.Sh., H.M., M.A.; Performed technical support and provided conceptual advice. All authors contributed to the

drafted the manuscript, revised it critically and approved the final version.

References

1. Dimitriadis E, Menkhorst E, Saito S, Kutteh WH, Brosens JJ. Recurrent pregnancy loss. Nat Rev Dis Primers. 2020; 6(1): 98.
2. D'Ippolito S, Ticconi C, Tersigni C, Garofalo S, Martino C, Lanzone A, et al. The pathogenic role of autoantibodies in recurrent pregnancy loss. Am J Reprod Immunol. 2020; 83(1): e13200.
3. Tsonis O, Balogun S, Adjei JO, Mogekwu O, Iliodromiti S. Management of recurrent miscarriages: an overview of current evidence. Curr Opin Obstet Gynecol. 2021; 33(5): 370-377.
4. Li S, Chen M, Zheng PS. Analysis of parental abnormal chromosomal karyotype and subsequent live births in Chinese couples with recurrent pregnancy loss. Sci Rep. 2021; 11(1): 20298.
5. Adib Rad H, Basirat Z, Mostafazadeh A, Faramarzi M, Bijani A, Nouri HR, et al. Evaluation of peripheral blood NK cell subsets and cytokines in unexplained recurrent miscarriage. J Chin Med Assoc. 2018; 81(12): 1065-1070.
6. Wang W, Sung N, Gilman-Sachs A, Kwak-Kim J. T Helper (Th) cell profiles in pregnancy and recurrent pregnancy losses: Th1/Th2/Th9/Th17/Th22/Tfh cells. Front Immunol. 2020; 11: 2025.
7. Zammiti W, Mtiraoui N, Cochery-Nouvellon E, Mahjoub T, Almawi WY, Gris JC. Association of -592C/A, -819C/T and -1082A/G interleukin-10 promoter polymorphisms with idiopathic recurrent spontaneous abortion. Mol Hum Reprod. 2006; 12(12): 771-776.
8. Baqer NN, Saheb EJ, Ahmed NS. Genetic polymorphism of IL-17A (rs2275913) in Iraqi women with recurrent abortion and its relationship with susceptibility to toxoplasmosis. Meta Gene. 2021; 29: 100939.
9. Gaffen SL. Structure and signalling in the IL-17 receptor family. Nat Rev Immunol. 2009; 9(8): 556-567.
10. Ghosh R, Mitra P, Kumar PVSNK, Goyal T, Sharma P. T helper cells in depression: central role of Th17 cells. Crit Rev Clin Lab Sci. 2022; 59(1): 19-39.
11. González-Fernández C, Chaves-Pozo E, Cuesta A. Identification and regulation of interleukin-17 (IL-17) family ligands in the teleost fish european sea bass. Int J Mol Sci. 2020; 21(7): 2439.
12. Lee SK, Kim JY, Hur SE, Kim CJ, Na BJ, Lee M, et al. An imbalance in interleukin-17-producing T and Foxp3- regulatory T cells in women with idiopathic recurrent pregnancy loss. Hum Reprod. 2011; 26(11): 2964-2971.
13. Ocejo-Vinyals JG, de Mateo EP, Hoz MÁ, Arroyo JL, Agüero R, Ausín F, et al. The IL-17 G-152A single nucleotide polymorphism is associated with pulmonary tuberculosis in northern Spain. Cytokine. 2013; 64(1): 58-61.
14. Hayashi R, Tahara T, Shiroeda H, Saito T, Nakamura M, Tsutsumi M, et al. Influence of IL17A polymorphisms (rs2275913 and rs3748067) on the susceptibility to ulcerative colitis. Clin Exp Med. 2013; 13(4): 239-244.
15. Duan Y, Shi JN, Pan C, Chen HL, Zhang SZ. Association between the interleukin-17A -197G>A (rs2275913) polymorphism and risk of digestive cancer. Asian Pac J Cancer Prev. 2014; 15(21): 9295-9300.
16. Rolandelli A, Hernández Del Pino RE, Pellegrini JM, Tateosian NL, Amiano NO, de la Barrera S, et al. The IL-17A rs2275913 single nucleotide polymorphism is associated with protection to tuberculosis but related to higher disease severity in Argentina. Sci Rep. 2017; 7: 40666.
17. Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gøtzsche PC, Ioannidis JP, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. J Clin Epidemiol. 2009; 62(10): e1-34.
18. Najafi S, Hadinedoushan H, Eslami G, Aflatoonian A. Association of IL-17A and IL-17 F gene polymorphisms with recurrent pregnancy loss in Iranian women. J Assist Reprod Genet. 2014; 31(11): 1491-1496.
19. Alkhuriji A. Association of maternal IL-17 and IL-23R polymorphisms with risk of recurrent spontaneous abortion in women in Saudi Arabia. Ital J Gynaecol Obstet. 2017; 29(1): 7-12.
20. Bahadori M, Zarei S, Zarnani AH, Zarei O, Idali F, Hadavi R, et al. IL-6, IL-10 and IL-17 gene polymorphisms in Iranian women with recurrent miscarriage. Iran J Immunol. 2014; 11(2): 97-104.
21. Zidan HE, Rezk NA, Alnemr AA, Moniem MI. Interleukin-17 and leptin genes polymorphisms and their levels in relation to recurrent pregnancy loss in Egyptian females. Immunogenetics. 2015; 67(11-12): 665-673.
22. Ford HB, Schust DJ. Recurrent pregnancy loss: etiology, diagnosis, and therapy. Rev Obstet Gynecol. 2009; 2(2): 76-83.
23. Li L, Liu Y, Feng T, Zhou W, Wang Y, Li H. The AHNAK induces increased IL-6 production in CD4+ T cells and serves as a potential diagnostic biomarker for recurrent pregnancy loss. Clin Exp Immunol. 2022; 209(3): 291-304.
24. Ewington LJ, Tewary S, Brosens JJ. New insights into the mechanisms underlying recurrent pregnancy loss. J Obstet Gynaecol Res. 2019; 45(2): 258-265.
25. Zhang M, Xu J, Bao X, Niu W, Wang L, Du L, et al. Association between genetic polymorphisms in interleukin genes and recurrent pregnancy loss - a systematic review and meta-analysis. PLoS One. 2017; 12(1): e0169891.
26. Espinoza JL, Takami A, Nakata K, Onizuka M, Kawase T, Akiyama H, et al. A genetic variant in the IL-17 promoter is functionally associated with acute graft-versus-host disease after unrelated bone marrow transplantation. PLoS One. 2011; 6(10): e26229.
27. Dai ZM, Zhang TS, Lin S, Zhang WG, Liu J, Cao XM, et al. Role of IL-17A rs2275913 and IL-17F rs763780 polymorphisms in risk of cancer development: an updated meta-analysis. Sci Rep. 2016; 6: 20439.
28. Shao M, Xu W, Yang H, Chen Y, Gao X, Xu S, et al. Interleukin-17 gene polymorphism (Rs2275913 G/A, Rs763780 C/T) in Rheumatoid arthritis: meta-analysis based on ethnicity. Immunol Invest. 2021; 50(6): 685-699.
29. Lang X, Liu W, Hou Y, Zhao W, Yang X, Chen L, et al. IL-17A polymorphism (rs2275913) and levels are associated with preeclampsia pathogenesis in Chinese patients. BMC Med Genomics. 2021; 14(1): 5.
30. Fu B, Tian Z, Wei H. TH17 cells in human recurrent pregnancy loss and pre-eclampsia. Cell Mol Immunol. 2014; 11(6): 564-570.
31. Nakashima A, Ito M, Yoneda S, Shiozaki A, Hidaka T, Saito S. Circulating and decidual Th17 cell levels in healthy pregnancy. Am J Reprod Immunol. 2010; 63(2): 104-109.

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Optimizing Immature Testicular Tissue and Cell Transplantation Results: Comparing Transplantation Sites and Scaffolds

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Abstract

For patients who had testicular tissue cryopreserved before receiving gonadotoxic therapies, transplantation of testicular tissues and cells has been recommended as a potential therapeutic option. There are no studies that indicate the generation of sperm after human immature testicular tissue (ITT) or spermatogonial stem cells (SSCs) transplantation. The use of releasing scaffolds and localized drug delivery systems as well as the optimizing transplantation site can play an effective role in increasing the efficiency and improving the quality of testicular tissue and cell transplantation in animal models. Current research is focused on optimizing ITT and cell transplantation, the use of releasing scaffolds, and the selection of the right transplantation site that might restore sperm production or male infertility treatment. By searching the PubMed and Google Scholar databases, original and review papers were collected. Search terms were relevant for SSCs and tissue transplantation. In this review, we'll focus on the potential advantages of using scaffolds and choosing the right transplantation site to improve transplantation outcomes.

Keywords: Injection, Scaffold, Spermatogonial Stem Cells, Transplantation

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Introduction

Chemotherapeutic drugs and radiation are proven to damage the gonads of prepubertal boys, despite their growing effectiveness in treating childhood cancers. As long-term survival rates for children with cancer keep going up, methods to maintain fertility in prepubertal boys who do not so far produce spermatozoa are urgently needed (1). According to a survey among adolescent cancer patients between the ages of 14 and 40 at the time of diagnosis, 51% wished to have their own children in the future (2). Prepubertal boys can't freeze a sample of their semen before beginning fertility-threatening therapies, while men can freeze a sample of their sperm (3). Numerous strategies utilizing the patient's own frozen-thawed immature testicular tissue (ITT) have been suggested to restore fertility after treatment, including autotransplantation of testicular tissue fragments or spermatogonial stem cells (SSC), *in vitro* maturation, and production of testicular organoids for use *in vivo* or *in vitro* (4). When a patient undergoes therapy, the transplantation of tissue or cells into the patient provides a promising method for the restoration of fertility. Cryopreservation of ITT for future transplantation has been recommended for a variety of patient populations; nevertheless, considerable obstacles must be overcome before this becomes a reality in clinical practice for fertility restoration. Today, there are no studies demonstrating the production of sperm following the transplantation of human ITT or SSC (5). ITT transplantation is one of the most effective methods for maintaining SSC in their niche and ensuring their interaction with germ cells and other supporting cells, resulting in a suitable environment for cell maturation, growth, and differentiation (6).

This review summarizes the progress made in SSC and tissue transplantation, particularly in animal models. The prospects for developing a transplantable scaffold to assist the development and differentiation of isolated testicular cells are also discussed in this study.

The PRISMA 2020 guideline forms the basis for the present narrative review. We conducted a literature search on testicular tissue and SSC transplantation as a treatment for male infertility. From 1938 until 2021, PubMed and Google Scholar were utilized as search engines. The search terms included: terms 'Immature testicular tissue', 'Injection', '*In vivo* spermatogenesis', 'Scaffold', 'Spermatogonial stem cells', 'Transplantation'' and a combination of words with the AND, OR functions, along with their equivalents in Mesh. Original and review studies

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were both included in this study. In addition to manually searching the reference lists of relevant papers to find additional studies that the electronic search missed, all of the aforementioned databases were searched for articles using the appropriate keywords. The following was the research question: Which scaffolds and transplanting sites are best?

The selection of the papers was presented using the PRISMA 2020 flowchart for narrative reviews (Fig.1).

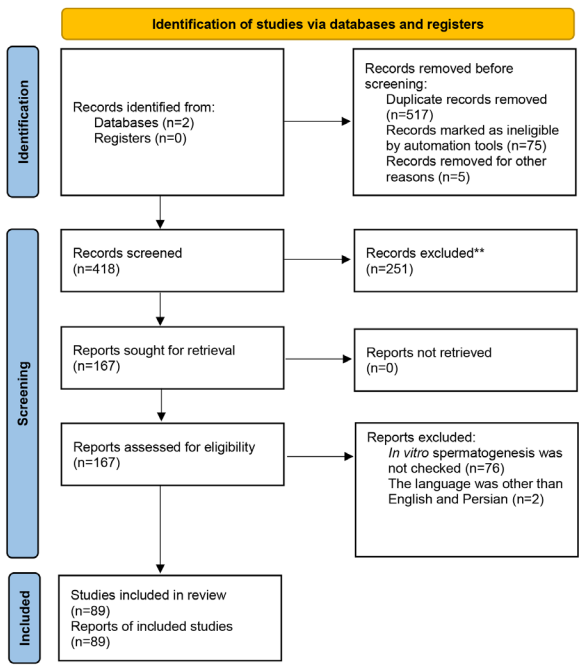

Fig.1: PRISMA 2020 Flow diagram of the study selection for narrative review.

SSC transplantation has recently been proposed as a possible therapeutic option for preserving fertility. Implanting SSC from a fertile donor into an infertile recipient is one therapeutic strategy. The method of transplanting SSC into seminiferous tubules has been extensively studied, and it is helpful and suitable for a wide range of species (Fig.2A) (7). In another approach, after collecting samples of testicular tissue, SSC are isolated and cultured in vitro before being injected into the patient's testes (8). SSC collected from cell suspensions could be cultured in vitro to generate a sufficient number of spermatogonial cells for a clinical application of SSC transplantation (9). Another hybrid procedure is *in vitro* transplantation, in which donor SSC are cultivated *in vitro*, then injected into the recipient testis, and ultimately re-cultured *in vitro* as a donor-host mix of tissue fragments. This technique allows relatively easier cell observation than pure *in vivo* xenografting (10).

In 1994, Brinster and Avarbock (11) and Brinster and Zimmermann (12) were the first to perform SSC transplantation in mice. They discovered that SSCs from donors could resumption spermatogenesis in host mice that have been treated with chemotherapy, resulting in the birth of offspring. Anjamrooz et al. (13) transplanted SSC from newborn mice into the seminiferous tubules of host mice two weeks after culture. According to their findings, enrichment of type A spermatogonial cells through an *in vitro* co-culture method can enhance recipient mice's epididymal sperm count. Koruji et al. (14) isolated Sertoli and spermatogonial cells, which were subsequently transferred, through rete testis, into the other irradiated testis of the same mouse. The results demonstrated that autotransplantation of SSC could result in sperm generation in the recipient's testes.

Experiments on animals, especially non-human primates, have shown progress in the field of SSC graft. In numerous rodent species, SSC graft has proven to be effective, including the hamster (15), rabbit (16), mouse (8, 11, 17, 18), and rat (19-22). Other animals utilized for donor cell injection into rete testis that colonized and differentiated successfully were goats (23, 24), sheep (25-27), and dogs (Table 1) (16, 28, 29).

Table 1: Spermatogonial stem cell transplantation

Doner	Recipient	Grafting site	Outcome: germ cell maturation/ offspring/ others	References
Mice	Mice	Seminiferous tubules	Offspring generation	Brinster (11, 12)
Mice	Mice	Rete testis	Sperm generation	Koruji et al. (14)
Hamster	Mice	Seminiferous tubules	Sperm generation	Ogawa et al. (15)
Rat	Mice	Seminiferous tubules	Sperm generation	Clouthier et al. (19)
Goats	Goats	Rete testis	Offspring generation	Honaramooz et al. (23)
Sheep	Sheep	Rete testis	Sperm generation	Herrid et al. (25)
Dog	Dog	Rete testis	Sperm generation	Kim et al. (28)
Rhesus	Rhesus	Rete testis	Sperm generation	Hermann et al. (30)
Rhesus	Rhesus	Rete testis	Offspring generation	Goossens and Tournaye (31)
Human	Mice	Rete testis	SSC survived in the mouse testis for as long as six months without showing any signs of differentiation	Nagano et al. (32)
Human	Mice	Rete testis	Human SSC was effectively transplanted into azoospermic mouse testis *in vitro*, and tissue culture conditions supported the homing of human SSC in the testis	Mohaqiq et al. (33)

SSC; Spermatogonial stem cell.

Studies on non-human primates provide an extremely useful insight into clinical transplantation (34). Using SSC injected into the recipient's seminiferous tubules, sperm was successfully produced in monkeys (30). In 1999, the first SSC transplants were performed on monkeys. The injection procedure has been used on bigger animals with great effectiveness. Injecting SSC into the efferent duct in the rete testis in small animals, the testes must be exposed. However, the technique is less invasive for larger animals (35). In the testes of macaques, spermatogenesis was resumed after both autologous and allogeneic SSC transplantations. The generated spermatozoa were capable of fertilizing oocytes. This study indicates the ability of SSC grafting in such a primate species, increasing the feasibility that this technique may be used in clinical practice in the future (31).

The first case of human SSC xenografting was reported in 2002. Nagano et al. (32) isolated human SSC from six infertile men and injected them into nude mice treated with busulfan. It was demonstrated that human SSC migrated into the basal membrane of mouse testicular seminiferous tubules, although their numbers significantly decreased one month after transplantation. However, some SSC survived in the mouse testis for as long as six months without showing any signs of differentiation. In a study, after xenotransplantation to mouse testes, SSCs were obtained from prepubescent boys with cancer and showed stem cell activity similar to that of human adult SSC (36). Mohaqiq et al. (33) transplanted human SSC into the testes of azoospermic adult mice. According to their findings, human SSC were effectively transplanted into azoospermic mouse testis in vitro, and tissue culture conditions supported the homing of human SSC in the testis (Table 1).

There are several examples of xenotransplantation that utilize *in vitro* procedures. SSC samples are typically cultured *in vitro* before being injected into the recipient. This method has also been tested on humans. In one experiment, human SSCs were cultured *in vitro* and subsequently injected into the rete testis of immune-deficient mice. The results revealed that SSC proliferated throughout the basement membrane of the host, but no sperm were identified. This suggests that human signaling factors may be essential for full differentiation (37).

In vitro proliferation and differentiation of SSC utilizing exogenous factors offers a platform for researching germ cell biology and enables germ-line transplantation for the treatment of infertility. In vitro, the differentiation of embryonic stem cells (ESC) into SSC and the ability to use transplantation techniques can help find ways to treat infertility (38). According to the findings of Rahmani et al. (39), it is possible to induce SSC-like cell differentiation from induced pluripotent stem cells (iPSC) after *in vitro* transplantation, 3D testicular organ cultivation, and *in vivo* transplantation. Dashtaki et al. (40) demonstrated that the adipose tissue-derived mesenchymal stem cells (AT-MSCs) that were transplanted within seminiferous tubules were localized in the basement membrane, and the testicles of mice treated with AT-MSCs expressed spermatogenesis-specific markers. Overexpression of germ cell-specific markers was seen in mice receiving cells cultured in the presence of growth factors.

Because of the unestablished domain of human SSC proliferation *in vitro* and the poor co-transplanted microenvironment for maintaining SSC, there are various limitations for SSC transplantation in humans (41). However, considering the effectiveness of the SSC transplantation method in several large animal species and the fact that many patients have previously cryopreserved testicular tissue or cells, a clinical translation of the approach is imminent.

The best site for SSC injection in the host is one of several unanswered questions. The findings demonstrate that microinjection of cell suspensions into the efferent ducts seminiferous tubules, efferent ducts, or rete testis is equally successful in producing donor-cell-derived spermatogenesis in the host. Each method is advantageous in a variety of species for different experimental reasons (42). Since ultrasonography can detect the rete testis while avoiding open surgery, it has become the most successful injection location. SSC transplantation in various big animal species, including nonhuman primates, has now been performed via ultrasound-guided rete testis injection (25, 35, 43, 44). There is no need for surgery when doing an ultrasound-guided rete testis injection. To summarize, the rete testis is seen via ultrasonography, and the injection needle is directed into the space of the rete testis through the scrotal skin (30). In the only human study, it was shown that the injection of contrast material into the rete testis, near the epididymis's caput, was the most effective site (45). Therefore, it seems that one of the suitable sites for SSC transplantation is rete testis.

SSC suspensions can be replaced with testicular tissue fragments transplanted into the recipient. This strategy preserves the reaction between germ cells and somatic cells by maintaining the SSC within their native environment (46). It appears that the most promising option for providing these patients with the opportunity to become the biological parents of their own kids is to transplant the cryopreserved patient's testicular tissue back into the patient after they have been cured (4). In several studies, orthotopic or heterotopic grafting of ITT from various animal species was performed on immunodeficient mice, which resulted in tissue maturation and full spermatogenesis (47-52). For the first time, Liu et al. (53) demonstrated that sperm from adult monkey testis xenotransplants can be used to produce non-human primate offspring. In 2019, Fayomi et al. (54) allotransplanted Rhesus macaque ITT into the scrotal and dorsal skin. According to the findings, grafts grew and released testosterone. This procedure requires appropriate vascularization of transplanted tissue to be successful (55). During transplantation, the supply of nutrition, oxygen, and factors that promote cell survival, proliferation, and differentiation is essential (56). It has yet to be demonstrated that xenotransplantation of

human ITT obtained from human fetuses or prepubertal boys has resulted in sperm production (57-65). After xenografting human ITT into mice, differentiation up to the pachytene spermatocyte stage was seen (Table 2) (58-64). Transplantation of ITT generally leads to the resumption of spermatogenesis, which may be significant when considering the possible future applications of transplanting testicular tissue from pubertal patients.

It will be critical to develop successful testicular transplantation protocols that will most probably include biomaterial scaffolds for providing a suitable microenvironment for testicular cells and *in vivo* tissue development inside microfluidic devices. To date, in comparison to ovarian transplantation, the application of modified biomaterials to enhance transplant results has been comparatively underrepresented in testicular transplantation (66).

Before a pilot clinical trial and clinical implementation of ITT transplantation, the viability of the procedure and optimum environment for producing spermatogenesis utilizing ITT transplants, including the transplantation site, must be investigated in a human-relevant preclinical model (6). In ITT transplantation, temperature and the grafting site are two crucial parameters. Xenografts could be transplanted into the testis or scrotum, known as orthotopic or homotopic grafting, or into other parts of the body, such as the intra-abdominal cavity or subcutaneous, known as ectopic or heterotopic grafting (69).

Full spermatogenesis has resulted from the transplantation of testicular tissue from various animal species into mice or rats in a variety of locations, including the back skin (15, 51, 70-72), the ear tip (67, 72), the intratesticular testis (6, 47, 73), scrotum (54, 74), and the anterior chamber of the eye (75). Recent research by Eyni et al. (68) demonstrated that when mouse ITT is transplanted into the epididymal fat of castrated adult mice, it survives for 8 weeks and spermatogenesis can be identified up to the level of elongated spermatids (Table 2).

For the first time, Luetjens and colleagues (76) demonstrated that autologous transplantation of nonhuman monkey testicular tissues arranged similarly to human testes results in complete spermatogenesis in the scrotum of immature animals. In addition, the transplantation of cryopreserved ITT from a rhesus monkey into the scrotum led to full spermatogenesis (74). The first non-human monkey born from sperm produced after ITT transplantation was a significant milestone in the clinical translation of ITT transplantation. In this research, fresh and frozen ITT of rhesus monkeys were grafted into the scrotum and the dorsal skin of castrated males. The recovery rate of testicular transplants was 100 percent after an 8 to 12-month transplantation interval, and full spermatogenesis was proven in all transplants (54).

Human ITT xenografted into the scrotum (54-56), intratesticular (58), and dorsal skin (61) of castrated nude mice demonstrated spermatogonia's capability to survive, proliferate, and even differentiate up to the stage of pachytene spermatocyte. The neovascularization and formation of a vascular system to optimize blood flow between the transplant and the recipient are critical for the transplant's survival. Reperfusion damage and ischemia, the preservation of early spermatogonial cells, and insufficient testicular transplant neovascularization are the main challenges of human ITT transplantation (60). When comparing the temperatures of ectopic and orthotopic transplantation locations, orthotopic transplantation has a significantly lower temperature. Thus, the increased temperature at the site of ectopic transplantation may be a contributing factor to spermatogenic arrest (76, 77). Orthotopic transplantation into the intratesticular site may provide intensive blood flow and hormonal support for testicular transplants (72). Testicular tissue might be lost in heterotopic transplantation because of probable degenerative processes and ischemia as well as immune reactions. This should lead to more research on biocompatible devices that can protect the testicular tissue's structure (77).

These findings support the development of orthotopic tissue transplantation as a prospective clinical option for restoring fertility in individuals who have had gonadotoxic therapies, and they urge the conduct of additional research on human ITT transplantation.

Table 2: Immature testicular tissue transplantation

Doner	Recipient	Grafting site	Outcome: germ cell matura-tion/offspring	References
Rat	Rat	Ear tip	Full spermatogenesis	Johnson et al. (67)
Piglets/goats/mice	Mice	Back skin	Sperm generation	Honaramooz et al. (48)
Mice/rabbit	Mice	Testis	Offspring generation	Shinohara et al. (47)
Cat	Mice	Back skin	Full spermatogenesis	Snedaker et al. (51)
Macaques	Mice	Back skin	Offspring generation	Liu et al. (53)
Human	Mice	Scrotal bursa	Pachytene spermatocyte generation	Wyns et al. (58)
Human	Mice	Back skin	Pachytene spermatocyte generation	Sato et al. (10)
Human	Mice	Intratesticular	Pachytene spermatocyte generation	Van Saen et al. (55)
Macaques	Macaques	Back skin, scrotal skin	Offspring generation	Fayomi et al. (54)
Mice	Mice	Epididymal fat	Elongated spermatids generation	Eyni et al. (68)

Treatment of both male and female infertility using scaffolds has shown significant potential. Because biodegradable scaffolds can mimic extracellular matrix (ECM) conditions and provide a suitable microenvironment for cell proliferation and differentiation, they are appropriate for cell delivery systems. Additionally, these scaffolds degrade over time, reducing the possibility of immunological response in the body (76). Controlled drug delivery system (DDS) is a new strategy for regulating the bioavailability of therapeutic substances. Using controlled DDS, drugs are loaded into a polymeric network structure and released in a definite way from the polymer (78). The most basic of recent advances is the embedding of testicular cells or tissues within a scaffold for in vivo transplantation (Fig.2B).

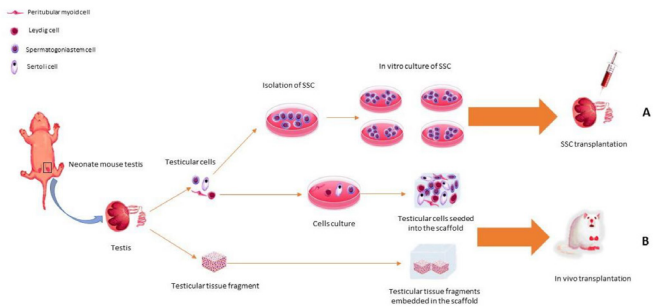

Fig.2: Schematic representation of testicular tissue and cell transplantation. **A.** In the past, spermatogonial stem cells (SSCs) were isolated and cultured before being injected into testicular azoospermia. **B.** With the development of tissue engineering and the establishment of releasing and non-releasing scaffolds, testicular tissue fragments or cells with the scaffold were transplanted into the recipient.

Hydrogels made of synthetic and natural polymers have attracted continuing interest in cell seeding, and recent advances in the field of tissue engineering have made such hydrogels particularly appealing as matrices for regenerating and repairing a broad range of organs and tissues (79). Wang et al. (80) seeded Leydig cells onto polyglycolic acid fiber scaffolds, cultivated them in vitro for 7 days and grafted the cell-scaffold structures into adult castrated rats' tunica vaginalis cavity or gastrocolic omentum. Using cells and a scaffold, a vascularized testosterone-secreting tissue was formed in both of the transplantation sites two months after transplantation. When a variety of testicular cell types, such as Leydig cells, were seeded, the serum testosterone level was raised much more. However, spermatogenesis was not achieved when a mixed community of testicular cells was used. Consequently, this strategy provided a strategy for the synthesis of androgen-secreting tissue for transplantation applications. Matrigel has also been used to encapsulate testicular cells in of several experiments (Table 3). Studies showed that seminiferous tubules were formed from testicular cells encapsulated in Matrigel, but only a small number of germ cells were found (54, 81, 82). Dores and Dobrinski (83) investigated graft development using Matrigel as a support structure to keep cells in close proximity. The improved method was then put to the test as a functional experiment to see how vascular endothelial growth factor-165 (VEGF165) affected blood supply and testicular tissue reorganization. Their findings revealed that adding Matrigel to the cell suspension improved tubule development and increased the efficiency of newly generated testis tissue, probably by promoting cell-to-cell interaction while reducing cell loss. However, adding VEGF165 to the cell suspension did not enhance blood vessel or tubule development, but it did increase the number of tubules containing spermatogonia. They found that adding Matrigel enhanced spermatogenic efficiency and that VEGF165 could preserve germ cells by promoting their survival in their niche.

Development of bioengineered scaffolds able to support testicular cells and improve ITT grafting in different species, it seems that may be a promising way to preserve fertility in humans.

To develop intelligent DDS, researchers have worked to engineer the chemical and physical features of DDS to optimize their biodegradability, surface functionality, environmental reaction, permeability, biorecognition, and biorecognition places (78). Hydrogels are an example of a DDS class that has achieved significant success in the field of intelligent drug delivery (51). It is possible to support the proliferation and differentiation of cells in newly generated tissues by using hydrogels containing growth factors. The use of hydrogels is frequently advantageous because of their high water content, quick nutrition transport, and ability to aid cell migration and angiogenesis (84). Numerous studies in regenerative medicine have investigated the advantages of local drug delivery through hydrogels (Table 3). Poels et al. (85) after transplantating testicular tissue of mice embedded in alginate hydrogel containing VEGF-nanoparticles into the scrotum, reported that VEGF-NP significantly enhanced angiogenesis but had no effect on spermatogenic cell survival, while alginate alone improved cell survival of spermatogonia. Furthermore, during long-term xenotransplantation, culture of human ITT with human VEGF-165 enhances spermatogonial viability, seminiferous tubule structure, and vascularization (56). When compared to just alginate encapsulation, testicular tissue embedded in alginate hydrogel loaded with necrosis inhibitor nanoparticles (NECINH-NP) led to a considerable enhancement in spermatogonial viability and tissue integrity after orthotopic autotransplantation (4). Alginate hydrogels supplemented with platelet-derived growth factor (PDGF)-delivery nanoparticles enhanced vascularization and vascular development in testicular tissue grafts in comparison to VEGF-only supplementation, although possible interactions with NECINH should be investigated further (86).

The successful development of bioengineered scaffolds with DDS would certainly be a significant advance toward restoring male fertility.

Table 3: Cell/tissue transplantation with scaffold

Tissue/Cell	Doner	Recipient	Grafting site	Scaffold	Factors	Outcome	References
Leydig cells	Rats	Rats	gastrocolic omentum/cavity of tunica vaginalis	Polyglycolic acid	-----	Vascularized testosterone-secreting tissue was formed and the serum testosterone level was raised much more	Wang et al. (80)
Testicular cell	Rat	Mice	back skin	Matrigel	-----	The grafts were vascularized and contained elongated seminiferous tubules, there were a few integra-tions of putative spermatogonia into the seminiferous epithelium, the development of tubule lumen, and putative Leydig cells	Gassei et al. (81)
Testicular cell	Canine	Mice	Seminiferous tubules	Matrigel	-----	De novo seminiferous tubules were formed and some germ cells were localized in the basement membrane of seminiferous tubules	Lee et al. (82)
Testicular cell	Porcine	Mice	Back skin	Matrigel	-----	Adding matrigel to the cell suspen-sion improved tubule development and increased the efficiency of newly generated testis tissue	Dores and Dobrinski. (83)
Immature testicular tissue	Mice	Mice	Scrotum	Alginate hydrogel	VEGF-NPs	VEGF-NP significantly enhanced angiogenesis but did not affect spermatogenic cell survival, while alginate alone improved cell sur-vival of spermatogonia	Poels et al. (85)
Immature testicular tissue	Mice	Mice	Scrotum	Alginate hydrogel	NECINH-NP	Testicular tissue embedded in alginate hydrogel loaded with NECINH-NP led to a considerable enhancement in spermatogonial viability and tissue integrity a	Del Vento et al. (4)
Testicular tissue frag-ments	Mice	Mice	Scrotum	Alginate hydrogel	NECINH, VEGF and PDGF-NP	PDGF-delivery nanoparticles enhanced vascularization and vascular development in testicular tissue grafts in comparison to VEGF-only supplementation although possible interactions with NECINH should be investigated further	Del Vento et al. (86)

Conclusion

Under experimental conditions, there has always been an obstacle to differentiation at the diploid cell stage so far. With the assistance of testicular tissue and cell transplantation, significant progress has been made toward the creation of therapeutic solutions for human fertility preservation. Animal studies demonstrate the feasibility of autologous transplanting of SSC or testicular tissue to produce gametes for the development of healthy offspring. The purpose of the current study is to apply this data in the clinic, especially for patients who will be receiving gonadotoxic treatments. However, for the method to be considered effective, differentiation must be achieved up to the haploid cell stage. Thus, further research is required to investigate the biology of spermatogenesis and to determine whether procedures that have proven successful with animal models may also be applied to human tissue. Tissue engineering and bioengineering could be viable options. Reproductive tissues can be designed to act as *in vitro* experimental research as well as substitute or regenerate damaged tissues to maintain reproductive ability. However, the risk of deleterious impacts of novel biomaterials on reproductive health, such as the quality and development of gametes, is typically disregarded. Hopefully, the discovery of new information from the engineered procedure mentioned here will make it possible to meet the reproductive demands of present patients and future generations.

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Authors' Contributions

A.A.; Contributed to data collection, Classification, Paper Summarization, Explicit literature search, and Manuscript writing. M.M.; Supervised the research and Contributed to writing the manuscript and Revision. M.H.; Contributed to manuscript writing and Paper summarization. All authors read and approved the final manuscript.

References

1. Giudice MG, De Michele F, Poels J, Vermeulen M, Wyns C. Update on fertility restoration from prepubertal spermatogonial stem cells: how far are we from clinical practice? Stem Cell Res. 2017; 21: 171-177.
2. Yokonishi T, Ogawa T. Cryopreservation of testis tissues and in vitro spermatogenesis. Reprod Med Biol. 2016; 15(1): 21-28.
3. Vermeulen M, Del Vento F, De Michele F, Poels J, Wyns C. Development of a cytocompatible scaffold from pig immature testicular tissue allowing human sertoli cell attachment, proliferation and functionality. Int J Mol Sci. 2018; 19(1): 227.
4. Del Vento F, Vermeulen M, Ucakar B, Poels J, des Rieux A, Wyns C. significant benefits of nanoparticles containing a necrosis inhibitor on mice testicular tissue autografts outcomes. Int J Mol Sci. 2019; 20(23): 5833.
5. Kilcoyne KR, Mitchell RT. Fertility preservation: testicular transplantation for fertility preservation: clinical potential and current challenges. Reproduction. 2019; 158(5): F1-F14.
6. Ntemou E, Kadam P, Van Saen D, Wistuba J, Mitchell R, Schlatt S, et al. Complete spermatogenesis in intratesticular testis tissue xenotransplants from immature non-human primate. Hum Reprod. 2019; 34(3): 403-413.
7. Ma L, Li B, Li L, Wang X, Liu C, Ding Q. Modified technique for spermatogonial stem cell transplantation into the seminiferous tubules in mouse model. Syst Biol Reprod Med. 2013; 59(2): 108-116.
8. Kanatsu-Shinohara M, Morimoto H, Shinohara T. Fertility of male germline stem cells following spermatogonial transplantation in infertile mouse models. Biol Reprod. 2016; 94(5): 112.
9. Sadri-Ardekani H, Akhondi MA, van der Veen F, Repping S, van Pelt AM. In vitro propagation of human prepubertal spermatogonial stem cells. JAMA. 2011; 305(23): 2416-2418.
10. Sato T, Katagiri K, Kubota Y, Ogawa T. In vitro sperm production from mouse spermatogonial stem cell lines using an organ culture method. Nat Protoc. 2013; 8(11): 2098-2104.
11. Brinster RL, Avarbock MR. Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci. 1994; 91(24): 11303-11307.
12. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci USA. 1994; 91(24): 11298-11302.
13. Anjamrooz SH, Movahedin M, Tiraihi T, Mowla SJ. Graft efficiency of co-cultured spermatogonial cells using sperm assay in epididymal lumen of recipient mice. Cell J. 2006; 7(4): 242-249.
14. Koruji M, Movahedin M, Mowla SJ, Gourabi H, Pour-Beiranvand S, Arfaee AJ. Autologous transplantation of adult mice spermatogonial stem cells into gamma irradiated testes. Cell J. 2012; 14(2): 82-89.
15. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. Biol Reprod. 1999; 60(2): 515-521.
16. Dobrinski I, Avarbock MR, Brinster RL. Transplantation of germ cells from rabbits and dogs into mouse testes. Biol Reprod. 1999; 61(5): 1331-1339.
17. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med. 2000; 6(1): 29-34.
18. Kanatsu-Shinohara M, Ogonuki N, Inoue K, Ogura A, Toyokuni S, Honjo T, et al. Allogeneic offspring produced by male germ line stem cell transplantation into infertile mouse testis. Biol Reprod. 2003; 68(1): 167-173.
19. Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL. Rat spermatogenesis in mouse testis. Nature. 1996; 381(6581): 418-421.
20. Ogawa T, Dobrinski I, Brinster RL. Recipient preparation is critical for spermatogonial transplantation in the rat. Tissue Cell. 1999; 31(5): 461-472.
21. Ryu BY, Orwig KE, Avarbock MR, Brinster RL. Stem cell and niche development in the postnatal rat testis. Dev Biol. 2003; 263(2): 253-263.
22. Zhang D, Liu X, Peng J, He D, Lin T, Zhu J, et al. Potential spermatogenesis recovery with bone marrow mesenchymal stem cells in an azoospermic rat model. Int J Mol Sci. 2014; 15(8): 13151-13165.
23. Honaramooz A, Behboodi E, Megee SO, Overton SA, Galantino-Homer H, Echelard Y, et al. Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol Reprod. 2003; 69(4): 1260-1264.
24. Honaramooz A, Behboodi E, Blash S, Megee SO, Dobrinski I. Germ cell transplantation in goats. Mol Reprod Dev. 2003; 64(4): 422-428.
25. Herrid M, Olejnik J, Jackson M, Suchowerska N, Stockwell S, Davey R, et al. Irradiation enhances the efficiency of testicular germ cell transplantation in sheep. Biol Reprod. 2009; 81(5): 898-905.
26. Herrid M, Davey R, Stockwell S, Olejnik J, Schmoelzl S, Suchowerska N, et al. A shorter interval between irradiation of recipient testis and germ cell transplantation is detrimental to recovery of fertility in rams. Int J Androl. 2011; 34(5pt1): 501-512.
27. Stockwell S, Hill JR, Davey R, Herrid M, Lehnert SA. Transplanted germ cells persist long-term in irradiated ram testes. Anim Reprod Sci. 2013; 142(3-4): 137-140.
28. Kim Y, Turner D, Nelson J, Dobrinski I, McEntee M, Travis AJ. Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog. Reprod. 2008; 136(6): 823-831.
29. Harkey MA, Asano A, Zoulas ME, Torok-Storb B, Nagashima J, Travis A. Isolation, genetic manipulation, and transplantation of canine spermatogonial stem cells: progress toward transgenesis through the male germ-line. Reproduction. 2013; 146(1): 75-90.
30. Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, et al. Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell. 2012; 11(5): 715-726.
31. Goossens E, Tournaye H. Functional sperm produced after spermatogonial stem cell transplantation into rhesus. Asian J Androl. 2013; 15(2): 216-217.
32. Nagano M, Patrizio P, Brinster RL. Long-term survival of human spermatogonial stem cells in mouse testes. Fertil Steril. 2002; 78(6): 1225-1233.
33. Mohaqiq M, Movahedin M, Mazaheri Z, Amirjannati N. Successful human spermatogonial stem cells homing in recipient mouse testis after in vitro transplantation and organ culture. Cell J. 2019; 20(4): 513-520.
34. Phillips KA, Bales KL, Capitanio JP, Conley A, Czoty PW, 't Hart BA, et al. Why primate models matter. Am J Primatol. 2014; 76(9): 801-827.
35. Schlatt S, Rosiepen G, Weinbauer GF, Rolf C, Brook PF, Nieschlag E. Germ cell transfer into rat, bovine, monkey and human testes. Hum Reprod. 1999; 14(1): 144-150.
36. Wu X, Schmidt JA, Avarbock MR, Tobias JW, Carlson CA, Kolon TF, et al. Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. Proc Natl Acad Sci USA. 2009; 106(51): 21672-21677.
37. Mirzapour T, Movahedin M, Koruji M, Nowroozi MR. Xenotransplantation assessment: morphometric study of human spermatogonial stem cells in recipient mouse testes. Andrologia. 2015; 47(6): 626-633.
38. Makoolati Z, Movahedin M, Forouzandeh-Moghadam M, Naghdi M, Koruji M. Embryonic stem cell derived germ cells induce spermatogenesis after transplantation into the testes of an adult mouse azoospermia model. Clin Sci (Lond). 2017; 131(18): 2381-2395.
39. Rahmani F, Movahedin M, Mazaheri Z, Soleimani M. Transplantation of mouse iPSCs into testis of azoospermic mouse model: in vivo and in vitro study. Artif Cells Nanomed Biotechnol. 2019; 47(1): 1585-1594.
40. Dashtaki ME, Hemadi M, Saki G, Mohammadiasl J, Khodadadi A. Spermatogenesis recovery potentials after transplantation of adipose tissue-derived mesenchymal stem cells cultured with growth factors in experimental azoospermic mouse models. Cell J. 2020; 21(4): 401-409.
41. Gül M, Dong L, Wang D, Diri MA, Andersen CY. Surrogate testes: Allogeneic spermatogonial stem cell transplantation within an encapsulation device may restore male fertility. Med Hypotheses. 2020; 139: 109634.
42. Ogawa T, Arechaga JM, Avarbock MR, Brinster RL. Transplantation of testis germinal cells into mouse seminiferous tubules. Int J Dev Biol. 2003; 41(1): 111-122.
43. Schlatt S, Foppiani L, Rolf C, Weinbauer GF, Nieschlag E. Germ cell transplantation into X-irradiated monkey testes. Hum Reprod. 2002; 17(1): 55-62.
44. Jahnukainen K, Ehmcke J, Quader MA, Saiful Huq M, Epperly MW, Hergenrother S, et al. Testicular recovery after irradiation differs in prepubertal and pubertal non-human primates, and can be enhanced by autologous germ cell transplantation. Hum Reprod. 2011; 26(8): 1945-1954.
45. Ning L, Meng J, Goossens E, Lahoutte T, Marichal M, Tournaye H. In search of an efficient injection technique for future clinical application of spermatogonial stem cell transplantation: infusion

of contrast dyes in isolated cadaveric human testes. Fertil Steril. 2012; 98(6): 1443-1448. e1.
46. Goossens E, Jahnukainen K, Mitchell RT, Van Pelt A, Pennings G, Rives N, et al. Fertility preservation in boys: recent developments and new insights. Hum Reprod Open. 2020; 2020(3): hoaa016.
47. Shinohara T, Inoue K, Ogonuki N, Kanatsu-Shinohara M, Miki H, Nakata K, et al. Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination. Hum Reprod. 2002; 17(12): 3039-3045.
48. Honaramooz A, Snedaker A, Boiani M, Schöler H, Dobrinski I, Schlatt S. Sperm from neonatal mammalian testes grafted in mice. Nature. 2002; 418(6899): 778-781.
49. Honaramooz A, Li MW, Penedo MCT, Meyers S, Dobrinski I. Accelerated maturation of primate testis by xenografting into mice. Biol Reprod. 2004; 70(5): 1500-1503.
50. Schlatt S, Honaramooz A, Boiani M, Schöler HR, Dobrinski I. Progeny from sperm obtained after ectopic grafting of neonatal mouse testes. Biol Reprod. 2003; 68(6): 2331-2335.
51. Snedaker AK, Honaramooz A, Dobrinski I. A game of cat and mouse: xenografting of testis tissue from domestic kittens results in complete cat spermatogenesis in a mouse host. J Androl. 2004; 25(6): 926-930.
52. Ohta H, Wakayama T. Generation of normal progeny by intracytoplasmic sperm injection following grafting of testicular tissue from cloned mice that died postnatally. Biol Reprod. 2005; 73(3): 390-395.
53. Liu Z, Nie YH, Zhang CC, Cai YJ, Wang Y, Lu HP, et al. Generation of macaques with sperm derived from juvenile monkey testicular xenografts. Cell Res. 2016; 26(1): 139-142.
54. Fayomi AP, Peters K, Sukhwani M, Valli-Pulaski H, Shetty G, Meistrich ML, et al. Autologous grafting of cryopreserved prepubertal rhesus testis produces sperm and offspring. Science. 2019; 363(6433): 1314-1319.
55. Van Saen D, Goossens E, Aerts JL, Haentjens P, Tournaye H. Does early cell death cause germ cell loss after intratesticular tissue grafting? Fertil Steril. 2013; 99(5): 1264-1272. e1.
56. Ntemou E, Kadam P, Van Laere S, Van Saen D, Vicini E, Goossens E. Effect of recombinant human vascular endothelial growth factor on testis tissue xenotransplants from prepubertal boys: a three-case study. Reprod Biomed Online. 2019; 39(1): 119-133.
57. Yu J, Cai ZM, Wan HJ, Zhang FT, Ye J, Fang JZ, et al. Development of neonatal mouse and fetal human testicular tissue as ectopic grafts in immunodeficient mice. Asian J Androl. 2006; 8(4): 393-403.
58. Wyns C, Curaba M, Martinez-Madrid B, Van Langendonckt A, François-Xavier W, Donnez J. Spermatogonial survival after cryopreservation and short-term orthotopic immature human cryptorchid testicular tissue grafting to immunodeficient mice. Hum Reprod. 2007; 22(6): 1603-1611.
59. Goossens E, Geens M, De Block G, Tournaye H. Spermatogonial survival in long-term human prepubertal xenografts. Fertil Steril. 2008; 90(5): 2019-2022.
60. Wyns C, Van Langendonckt A, Wese FX, Donnez J, Curaba M. Long-term spermatogonial survival in cryopreserved and xenografted immature human testicular tissue. Hum Reprod. 2008; 23(11): 2402-2414.
61. Sato Y, Nozawa S, Yoshiike M, Arai M, Sasaki C, Iwamoto T. Xenografting of testicular tissue from an infant human donor results in accelerated testicular maturation. Hum Reprod. 2010; 25(5): 1113-1122.
62. Van Saen D, Goossens E, Bourgain C, Ferster A, Tournaye H. Meiotic activity in orthotopic xenografts derived from human postpubertal testicular tissue. Hum Reprod. 2011; 26(2): 282-293.
63. Poels J, Van Langendonckt A, Many MC, Wese FX, Wyns C. Vitrification preserves proliferation capacity in human spermatogonia. Hum Reprod. 2013; 28(3): 578-589.
64. Van Saen D, Goossens E, Haentjens P, Baert Y, Tournaye H. Exogenous administration of recombinant human FSH does not improve germ cell survival in human prepubertal xenografts. Reprod Biomed Online. 2013; 26(3): 286-298.
65. Valli H, Phillips BT, Shetty G, Byrne JA, Clark AT, Meistrich ML, et al. Germline stem cells: toward the regeneration of spermatogenesis. Fertil Steril. 2014; 101(1): 3-13.
66. Gargus ES, Rogers HB, McKinnon KE, Edmonds ME, Woodruff TK. Engineered reproductive tissues. Nat Biomed Eng. 2020; 4(4): 381-393.
67. Johnson L, Suggs LC, Norton YM, Zeh WC. Effect of developmental age or time after transplantation on Sertoli cell number and testicular size in inbred Fischer rats. Biol Reprod. 1996; 54(5): 948-959.
68. Eyni H. Evaluation of spermatogenesis after graft of fresh and frozen-thawed neonatal mouse testicular tissue fragments to epididymal fat region of bilateral castrated adult mouse. Presented for the MSc., Tehran. Tarbiyat Modarres University. 2017.
69. Arregui L, Dobrinski I. Xenografting of testicular tissue pieces: 12 years of an in vivo spermatogenesis system. Reproduction. 2014; 148(5): R71-R84.
70. Kaneko H, Kikuchi K, Nakai M, Somfai T, Noguchi J, Tanihara F, et al. Generation of live piglets for the first time using sperm retrieved from immature testicular tissue cryopreserved and grafted into nude mice. PLoS One. 2013; 8(7): e70989.
71. Rathi R, Honaramooz A, Zeng W, Turner R, Dobrinski I. Germ cell development in equine testis tissue xenografted into mice. Reproduction. 2006; 131(6): 1091-1098.
72. Chan F, Allison JE, Stanley AJ, Gumbreck LG. Reciprocal transplantation of testes between normal and pseudohermaphroditic male rats. Fertil Steril. 1969; 20(3): 482-494.
73. Van Saen D, Goossens E, De Block G, Tournaye H. Regeneration of spermatogenesis by grafting testicular tissue or injecting testicular cells into the testes of sterile mice: a comparative study. Fertil Steril. 2009; 91 Suppl 5: 2264-2272.
74. Jahnukainen K, Ehmcke J, Nurmio M, Schlatt S. Autologous ectopic grafting of cryopreserved testicular tissue preserves the fertility of prepubescent monkeys that receive sterilizing cytotoxic therapy. Cancer Res. 2012; 72(20): 5174-5178.
75. Turner CD. Intra-ocular homotransplantation of prepuberal testes in the rat. Am J Anat. 1938; 63(1): 101-159.
76. Luetjens CM, Stukenborg JB, Nieschlag E, Simoni M, Wistuba J. Complete spermatogenesis in orthotopic but not in ectopic transplants of autologously grafted marmoset testicular tissue. Endocrinology. 2008; 149(4): 1736-1747.
77. Benvenutti L, Salvador RA, Til D, Senn AP, Tames DR, Amaral NLL, et al. Wistar rats immature testicular tissue vitrification and heterotopic grafting. JBRA Assist Reprod. 2018; 22(3): 167-173.
78. Bhattarai N, Gunn J, Zhang M. Chitosan-based hydrogels for controlled, localized drug delivery. Adv Drug Deliv Rev. 2010; 62(1): 83-99.
79. Hoffman AS. Hydrogels for biomedical applications. Ann N Y Acad Sci. 2001; 944(1): 62-73.
80. Wang XY, Xing X, Zhou GD, Liu W, Cao YL. Androgen-secreting tissue construction with co-cultured rat testes somatic cells by tissue engineering technique. Zhonghua Yi Xue Za Zhi. 2007; 87(31): 2223-2227.
81. Gassei K, Schlatt S, Ehmcke J. De novo morphogenesis of seminiferous tubules from dissociated immature rat testicular cells in xenografts. J Androl. 2006; 27(4): 611-618.
82. Lee KH, Lee WY, Kim DH, Lee SH, Do JT, Park C, et al. Vitrified canine testicular cells allow the formation of spermatogonial stem cells and seminiferous tubules following their xenotransplantation into nude mice. Sci Rep. 2016; 6: 21919.
83. Dores C, Dobrinski I. De novo morphogenesis of testis tissue: an improved bioassay to investigate the role of VEGF165 during testis formation. Reproduction. 2014; 148(1): 109-117.
84. Dhandayuthapani B, Yoshida Y, Maekawa T, Kumar S. Polymeric scaffolds in tissue engineering application: a review. Int J Polym Sci. 2011; 2011: 1-19.
85. Poels J, Abou-Ghannam G, Decamps A, Leyman M, des Rieux A, Wyns C. Transplantation of testicular tissue in alginate hydrogel loaded with VEGF nanoparticles improves spermatogonial recovery. J Control Release. 2016; 234: 79-89.
86. Del Vento F, Poels J, Vermeulen M, Ucakar B, Giudice MG, Kanbar M, et al. Accelerated and improved vascular maturity after transplantation of testicular tissue in hydrogels supplemented with VEGF-and PDGF-loaded nanoparticles. Int J Mol Sci. 2021; 22(11): 5779.

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An Analysis of pre and post-Processing Semen Parameters at The Time of Intrauterine Insemination; and The Confounding Effects of Total Motile Sperm Counts on Pregnancy Outcome: A Prospective Cohort Study

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Abstract

Background: This study aims to determine whether pre or post-processing semen parameters obtained during intrauterine insemination (IUI) predict pregnancy when controlling for confounding effects.

Materials and Methods: A prospective cohort study of 2231 semen analyses was conducted at McGill University of IVF center. Any couples who underwent IUI with partner sperm, over a 2.5-year period, were included. Controlled ovarian stimulation was done with Clomiphene Citrate, Letrozole, or Gonadotropins. Statistical analysis was performed using t tests, two types of stepwise logistic regression, and stepwise discriminant analysis. A comparison of pre and post-processing semen parameters was undertaken to determine the probability of pregnancy.

Results: There were significant differences between pregnant and non-pregnant women in post-processing concentration (P=0.043), post-processing total motile sperm count (TMSC) (P=0.049), and post-linearity (P=0.012). However, when variable out-of-the-equation logistic regression or discriminant analysis, which controls for confounding effects between variables, were used, the findings were no longer significant. It was statistically proven that when a variable in the equation logistic regression was employed, post-processing concentration (P=0.005) and post-processing TMSC (P=0.009) remained reliable predictors of pregnancy.

Conclusion: Two of three prediction models suggested that TMSC's relationship with pregnancy is due to confounding factors. One model maintained the validity of the TMSC. While TMSC has always been studied as an important predictor of insemination pregnancies, this finding may be due to confounding effects between semen parameters and therefore requires further investigation as to this relationship.

Keywords: Artificial Insemination, Pregnancy, Semen Analysis

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Introduction

The Centers for Disease Control and Prevention (CDC) defines infertility as the inability to conceive after a year of unprotected intercourse (1). Couples who present for an infertility evaluation complete a semen analysis as part of their initial evaluation (2, 3). Of all factors, the most important is felt to be the total motile sperm count (TMSC) when it comes to predicting pregnancy (3, 4). However, the value of TMSC has become controversial (5) with other studies not finding a relationship with intrauterine insemination (IUI) outcomes. Since semen processing can alter the specimen, the post-processing parameters are hypothesized to be more prognostic than the pre-processing values in predicting the likelihood of pregnancy (4, 6). Most studies have found TMSC to predict pregnancy outcomes (7, 8). Furthermore, a systematic review published in 2014 by Ombelet et al. (8) analyzed the literature published and established that the TMSC was a tool with substantial discriminatory ability. In a longitudinal cohort study conducted in three Dutch hospital sites, it was found that TMSC was a better correlator of spontaneous pregnancy than the 2010 World Health Organization's (WHO) classifications. Furthermore, the article's data

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suggests that TMSC should be used as an indicator when defining the severity of male infertility as it is a more exact parameter (3). Similarly, a 2016 study found that TMSC was more predictive than the WHO 2010 cut-off values for pregnancy outcomes in couples undergoing inta-cytoplasmic sperm injection (ICSI) (9). A 2021 study found that in mild male factor infertility, TMSC is related to pregnancy outcomes (10).

However, a retrospective analysis from China found that a decrease in TMSC did not affect pregnancy outcomes at IUI (11). Other recent studies have also failed to confirm this relationship (5). Why some studies find a value in the TMSC and others do not about pregnancy parameters is unknown. Few if any studies have evaluated the confounding effects of other semen analysis parameters on the TMSC, which may be an explanation for the conflicting results in the literature as related to TMSC and pregnancy outcomes. This study was conducted to further analysis the effect of all analyzed parameters on pregnancy results, and whether pre or post-processing semen analysis results are in fact more predictive of pregnancy.

Materials and Methods

Study design

In this a prospective cohort study, all the pre and post-processing semen analysis results were performed before the insemination at the institution over 2.5 years and were prospectively enrolled in this database to be studied. This amounted to 2231 semen analyses at the time of IUI from 2227 patients. Fresh partners' semen was included in the analysis. Donor IUI semen results were excluded. No patients during this period opted out of the analysis.

Participants

Infertility was defined as a minimum of 1 year of unprotected intercourse without achieving pregnancy per the Centers for Disease Control (1). The duration of infertility amongst the couples ranged from one to seven years. All female partners required unilateral fallopian tube patency, which was tested by either hysterosalpingography or laparoscopy with chromopertubation. No subjects had untreated intra-cavitary lesions including polyps or fibroids, hydrosalpinges in-situ, thyroid, or prolactin abnormalities. The couples' indications for IUI included: male factor subfertility, ejaculation dysfunction, endometriosis, ovulatory dysfunction, and unexplained infertility. The women in the study range between 21- 42 years old. The fertility workup among the couples included the following for women: complete medical history, physical exam, complete blood count, thyroid function test, serum hormone levels on day 2-5 of their spontaneous or progesterone-provoked menstrual cycle (estradiol, total testosterone, prolactin and Follicle Stimulating Hormone), a transvaginal ultrasound, and hysterosalpingography on cycle day 6 to 11 or a laparoscopic demonstrating tubal patency. The male fertility workup included: a complete medical history, a physical exam, serologies, and a semen analysis.

Subjects with less than 5 million total motile sperm count or teratozoospermia (<4 % normal strict morphology), long histories of infertility greater than 3 years duration, stage 3 or 4 endometriosis, or histories suggestive of extensive pelvic adhesions were recommended to go to *in vitro* fertilization (IVF) and avoid insemination however, this was not mandatory. Table 1 outlines the participants' inclusion and exclusion criteria.

Table 1: Inclusion and exclusion criteria

Inclusion criteria	Exclusion criteria
One year of infertility	Untreated intra-cavitary lesions
Unilateral/bilateral fallopian tube patency	Uterine polyps or fibroids
IUI indications: male factor subfertility, ejaculation dysfunction, endometriosis, ovulatory dysfunction, and unexplained infertility	Thyroid or prolactin abnormalities
Women between 21-42 years old	

Procedure

The semen analysis was performed in conjunction with the WHO laboratory manual for the examination and processing of human semen - 5th ed (12).

The IUI procedure required a fresh semen collection, in which individuals were asked to refrain from ejaculation for two days before collection of the specimen, but not more than four days. Specimens were produced by masturbation in a collection room next to our laboratory or at the patient's homes. If semen collection was performed in the patients' home it needed to be delivered to the clinic no more than 30 minutes later, to maintain fresh and viable semen.

The semen analysis was performed in the following manner. Ejaculated sperm were permitted to liquefy before initial analysis. Liquefied semen was mixed before being placed on a standard count slide (Leja Products BV, Nieuw-Vennep, the Netherlands). The loaded slide was placed on a 37°C stage of an integrated visual optical system (IVOS) computer-assisted semen analyzer (Hamilton Thorn Biosciences, Beverly, MA) for every analysis. A minimum of three random fields were checked for each analysis. After density gradient separation of Pure Sperm (Nidacon, Molndol, Sweden), specimens were washed and concentrated to approximately 0.5 ml, and an aliquot of the concentrate was analyzed by computer-aided sperm analysis (CASA) ("post"-assessment). The results of the CASA were validated by manually examining a slide of sperm under high-power field microscopy by an andrologist. Intra and inter-assay coefficients of variation were below 10%.

For any questions related to the measurement or meaning of the parameters by the Hamilton Thorn CASA system, we refer you to https://www.hamiltonthorne.com/index.php/71-documentation/manuals.

Semen processing was performed in the following manner. Following liquefaction and semen analysis ("pre"), a maximum of 4 ml of raw semen is placed on a differential density gradient column consisting of 1 ml, 40%, and 1 ml, 80% Pure Sperm (Nidacon, Molndol, Sweden). The column was centrifuged for 20 minutes at 350 × g. Following centrifugation, the 40% layer and the seminal plasma fractions were removed from the test tube, and the 80% layer was left. About 6-8 ml of sperm washing medium and 5% human serum albumin (HAS, Cooper Surgical, USA) were mixed with the 80% layer and centrifuged for 10 more minutes at 550 × g. After centrifugation, the sperm pellet was recalibrated to contain about 0.5 ml, and a portion was analyzed for the "post" assessment.

Strict morphology was not included because to perform this analysis part or all the specimen needs to be killed and stained and since this specimen was being used to perform the insemination, strict morphology was not analyzed.

Specimens with levels of leukocytes indicative of an acute infection were not inseminated. Couples in this case were informed to undergo testing for the cause of this infection.

A positive pregnancy test (A pregnancy), and not a clinical pregnancy or a live birth, was selected as the outcome measured as "success". A positive pregnancy test is described as an increased level of β-human chorionic gonadotropin (β-hCG), which is released during the early weeks of pregnancy. In contrast with a clinical pregnancy, which is confirming the pregnancy visually- by ultrasound. A positive pregnancy test was felt to reflect the sperm's capacity to fertilize the oocyte. The presence of an ultrasound confirmed clinical pregnancy would have been modulated by aneuploidy and other genetic abnormalities, as well as endometrial factors, which are sperm independent. Had we selected live birth as the outcome measured, it would have been further modulated by the maternal environment and pregnancy complications. As such neither live birth nor clinical pregnancy was selected as the outcome of interest.

The goal was to understand the role of semen parameters on pregnancy outcomes, irrespective of other female or male factors, and help the physician guide the patients in terms of pregnancy outcomes at the time of insemination. Although factors such as male age, female age, and ovarian reserve parameters may play a role in pregnancy outcomes at insemination, we did not attempt to analyze the impact of these variables. When the couple undergoing IUI, asks the physician what the likelihood of pregnancy is, the physician does not consider any factors at that time beyond the quality of the sperm. As such, this study will help physicians counsel patients, and educate them on pregnancy outcomes based on our findings.

The IUI procedure was conducted 24 hours after a urinary lutenizing hormone (LH) surge, or 36 hours after β-hCG injection (10,000 IU, Merck and Co, USA, or Ferring Pharmaceuticals, USA or Ovidrel 250 mcg, Merck-Serono, USA). β-hCG was administered when the transvaginal ultrasound measured the largest follicle diameter to be ≥ 18 mm. Next, insemination was conducted in a sterile manner. A flexible plastic catheter was inserted into the female, while she lay in the dorsal lithotomy position. Post-insemination, the patient lay down for about ten minutes, to allow gravity to help the sperm move upwards through the uterus. Serum β-hCG levels were drawn from the patient around 16 days post-IUI, in order to establish pregnancy status and a baseline β-hCG level. A positive pregnancy was defined as β-hCG higher than 10 mIU/ml.

Statistical analysis

All statistical analyses were performed using the statistical package for Social Sciences 23.0 (SPSS Inc., Chicago, IL). Continuous variables were assessed for normal distribution using the Kolmogorov-Smirnov test. Any non-parametric distributions were logarithmically transformed to obtain a normal distribution for analysis (Table 2). Results are reported as mean value ± standard deviation (SD). Discriminators (statistical determinates as measured by relevant variables) of pregnant versus not pregnant among the pre and post-processing semen analysis parameters were assessed using Student's t test, the two types of logistic regression analysis and stepwise discriminant analysis. Both stepwise logistic regression and stepwise discriminant analysis were used since these are different techniques that could verify the results of the other analysis. Two types of stepwise logistic regression were employed, the variable in the equation method and the variable out of the equation method. The confounding effects controlled for were all parameters listed in Table 2. Approval from Stanford University's committee for the protection of human research subjects was obtained for the collection and analysis of this study's data. It should be noted that the variable out-of-the-equation method of logistic regression does not generate an odds ratio or a confidence interval, and only a P value is provided.

A power analysis was performed to determine whether adequate study size was present. The values to calculate were the means and standard deviation obtained for the post-processing TMSC. MU1 33, MU2 29, sigma was 2, with a 5% alpha and 80 percent beta, and the number of IUI needed for significance was 40. Therefore 2231 IUI was an adequate enrollment. There is no technique for power analysis for stepwise logistic regression or discriminant analysis.

Table 2: Comparison of pre and post-processing semen analysis parameters in pregnant and none pregnant patients by Student's test

Parameters	Pregnant	Not pregnant	P value
Initial volume (ml)	2.9 ± 1.5	3.0 ± 1.6	0.492
Initial concentration (M/ml)[a]	56 ± 43	52 ± 42	0.086
Initial percent motile (%)	49 ± 21	48 ± 22	0.319
Initial concentration motile (M/ml)[a]	32 ± 35 (not transformed values)	31 ±42 (not transformed values)	0.070 (from Log transformation)
Initial total motile sperm count (M)[a]	89 ± 107	84 ± 101	0.349
Initial progression (U/seconds)	44 ± 9	45 ± 10	0.171
Initial path speed (U/seconds)	76 ± 19	78 ± 35	0.272
Initial linearity (0-100)	58 ± 9	58 ± 9	0.460
Initial lateral head displacement (U)	3.4 ± 2.8	3.4 ± 2.5	0.920
Initial velocity average path (U/seconds)	52 ± 11	53 ± 12	0.240
Post volume (ml)	0.51 ± 0.08	0.52 ± 0.16	0.684
Post concentration (M/ml)[a]	75 ± 81	67 ± 73	0.043[a]
Post percent motile (%)	72 ± 25	72 ± 24	0.721
Post concentration motile (M/ml)[a]	64 ± 78	58 ± 70	0.089
Post total motile sperm count (M)[a]	33 ± 45	29 ± 37	0.049[a]
Post progression (U/seconds)	63 ± 15	63 ± 16	0.453
Post path speed (U/seconds)	113 ± 29	113 ± 30	0.908
Post linearity (0-100)	58 ± 28	57 ± 8	0.012[a]
Post lateral head displacement (U)	5.0 ± 5.7	4.8 ± 3.3	0.349
Post velocity average path (U/seconds)	73 ± 19	73 ± 19	0.971

Data are presented as mean ± SD. M; Million and [a]; <0.05 statistically significant when using t tests which do not control for confounding effects of the other variables analyzed, post-processing concentration (P=0.043), post-processing total motile sperm count (P=0.049), and post linearity (P=0.012) all are significant discriminators between the pregnant and not pregnant group. While preprocessing total motile sperm count and sperm concentration, among the other factors failed to be related to pregnancy outcome.

Ethical considerations

The Stanford University Committee for the Protection of human research subjects' approval has been obtained for the collection and analysis of this data (IRB 284365). Patients' written consent was obtained.

Results

All continuous variables were normally distributed except for the initial concentration of motile sperm which was logarithmically transformed for all statistical analysis.

Twenty-two percent of IUI's achieved a pregnancy. A comparison using the student's t test of the semen parameters in the group that conceived and the group that did not can be seen in Table 2. It can be noted, that when using the t test which does not control for confounding effects of the other variables analyzed, post-processing concentration, post-processing total motile sperm count, and post linearity all are significant discriminators between the pregnant and not pregnant group.

Stepwise discriminant analysis was performed since it is a technique to detect differences that predict inclusion in one of two groups while controlling for confounding effects (Table 3). The minimum F to enter the computation was 3.84 which is the minimum value to result in a statistically significant result. None of the variables reached the minimum F which could result in a statistically significant comparison.

Table 3: Evaluation of the ability of pre and post-processing semen analysis parameters to predict pregnancy by stepwise discriminant analysis

Parameter	F to enter
Initial volume (ml)	0.16
Initial concentration (M/ml)[a]	2.75
Initial percent motile (%)	0.74
Initial concentration motile (M/ml)[a]	0.31
Initial total motile sperm count (M)[a]	1.22
Initial progression (U/seconds)	1.91
Initial path speed (U/seconds)	1.22
Initial linearity (0-100)	0.67
Initial lateral head displacement (U)	0.45
Initial velocity average path (U/seconds)	1.39
Post volume (ml)	0.17
Post concentration (M/ml)[a]	3.11
Post percent motile (%)	0.001
Post concentration motile (M/ml)[a]	2.34
Post total motile sperm count (M)[a]	3.35
Post progression (U/seconds)	0.56
Post path speed (U/seconds)	0.50
Post linearity (0-100)	1.86
Post lateral head displacement (U)	0.57
Post velocity average path (U/seconds)	0.001

For the Stepwise discriminant analysis, the minimum F to enter the computation was 3.84 which is the minimum value to result in a statistically significant result. As can be noted above, none of the variables reached the minimum F which could result in a statistically significant comparison. In other words, none of the sperm parameters predicted a pregnancy using discriminant analysis. M; Million and [a]; <0.05 statistically significant.

Table 4: Evaluation of the ability of pre and post-processing semen analysis parameters to predict pregnancy by stepwise logistic regression analysis

Variable out of the equation						Variable in the equation							
Variable	P value	Variable	P value	Variable	P value	OR	95% CI		Variable	P value	OR	95% CI	
							Lower	Upper				Lower	Upper
Initial volume (ml)	0.692	Post volume (ml)	0.680	Initial volume (ml)	0.991	1.001	0.906	1.086	Post volume (ml)	0.063	0.055	0.003	1.132
Initial concentration (M/ml)[a]	0.097	Post concentration (M/ml)[a]	0.080	Initial concentration (M/ml)[a]	0.008	1.014	1.004	1.024	Post concentration (M/ml)[a]	0.065	1.01	0.999	1.021
Initial percent motile (%)	0.388	Post percent motile (%)	0.972	Initial percent motile (%)	0.430	1.009	1.000	1.018	Post percent motile (%)	0.839	1.001	0.994	1.008
Initial concentration motile (M/ml)[a]	0.154	Post concentration motile (M/ml)[a]	0.129	Initial concentration motile (M/ml)a	0.008	0.981	0.967	0.995	Post concentration motile (M/ml)[a]	0.005	0.975	0.958	0.992
Initial total motile sperm count (M)[a]	0.267	Post total motile sperm count (M)[a]	0.069	Initial total motile sperm count (M)[a]	0.653	1.001	0.998	1.003	Post total motile sperm count (M)[a]	0.009	1.031	1.008	1.055
Initial progression (U/seconds)	0.180	Post progression (U/seconds)	0.460	Initial progression (U/seconds)	0.541	0.988	0.950	1.027	Post progression (u/sec)	0.190	1.016	0.992	1.039
Initial path Speed (U/seconds)	0.270	Post path speed (U/seconds)	0.823	Initial path speed (U/seconds)	0.212	0.99	0.974	1.006	Post path speed (U/seconds)	0.688	1.003	0.989	1.017

Using the variables out of the equation method, none of the variables are significant predictors of pregnancy. However, when the variable in the equation method was used several discriminators of pregnancy did occur. These included initial concentration and initial concentration motile, post-processing concentration motile, and post processing total motile sperm count. Pre-processing total motile sperm count was not a predictor of pregnancy in the variable in or out of the equation models. M; Million, OR; Odds ration, CI; Confidence interval, [a]; <0.05 statistically significant.

Next, stepwise logistic regression analysis was performed (Table 4). Using variables out of the equation method, none of the variables are significant predictors of pregnancy. However, when the variable in the equation method was used several discriminators of pregnancy did occur. However, pre-processing TMSC was not one of these discriminators. Post-processing TMSC remained a significant predictor of pregnancy with this modality (P=0.009). To further evaluate whether TMSC was not a significant predictor of results because it depends on sperm volume, concentration, and motility, the analysis was repeated without these three parameters. However, pre-processing TMSC failed to reach significance in this analysis P=0.653, odds ratio (OR)=1.001, confidence interval (CI)=0.999 to 1.002

Discussion

Many studies have found the pre-processing and post-processing TMSC to be a significant predictor of pregnancy, while other studies have not detected a difference (3-8, 11). The results of these studies have found that a TMSC between 1 million and 20 million is a cut-off for pregnancy after IUI (3, 4, 6-8). This discrepancy in TMSC cut-off required investigation. The results of this study suggest that any difference detected may be the result of confounding effects between sperm parameters. With this study only 1 out of 3 methods used to predict pregnancy found TMSC to be a significant predictor of pregnancy, and this was only considering post-processing analyses. If a statistical test such as the student's t test is performed significant differences are found, which may become insignificant when controlling for confounding effects between semen analysis results. It could be hypothesized that based on the variable in the equation method of stepwise logistic regression some variables do predict pregnancy outcomes. In that case, only post-processing semen analysis results should be considered significant. It should be noted that the post-processing TMSC with the variable out of the equation method of stepwise logistic regression analysis trended towards being a significant predictor of pregnancy. It may remain a significant predictor of pregnancy in a larger study, although with 2231 IUIs this study is robust. (This can be confirmed by the small CI generated with the logistic regression analysis). Hamilton et al. (3) argued that a semen analysis is only valid if it is correlated with pregnancy and not with other factors, which supports our use of pregnancy as the predictor. It should be considered, that since certainly pre-processing TMSC and likely post-processing TMSC only can predict pregnancy due to confounding effects, we have likely found the explanation of why the predictive cut-off for TMSC varies so substantially in different studies and why certain studies have failed to detect TMSC as a predictor of pregnancy outcomes at IUI (3-11).

When running a logistic regression (which predicts the correlation of the sperm parameters being studied to pregnancy outcome) the variable in the method established no correlations, and as such no significant results. The variable out method established a significant correlation between post-processing TMSC and pregnancy outcome. A possible explanation for the different outcomes of the two logistic regression analyses can be explained by the variable selection problem. The variable selection

problem explains that when computation of the linear regressions occurs, the computer determines which variable to add into the equation (variable in method), or which variable to take out (variable out method) to compute the smallest P value possible (13). Therefore, the variable in the method begins with no variables and adds a variable one by one, computing a P value for each variable when added into the equation. On the other hand, different results were calculated by beginning with all the variables and removing one variable at a time, again computing a P value for each variable. These variables received different P values in each method since the variable was added or removed, by the program computing the logarithmic regression. Therefore, the same variable generated different p values based on when it was added or removed from the equation. It should be noted that either the variable out of the equation or the variable in the equation method are acceptable tests to generate prediction models. It is up to the researcher a priori to determine which will be used.

The strengths of this study include the prospective nature and inclusion of a moderately robust patient population. The weaknesses of this study include that: semen analyses are well known for variations between tests, and the use of the CASA also has important inherent limitations- such as the inability to obtain accurate counts and percent motilities when the concentration of specimens is very high or quite low, or when a specimen is contaminated with debris. Another weakness is that this study does not contain data on 100,000 or more insemination cycles. However, if that had been done, we may have been able to generate statistical significance with all variables, which would not have represented a true clinical significance, a risk with ultra-large data.

Conclusion

This study suggests that the value of TMSC in predicting pregnancy may be due to confounding effects. This would imply that as other parameters change in the semen analysis, the total motile sperm count may lose its significance and legitimacy as a predictor of pregnancy. This finding of confounding effects may explain the diverse cut-off values of TMSC as a predictor of pregnancy in the medical literature ranging from 1 million to 20 million depending on the study reviewed or the failure to detect TMSC as a predictor of pregnancy in other studies. If any parameters do predict pregnancy, they are the post-processing results. In conclusion, at the time of insemination, based on the semen parameters, it is unlikely that a physician can adequately counsel a couple on the likelihood of success.

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Authors' Contributions

M.H.D.; Was involved in the data collection. M.H.D., T.F.I.; Were involved in the planning of the analysis, the analysis of the data and wrote the initial manuscript. M.H.D., T.F.I., S.N., S.-L.T.; Were involved in the interpretation of the data. S.-L.T., S.N.; Were involved in the editing of the manuscript. All authors approved of the final version of this manuscript.

References

1. Centers for Disease Control and Prevention. Infertility FAQs. 2023. Available from: https://www.cdc.gov/reproductivehealth/infertility/index.htm#:~:text=In%20general%2C%20infertility%20is%20defined,or%20longer)%20of%20unprotected%20sex (1 May 2023).
2. Salmon-Divon M, Shrem G, Balayla J, Nehushtan T, Volodarsky-Perel A, Steiner N, et al. An age-based sperm nomogram: the McGill reference guide. Hum Reprod. 2020; 35(10): 2213-2225
3. Hamilton JA, Cissen M, Brandes M, Smeenk JM, de Bruin JP, Kremer JA, et al. Total motile sperm count: a better indicator for the severity of male factor infertility than the WHO sperm classification system. Hum Reprod. 2015; 30(5): 1110-1121.
4. Madbouly K, Isa A, Habous M, Almannie R, Abu-Rafea B, Binsaleh S. Postwash total motile sperm count: should it be included as a standard male infertility work up. Can J Urol. 2017; 24(3): 8847-8852.
5. Lin H, Li Y, Ou S, Jiao X, Wang W, Haahr T, et al. The relationship of total progressive motile sperm count with the outcome of IUI? An analysis of 5171 cycles. Gynecol Endocrinol. 2022; 38(11): 954-959.
6. Hansen KR, Peck JD, Coward RM, Wild RA, Trussell JC, Krawetz SA, et al. Intrauterine insemination performance characteristics and post-processing total motile sperm count in relation to live birth for couples with unexplained infertility in a randomised, multicentre clinical trial. Hum Reprod. 2020; 35(6): 1296-1305.
7. Mathes M, Kastrick E, Sayles H, Gustin S. How low is too low? Postwash total motile sperm count effect on pregnancy outcomes in intrauterine insemination. Hum Fertil (Camb). 2022: 1-6.
8. Ombelet W, Dhont N, Thijssen A, Bosmans E, Kruger T. Semen quality and prediction of IUI success in male subfertility: a systematic review. Reprod Biomed Online. 2014; 28(3): 300-309.
9. Borges E Jr, Setti AS, Braga DP, Figueira RC, Iaconelli A Jr. Total motile sperm count has a superior predictive value over the WHO 2010 cut-off values for the outcomes of intracytoplasmic sperm injection cycles. Andrology. 2016; 4(5): 880-886.
10. Lin H, Li Y, Ou S, Jiao X, Wang W, Humaidan P, et al. Role of the total progressive motile sperm count (TPMSC) in different infertility factors in IUI: a retrospective cohort study. BMJ Open. 2021; 11(2): e040563.
11. Luo Y, Liu M, Wu S, Zhang M, Yuan J, Zhong Y, et al. A comprehensive evaluation of pre- and post-processing sperm parameters for predicting successful pregnancy rate following intrauterine insemination with the husband's sperms. BMC Pregnancy Childbirth. 2022; 22(1): 703.
12. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. Geneva: WHO Press. Available from: https://www.who.int/docs/default-source/reproductive-health/srhr-documents/infertility/examination-and-processing-of-human-semen-5ed-eng.pdf (23 Aug 2022).
13. George EI. The variable selection problem. J Am Stat Assoc. 2000; 95(452): 1304-1308.

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Assessment of Expression Levels and Localization Patterns of Phospholipase C zeta in Different Grades of HOST in Human Sperm

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Abstract

Background: Phospholipase C zeta (PLC-ζ) deficiency in sperm can underlie oocyte activation failure after intracytoplasmic sperm injection (ICSI). The aim of this study was to determine PLC-ζ expression and location in individual spermatozoa in each host score so that a hypo-osmotic swelling test (HOST) may be used to help routine sperm selection for ICSI.

Materials and Methods: In this experimental study, fresh semen samples were randomly obtained from 30 men who were referred to the Andrology Unit of the Infertility Center. Samples were processed by density gradient centrifugation (DGC) and exposed to hypotonic conditions. Seven different tail patterns, classified from 'a' to 'g' can be detected according to World Health Organization (WHO) criteria. Then, the PLC-ζ protein localization pattern was assessed by quantitative Immunofluorescence in individual sperm Host grades. Moreover, the sperm content of PLC-ζ protein was evaluated by flow cytometry correlated with semen analysis parameters.

Results: In the present study, quantitive immunofluorescence analysis indicated that sperm from different host grades exhibited seven localization patterns of PLC-ζ of acrosomal (A); equatorial (EQ), and postacrosomal (PA) patterns. A+EQ=acrosomal and equatorial, A+PA=acrosomal and post-acrosomal, EQ+PA=equatorial and post-crosomal, and A+EQ+PA.

The sperm from HOST grade 'd' exhibited significantly higher PLC-ζ (A+PA) and (A+EQ+PA) staining compared to sperm from other grades (P=0.006). The sperm from grade 'd' exhibited higher PLC-ζ (EQ+PA) compared with other grades (P=0.001). However, grade 'd' was not significantly different from 'c' (P=0.087). Analysis of the combined results confirmed that there was a clear reduction in PLC-ζ immunofluorescence in Host grades 'a', 'f' and 'g' sperms.

Conclusion: Our data suggest that HOST may represent a useful diagnostic tool for the selection of sperms exhibiting a higher level of PLC-ζ expression.

Keywords: Infertility, Intracytoplasmic Sperm Injection, Phospholipase C Zeta, Sperm-Ovum Interactions

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Introduction

Fertilization failure during assisted reproductive technology (ART) occurs in about (1-3%) of couples with a history of infertility (1, 2). Oocyte activation deficiency may be the most frequent cause of fertilization failure (3, 4). The sperm-specific phospholipase C zeta (PLC-ζ) has been identified as one of the possible factors involved in oocyte activation (5). Some studies have related the reduction of protein expression levels and aberrant forms of PLC-ζ to some male infertility such as globospermia and varicocele (6, 7).

Previous research has shown that the expression and localization of PLC-ζ coincide with late spermatogenic events such as histone-protamine remodelling involved in the maintenance of sperm chromatin integrity (8). PLC-ζ with a molecular mass of around 70 KDa is delivered into the ooplasm by sperm, which activates the phosphoinositide pathway by hydrolyzing phosphatidylinositol 4, 5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1, 4, 5-triphosphate (IP3), which then induces Ca^{2+} oscillations by binding to its receptor on the endoplasmic reticulum. The activation of the oocyte is caused by Ca^{2+} oscillations. Oocyte stimulation causes cortical granule exocytosis, which prevents polyspermy, the release of oocyte meiotic arrest, and the creation of the female pronucleus (9).

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Same to sperm quality which differs in different men and even in the ejaculates of the same individuals, the basic pattern of PLC-ζ localization has been characterized as varying significantly in sperm from normal fertile males, and it may also vary between ejaculates from the same person (10, 11). This variability may explain the main differences in sperm to activate the oocyte between individuals or within an ejaculate (12). In recent years, seven localization patterns of PLC-ζ were identified in human sperm: acrosomal (A); equatorial (EQ); post acrosomal (PA); A+ PA; A+EQ; EQ+ PA; and (A+ EQ+ PA) (13, 14). Equatorial and post-acrosomal PLC-ζ localization is the most prominent in human sperm. After gamete fusion, equatorial and post-acrosomal regions would permit the rapid dispersion of PLC-ζ into the oocyte (14).

The PLC-ζ deficit in sperm with normal morphology is well-established to cause Oocyte activation failure following ICSI (15, 16). Finding a means to choose the appropriate sperm for ICSI bypasses the natural barrier is critical. According to some data, the hypo-osmotic swelling test (HOST) has the potential to be used as a non-destructive sperm viability test for ICSI sperm selection (17, 18). Upon exposure of spermatozoa to hypo-osmotic conditions, seven forms of sperm tail have been identified that are referred to as 'a' to g' (19).

Previous research has shown that the HOST value can indicate the degree of sperm DNA damage in an ejaculate and that tail swelling can predict the possibility of DNA damage in individual spermatozoa. Therefore, these authors conclude HOST grade 'd' and grade 'c' may be healthier spermatozoa (17, 20). Some sperm selection methods are introduced in studies (21). The clinical value of HOST is supported by other authors but there is less information on the status of PLC-ζ expression in sperm of different HOST scores. This study aimed to determine PLC-ζ expression and location in individual spermatozoa in each host score so that HOST may be used to help with routine sperm selection for ICSI.

Materials and Methods

Ethical Issue

This experimental study was approved by the Research Ethics Committee of Islamic Azad University, Flavarjan, Iran, and (IR.IAU.FAlA.REC.1401.017). All participants signed written informed consent. In this study, fresh semen samples were randomly obtained from 30 men referred to the Andrology Unit of the Infertility Center.

Patients and standard semen analyses

Fresh sperm samples were randomly collected from 30 males aged 24-45 who visited the Shahid Beheshti Hospital Fertility and Infertility Center's Andrology. Male recruitment criteria required a minimum sperm count of 5×10^6 spermatozoa/mL. Semen samples were collected in sterile containers by masturbating after 3-4 days of sexual abstinence. After the liquefaction of the sperm, the World Health Organization (19) guidelines for sperm analysis were used. Computer-assisted semen analysis (CASA) was used to determine sperm count and motility (Test Sperm 2.1; Video test, St. Petersburg, Russia). The Eosin-staining technique was used to test the viability of the sperm. Diff-Quik staining (Idehvarzan, Tehran, Iran) was used to analyze sperm morphology, and one hundred sperms were scored on each slide and graded in duplicate using Kruger's rigorous criteria according to the WHO 2010 guideline.

Sperm preparation and HOST procedure

A density gradient was used to process sperm samples. Two ml of liquefied sperm was stacked on a two-step discontinuous Pure Sperm concentration gradient 40:80 percent (Nidacon International AB), centrifuged at 300 g for 20 minutes at room temperature, then collected and washed twice.

As previously mentioned, each patient's HOST was prepared and performed on semen samples. First, 100 µl of washed sperm was mixed with 1 ml of warmed 150+5 m Osm hypo-osmotic swelling solution (Ham's medium diluted with an equal volume of sterile purified H_2O_2 and heated to 37°C for 5 minutes). The percentage of HOST-positive samples and their grades were then calculated using WHO guidelines (19).

Immunofluorescence

PLC-ζ rabbit polyclonal antibody (LS-C144827) was bought from Life Span BioSciences for this work (USA). Azad et al. (22) used immunoblot to determine the specificity of this antibody. Samples were pelleted by centrifugation at 1500 g for 5 minutes, washed in phosphate buffer saline (PBS), fixed with 4 percent paraformaldehyde in PBS (BDH, Lutterworth, UK), rinsed in PBS, and drawn on slides pre-coated with 0.01 percent poly-L-lysine (Sigma Aldrich, USA). The attached sperm were permeabilized for 30 minutes in PBS (Sigma Aldrich, USA) containing 0.5 percent Triton X-100. After blocking for 1 hour in 3 percent bovine serum albumin (Sigma-Aldrich, USA), the slides were incubated overnight at 4°C with a primary anti-human-PLC antibody diluted in 0.05 percent bovine serum albumin. After that, the samples were washed three times in PBS and incubated for one hour at room temperature with 5 mg/ml of diluted secondary donkey anti-rabbit antibodies conjugated with DYLight-488 (Thermo, USA). Finally, samples were washed three times in PBS and mounted for analysis (Invitrogen's Prolong Gold Antifade Mounting Reagent). A fluorescent microscope was used to study 1000 sperm (Olympus, BX51, Japan).

Statistical analysis

In each example, the data is shown as the mean ± SEM of the number of samples assessed. To compare the distribution of PLC-ζ in HOST grades, a statistical one-way analysis of variance (ANOVA) was used. The

significance level was P≤0.05. Statistical Package for Social Sciences version 22 was used to conduct all data analyses (SPSS Inc., Chicago, IL, USA).

Results

Semen analysis

The mean sperm concentration determined in the semen analysis was 69.56 ± 11.4 million/ml (mean ± SD) ranging from 8 to 233 million/ml means sperm motility was 40.08% ± 11.3. Further, means sperm normal morphology was 7.6 ± 0.39 with a minimum and a maximum of 5 to 12%.

The following are the average percentages for different degrees of sperm tail swelling: 44.2 ± 1.1 (grade a), 17.6 ± 0.35 (grade b), 9.3 ± 0.23 (grade c), 6.05 ± 0.12 (grade d), 5.5 ± 0.16 (grade e), 6.3 ± 023 (grade f) and 10.85 ± 0.25 (grade g).

Proportional analysis of PLC-ζ localization patterns in each HOST grade sperm

The present study reported that individual spermatozoa with PLCζ deficiency and altered localization patterns are identifiable regarding different grades of HOST (Figs.1, 2). As shown in Table 1, no significant difference in (A), and (EQ), localization was detected in sperm from host grades. The sperm from grade 'd' displayed higher PLC-ζ (PA) compared with grade a' (P=0.01) and grade 'f' (P=0.049). The results indicated that the sperm from grade'd' exhibited significantly higher PLC-ζ (A+EQ) staining compared with sperm from grade 'f' (P=0.023) and grade 'g' (P=0.012). Proportional analysis of sperm exhibiting the sperm from grade 'd' exhibited, significantly higher PLC-ζ (PA+EQ) staining compared with sperm from other grades (P=0.001). Grade 'c' was an exception and no significant difference was observed between grade'd' and 'c' (P=0.087). PLC-ζ immunoreactivity indicated that a significantly larger proportion of sperm of 'd' grades exhibited PLC-ζ (A+PA) and (A+EQ+PA) staining, compared with other grades (Table 1).

Analysis of the combined results confirmed that there was a clear reduction in PLC-ζ immunofluorescence in Host grades 'a', 'f ', and 'g' sperms. As shown in Table 1, grade 'f' displayed significantly lower 5 of the 7 localization patterns compared to grade 'd'. Also, grades 'a' and 'g' displayed significantly lower 4 of the 7 localization patterns compared to grade 'd'.

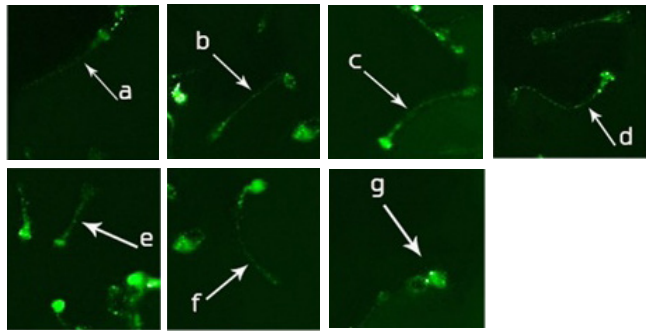

Fig.1: Representative PLC-ζ immunostaining of different PLC-ζ localization patterns obtained by immunofluorescence in Host grade. Grade 'a' sperm are HOST negative whiles grades 'b', 'c', 'd, 'e', 'f', and 'g' are HOST positive (scale bars: 2 µm).

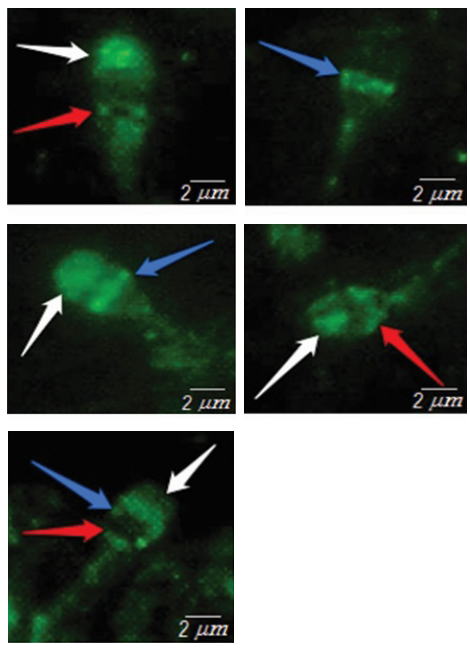

Fig.2: Representative image of phospholipase C zeta (PLC-ζ) immunofluorescence in human sperm. The white arrow indicates acrosomal localization; the blue arrow indicates equatorial localization; and the red arrows indicate postacrosomal localization (scale bars: 2 µm).

Table 1: Comparison of PLC-ζ localization patterns between grade'd' and other HOST grades

HOST grade	A	PA	EQ	A+PA	A+EQ	PA+EQ	A+PA+EQ
'a'	0.46 ± 0.3 P=0.16	0.6 ± 0.23 **P=0.01**	0.96 ± 0.83 P=0.25	3.3 ± 0.5 **P<0.001**	1.2 ± 0.8 P=0.13	3.7 ± 0.9 **P<0.001**	7.4 ± 0.7 **P<0.001**
'b'	0.46 ± 0.3 P=0.16	0.36 ± 0.23 P=0.87	2.17 ± 0.83 **P=0.01**	2.7 ± 0.5 **P<0.001**	1.3 ± 0.79 P=0.08	3 ±.9 **P=0. 001**	6.8 ± 0.7 **P<0.001**
'c'	0.36 ± 0.3 P=0.9	0.32 ± 0.23 P=0.17	0.64 ± 0.83 P=0.4	1.5 ± 0.5 **P=0.006**	0.75 ± 0.79 P=0.34	1.5 ± 0.9 P=0.087	3 ± 0.7 **P<0.001**
'e'	0.46 ± 0.3 P=0.16	0.39 ± 0.23 P=0.09	0.17 ± 0.83 P=0.83	2.6 ± 0.5 **P<0.001**	0.96 ± 0.79 P=0.22	3 ± 0.9 **P=0.001**	4.6 ± 0.7 **P<0.001**
'f'	0.5 ± 0.3 P=0.13	0.46 ± 0.23 **P=0.049**	0.2 ± 0.83 P=0.79	3 ± 0.5 **P<0.001**	1.8 ±.79 **P=0.023**	3.9 ± 0.9 **P<0.001**	6.1 ± 0.7 **P<0.001**
'g'	0.18 ± 0.3 P=0.6.3	0.36 ± 0.23 P=0.13	0.9 ± 0.83 P=0.3	3.5 ± 0.5 **P<0.001**	2 ± 0.79 **P=0.012**	4.3 ± 0.9 **P<0.001**	8 ± 0.7 **P<0.001**

The obtained P value of comparison of PLC-ζ localization patterns between grade 'd' and other HOST grades 'a', 'b', 'c', 'e', 'f', and 'g'. All data are presented as mean ± SE. Statistically significant (P≤0.05) differences are detailed in bold. PLC-ζ localization patterns: A; Acrosomal, E; Equatorial, PA; Post-acrosomal, A+EQ; Acrosomal and equatorial, A+PA; Acrosomal and post-acrosomal, EQ+PA; Equatorial and post-acrosomal, and A+EQ+PA; Acrosomal, equatorial, and post-acrosomal.

Discussion

While sperm staining should be avoided, the WHO has validated the Hypo osmotic Swelling Test as an alternate viability test (23). It has been claimed that the HOST is a supplemental test that is a simple, cost-effective, quick, and non-invasive method to select individual healthy spermatozoa (17, 18).

Some investigators reported that there is a correlation between HOST and other sperm parameters such as motility (24), aneuploidy (25), IVF outcome, and zona-free hamster ovum penetration assay (26). Also, HOST has been used to identify spermatozoa that appear to have minimal DNA damage (17). Recently, it was observed with normal semen parameters, the use of HOST in ICSI-Frozen ET cycles led to increases in the rate of live births in women aged 36-40 (27).

The present study reported that individual spermatozoa with PLCζ deficiency and altered localization patterns are identifiable regarding different grades of HOST. In this sense, all of the specimens investigated in this work were processed by density gradient and then submitted to HOST. It should be noted that the lowest occurrences of spontaneously developed tail swellings (SDTS) were found in DGW sperms (28).

Besides, for the first time, the results of this study showed that sperm HOST grades exhibit various expressions of PLC-ζ protein in the head. In addition, a significant PLC-ζ localization in the midpiece of human sperm was observed. This is in line with some other reports indicating PLC-ζ localization patterns were not restricted to the sperm head, but have also been observed in the sperm tail and midpiece (29, 30).

As previously mentioned PLC-ζ is a prognostic and diagnostic marker for ICSI outcome and repetitive ICSI failure depends on the localization patterns and the amount of PLC-ζ in the sperm head (14). Recent clinical reports showed a relationship between reduced protein expression levels and abnormal forms of PLC-ζ with human male infertility (31). PLC-ζ protein expression was considerably reduced or nonexistent in globozoospermia, which was characterized by low rates of oocyte activation (30). A recent study indicated that oocyte activation and clinical outcomes might not be related to PLC-ζ quantity alone (32).

Aarabi et al point to another sperm protein (PAWP) as a candidate for oocyte activation (33). When PLC-ζ is lacking or non-functional, PAWP is unable to promote the activation of human oocytes (34). Furthermore, in both mouse and human oocytes, injection of the recombinant protein PLC-ζ caused [Ca^{2+}] oscillations (35). Therefore, these studies confirmed the main role of PLC-ζ in the activation of mammalian oocytes.

Lee et al. (16) found that in patients with normal semen parameters that have low fertilization after ICSI, a few sperm expressing PLC-ζ and initiating robust calcium oscillation. Since, Semen samples contain a heterogeneous population of spermatozoa (32, 36) during the ICSI procedure identifying and using sperm that expresses higher-level PLC-ζ may increase the fertilization rate.

Indeed, earlier studies indicated that peripheral localization patterns of PLC-ζ were responsible for acrosome reaction and egg activation, whiles the post-acrosomal localization could modulate some aspect of pronuclear function (12). Higher levels of PLC-ζ (A+PA) were linked to ICSI success by Yelumalai et al. (14). Recent studies suggest successful fertilization was related to higher levels of (A+ EQ) PLC-ζ (29).

The results of the current study investigated seven localization patterns of PLC-ζ in seven Host scores: A=acrosomal, E=equatorial, PA=post-acrosomal, A+EQ=acrosomal and equatorial, A+PA=acrosomal and post-acrosomal, EQ+PA=equatorial and post-acrosomal, and A+EQ+PA. In the present study, grade 'f' displayed significantly lower 5 of the 7 localization patterns compared to grade 'd'. Also, grades 'a' and 'g' displayed significantly lower 4 of the 7 localization patterns compared to grade 'd'. Hence, the selection of sperm based on HOST may prevent insemination of sperm which is poor in PLC-ζ expression and localization.

Several reports showed PLC-ζ deficiency correlation with other defects in sperm. For instance, Kashir et al. study showed that cryopreservation could reduce the level of PLC-ζ expression in spermatozoa. It may be due to a change in membrane function that disturbs PLC-ζ localization and leak the sperm head (37). Park et al. (38) study showed a negative connection between sperm PLC-ζ immunoreactivity and an oxidation marker, 8-hydroxy-2'-deoxyguanosine (8-OHdG), and concluded that reduction of PLC-ζ expression in human sperm could be correlated to oxidative stress. Tavalaee et al. (39) demonstrated that DNA damage could lead to reduced expression of PLC-ζ in human sperm.

As a result, it has been proposed that the reduction of PLC-ζ expression in Host grades 'a', 'f', and 'g' in our study could be a consequence of the other problems in these sperm, such as DNA fragmentation or membrane function. This is in agreement with the Bassiri et al. (17, 20) study, which showed DNA fragmentation is higher in Host grades 'a' and 'g' grade sperms and recommended limiting the used sperm of these HOST grads in ICSI.

One of the limitations of the current study was the lack of evaluation of ICSI outcome for each Host graded sperm.According to the literature, the HOST may be helpful for screening paternal factors connected to repeat embryonic or early fetal loss and it can also be utilized in clinical laboratories (40). Analysis of the combined results confirmed that there was a clear reduction in PLC-ζ immunofluorescence in Host grades 'a', f ', and 'g' grade sperms. For the first time also our study showed that the sperm from grade 'd' exhibited significantly higher PLC-ζ (A+EQ+PA) and (A+PA) staining compared with sperm

from other grades. According to these results the sperm from HOST grade 'd' may be healthier with a greater capacity for inducing oocyte activation.

Conclusion

These findings promote the potential application of HOST as a useful method in selecting the most suitable sperm for ICSI that express PLC-ζ. Future studies could test these conclusions by looking at the particular Ca^{2+} oscillation signatures of each HOST grade sperm.

Acknowledgments

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Authors' Contributions

E.Y.; Contributed to conception, design, data analysis, interpretation, prepared samples, collected data, and manuscript preparation. A.A.; Contributed to manuscript writing and the final review of the manuscript and helped in the statistical analysis. All authors read and approved the final manuscript.

References

1. Lee SH, Lee JH, Park YS, Yang KM, Lim CK. Comparison of clinical outcomes between in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in IVF-ICSI split insemination cycles. Clin Exp Reprod Med. 2017; 44(2): 96-104.
2. Cardona Barberán A, Boel A, Vanden Meerschaut F, Stoop D, Heindryckx B. Diagnosis and treatment of male infertility-related fertilization failure. J Clin Med. 2020; 9(12): 3899.
3. Jones C, Meng X, Coward K. Sperm factors and egg activation: phospholipase C zeta (PLCZ1) and the clinical diagnosis of oocyte activation deficiency. Reproduction. 2022; 164(1): F53-F66.
4. Wang M, Zhu L, Liu C, He H, Wang C, Xing C, et al. A novel assisted oocyte activation method improves fertilization in patients with recurrent fertilization failure. Front Cell Dev Biol. 2021; 9: 672081.
5. Tavalaee M, Nomikos M, Lai FA, Nasr-Esfahani MH. Expression of sperm PLCζ and clinical outcomes of ICSI-AOA in men affected by globozoospermia due to DPY19L2 deletion. Reprod Biomed Online. 2018; 36(3): 348-355.
6. Haghighat S, Tavalaee M, Kouhkan A, Zakeri Z, Noureddini M, Shahverdi AH, et al. Reduction of truncated kit expression in men with abnormal semen parameters, globozoospermia and history of low or fertilization failure. Cell J. 2019; 21(3): 314-321.
7. Janghorban-Laricheh E, Ghazavi-Khorasgani N, Tavalaee M, Zohrabi D, Abbasi H, Nasr-Esfahani MH. An association between sperm PLCζ levels and varicocele? J Assist Reprod Genet. 2016; 33(12): 1649-1655.
8. Tavalaee M, Kiani-Esfahani A, Nasr-Esfahani MH. Relationship between phospholipase C-zeta, semen parameters, and chromatin status. Syst Biol Reprod Med. 2017; 63(4): 259-268.
9. Saleh A, Kashir J, Thanassoulas A, Safieh-Garabedian B, Lai FA, Nomikos M. Essential role of sperm-specific PLC-Zeta in egg activation and male factor infertility: an update. Front Cell Dev Biol. 2020; 8: 28.
10. Kumar N, Singh AK. Semen quality parameters in male partners of infertile couples and their correlation with socio-demographic features in rural tertiary care center of southern india: an observational study. J Infertil Reprod Biol. 2022; 10(1): 1-6.
11. Ferrer-Vaquer A, Barragan M, Freour T, Vernaeve V, Vassena R. PLCζ sequence, protein levels, and distribution in human sperm do not correlate with semen characteristics and fertilization rates after ICSI. J Assist Reprod Genet. 2016; 33(6): 747-756.
12. Grasa P, Coward K, Young C, Parrington J. The pattern of localization of the putative oocyte activation factor, phospholipase Czeta, in uncapacitated, capacitated, and ionophore-treated human spermatozoa. Hum Reprod. 2008; 23(11): 2513-2522.
13. Kashir J, Jones C, Mounce G, Ramadan WM, Lemmon B, Heindryckx B, et al. Variance in total levels of phospholipase C zeta (PLC-ζ) in human sperm may limit the applicability of quantitative immunofluorescent analysis as a diagnostic indicator of oocyte activation capability. Fertil Steril. 2013; 99(1): 107-117. e3.
14. Yelumalai S, Yeste M, Jones C, Amdani SN, Kashir J, Mounce G, et al. Total levels, localization patterns, and proportions of sperm exhibiting phospholipase C zeta are significantly correlated with fertilization rates after intracytoplasmic sperm injection. Fertil Steril. 2015; 104(3): 561-568. e4.
15. Chithiwala ZH, Lee HC, Hill DL, Jellerette-Nolan T, Fissore R, Grow D, et al. Phospholipase C-zeta deficiency as a cause for repetitive oocyte fertilization failure during ovarian stimulation for in vitro fertilization with ICSI: a case report. J Assist Reprod Genet. 2015; 32(9): 1415-1419.
16. Lee HC, Arny M, Grow D, Dumesic D, Fissore RA, Jellerette-Nolan T. Protein phospholipase C Zeta1 expression in patients with failed ICSI but with normal sperm parameters. J Assist Reprod Genet. 2014; 31(6): 749-756.
17. Bassiri F, Tavalaee M, Shiravi AH, Mansouri S, Nasr-Esfahani MH. Is there an association between HOST grades and sperm quality? Hum Reprod. 2012; 27(8): 2277-2284.
18. Stanger JD, Vo L, Yovich JL, Almahbobi G. Hypo-osmotic swelling test identifies individual spermatozoa with minimal DNA fragmentation. Reprod Biomed Online. 2010; 21(4): 474-484.
19. Lu JC, Huang YF, Lü NQ. WHO laboratory manual for the examination and processing of human semen: its applicability to andrology laboratories in China. Zhonghua Nan Ke Xue. 2010; 16(10): 867-871.
20. Bassiri F, Tavalaee M, Nasr Esfahani MH. Correlation between different patterns of hypo-osmotic swelling and sperm functional tests. Int J Fertil Steril. 2013; 7(3): 193-198.
21. Wildy ML, Boyd L, Fourie J, Makoyo O, Huyser C. The development of a simplified swim-up method for sperm processing. J Infertil Reprod Biol. 2021; 9 (4): 160-167.
22. Azad N, Nazarian H, Ghaffari Novin M, Masteri Farahani R, Piryaei A, Heidari MH. Phospholipase C zeta parameters in sperm from polymorphic teratozoospermic men. Ann Anat. 2018; 215: 63-70.
23. Hermann AJE, Sylvère NKG, Guillaume YY, Amadou CF, Marie-Florence N, Alexis BG, et al. The asthenozoospermia as a major sperm abnormality of people received in Institute Pasteur of Cote d'Ivoire for a semen analysis. J Infertil Reprod Biol. 2019; 7(3): 17-20.
24. Penitente-Filho JM, Dias JCO, Oliveira FA, Silveira CO, Torres CAA. Correlation between sperm motility and hypoosmotic swelling test on cryopreserved goat semen. Magistra. 2015; 27(3/4): 468-473.
25. Pang MG, You YA, Park YJ, Oh SA, Kim DS, Kim YJ. Numerical chromosome abnormalities are associated with sperm tail swelling patterns. Fertil Steril. 2010; 94(3): 1012-1020.
26. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJ. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil. 1984; 70(1): 219-228.
27. Ozdemir U, Atayurt Z, Sik A, Ozdamar O, Aba YA, Ozolcay O. Can hypo-osmotic swelling test (host) improve pregnancy outcomes in unexplained iinfertility patients with normal semen parameters undergoing ICSI-frozen embryo transfer cycles? Clin Exp Health Sci. 2021; 11(3): 536-542.
28. Hossain A, Osuamkpe C, Hossain S, Phelps JY. Spontaneously developed tail swellings (SDTS) influence the accuracy of the hypo-osmotic swelling test (HOS-test) in determining membrane integrity and viability of human spermatozoa. J Assist Reprod Genet. 2010; 27(2-3): 83-86.
29. Kashir J, Mistry BV, BuSaleh L, Abu-Dawas R, Nomikos M, Ajlan A, et al. Phospholipase C zeta profiles are indicative of optimal sperm parameters and fertilisation success in patients undergoing fertility treatment. Andrology. 2020; 8(5): 1143-1159.
30. Heytens E, Parrington J, Coward K, Young C, Lambrecht S, Yoon SY, et al. Reduced amounts and abnormal forms of phospholipase C zeta (PLCzeta) in spermatozoa from infertile men. Hum Reprod. 2009; 24(10): 2417-2428.
31. Kashir J, Konstantinidis M, Jones C, Lemmon B, Lee HC, Hamer R, et al. A maternally inherited autosomal point mutation in human

phospholipase C zeta (PLCζ) leads to male infertility. Hum Reprod. 2012; 27(1): 222-231.
32. Aras-Tosun D, Cakar Z, Can A, Ozkavukcu S, Kaplanoglu I, Cinar O. Phospholipase C-zeta levels are not correlated with fertilisation rates in infertile couples. Andrologia. 2022; 54(1): e14269.
33. Aarabi M, Balakier H, Bashar S, Moskovtsev SI, Sutovsky P, Librach CL, et al. Sperm content of postacrosomal WW binding protein is related to fertilization outcomes in patients undergoing assisted reproductive technology. Fertil Steril. 2014; 102(2): 440-447.
34. Escoffier J, Lee HC, Yassine S, Zouari R, Martinez G, Karaouzène T, et al. Homozygous mutation of PLCZ1 leads to defective human oocyte activation and infertility that is not rescued by the WW-binding protein PAWP. Hum Mol Genet. 2016; 25(5): 878-891.
35. Cox LJ, Larman MG, Saunders CM, Hashimoto K, Swann K, Lai FA. Sperm phospholipase Czeta from humans and cynomolgus monkeys triggers Ca2+ oscillations, activation and development of mouse oocytes. Reproduction. 2002; 124(5): 611-623.
36. Morrell JM, Alsina MS, Abraham MC, Sjunnesson Y. Practical applications of sperm selection techniques for improving reproduction efficiency. Anim Reprod. 2016; 13(3): 340-345.
37. Kashir J, Heynen A, Jones C, Durrans C, Craig J, Gadea J, et al. Effects of cryopreservation and density-gradient washing on phospholipase C zeta concentrations in human spermatozoa. Reprod Biomed Online. 2011; 23(2): 263-267.
38. Park JH, Kim SK, Kim J, Kim JH, Chang JH, Jee BC, et al. Relationship between phospholipase C zeta immunoreactivity and DNA fragmentation and oxidation in human sperm. Obstet Gynecol Sci. 2015; 58(3): 232-238.
39. Tavalaee M, Kiani-Esfahani A, Nasr-Esfahani MH. Relationship between potential sperm factors involved in oocyte activation and sperm DNA fragmentation with intra-cytoplasmic sperm injection clinical outcomes. Cell J. 2017; 18(4): 588-596.
40. Bhattacharya SM. Hypo-osmotic swelling test and unexplained repeat early pregnancy loss. J Obstet Gynaecol Res. 2010; 36(1): 119-122.

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The Decellularized Calf Testis: Introducing Suitable Scaffolds for Spermatogenesis Studies

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Abstract

Background: Men's infertility and lack of production of healthy and active sperm are concerns of recent years in most countries. Studies on the preparation of extracellular matrix (ECM) from decellularization of testis tissue and spermatogenesis could provide proper results to solve some of the men's infertility problems. This study aims to decellularize calf testis by different methods to reach a suitable scaffold and introduce it in spermatogenesis studies.

Materials and Methods: In this experimental study, calf testis were decellularized by a freeze-de freeze, 1% sodium deoxycholate (SD), 0.1% sodium dodecyl sulfate (SDS), 0.1% SDS-vacuum, 1% SDS, 1% SDS-vacuum, and Triton-X100 methods. The content of DNA, collagen, and glycosaminoglycan (GAG) was analyzed using the kit and staining with Hematoxylin-Eosin, Masson's trichrome, Alcian blue, and Orcein methods. The morphology of the scaffolds was analyzed with a scanning electron microscope (SEM).

Results: Methods of 1% SDS, 1% SDS-vacuum, and 1% SD completely removed the cells. The preservation of collagen and GAG was confirmed using the staining kit and methods. The use of a vacuum showed greater porosity in the SEM images. Toxicity and hemolysis were not observed in the scaffolds.

Conclusion: Testis decellularization with 1% SDS and 1% SD, in addition to cell removal, could maintain the ECM structure to a large extent without having cytotoxic and hemolysis effects.

Keywords: Decellularization, Scaffold, Testis

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Introduction

Reproductive disorders are shared all over the world, which causes many social problems and harms the individual economy and society. Today, the number of couples with male infertility factors is increasing due to diet changes, lifestyle, and occupational injuries. Assisted reproductive techniques (ART) offer many efforts to treat reproductive disorders, and they are improving day by day, but in many cases, saving fertility fails (1, 2). Spermatogenesis and germ cell differentiation are complex processes of reproduction that lead to the production of fertile sperm. Spermatogonia stem cells divide inside seminiferous tubules and produce progenitor cells. This process is prompted by hormonal factors, growth factors, cytokines, and extracellular matrix (ECM). Therefore, defects in the function of any of those factors could interfere with spermatogenesis and lead to male infertility (3, 4).

Regenerative medicine and tissue engineering with new approaches to the male fertility subject has provided a unique opportunity to offer practical solutions in this field. Tissue engineering is a science that has taken a big step in tissue repair and treatment by making natural and synthetic scaffolds alone or using cells. Scaffolds of natural origin, such as decellularized tissues, can effectively mimic the body's natural ECM structure (5). Decellularized scaffolds have the potential to simulate the physical microenvironment that is impacted cell fate and repair. Regenerative medicine and tissue engineering with new approaches to improve fertility have provided a unique opportunity to offer practical solutions in

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this field. These sciences have taken a big step in treatment by making natural and synthetic scaffolds alone or using cells (6, 7).

Preservation of the ECM is one of the critical cases of the tendency to use this technique in studies. These tissues are obtained from various sources such as corpses and animals and are examined in research works. Due to the decellularization of the tissue, rejection risks of the transplant are almost eliminated, and at the same time, if these tissues are obtained from unusable animal sources, the ethical problems of providing human corpses are eliminated (8).

Due to the increasing male infertility ratio across the world, more attention has been paid to the methods for treating this problem (9). The use of ECM prepared from decellularized tissues is one of the proposed and used methods for the treatment of infertility. In tissue engineering, testicular organoids are cellular groupings that mimic testicular architecture and function. This technology in science opens new horizons for the study and realization of spermatogenesis *in vitro*. In fact, these scaffolds create conditions similar to the bodies for the growth of sperm. These techniques enable pharmaceutical spermatogenesis research that addresses infertility and reduces animal usage (10). The testis tissue of several species has been decellularized by various methods and introduced for spermatogenesis studies. So far, this process has not been investigated in the decellularized tissue of calf testis. For this purpose, we used the calf testis and vacuum method for the first time in this study. Therefore, the current study aims to take a step towards spermatogenesis research and helping treat male infertility. The results of this study and the preparation of testicular scaffolds derived from ECM to support testicular cells could be valuable for spermatogenesis studies and drug screening.

Methods and Materials

This experimental study was accepted under the management of the Ethics Committee of Kermanshah University of Medical Sciences (IR.KUMS.MED.REC.1401.031).

Calf testis

Preparation of the calf testis

Calf meat is considered one of the food sources. After sacrificing, the testis was removed from the Holstein breed calf and placed in a phosphate-buffered saline (PBS) solution including 2% antibiotics penicillin-streptomycin (P/S) and transferred to the laboratory. Tissues were collected from 5 calf.

Decellularization methods

After transferring the tissue to the laboratory, the tunica albuginea was removed, and the testis was cut into small pieces (about 1 cm^3). Seven methods were used to decellularize the testis tissue in this study. In the freeze-de-freeze method, the tissues were placed in liquid nitrogen for 30 seconds (s) and then at room temperature for 10 minutes; this cycle was repeated 5 times. In 1% SD, 0.1% SDS, and 1% SDS methods, the tissues were immersed separately in the above solutions and agitated on a shaker at 90 revolutions per minute (rpm) for 24 hours , then the tissues were shaken in PBS for 12 hours. After the above time, decellularized tissues with 0.1% and 1% SDS are placed under a vacuum for 5 hours. To sterilize the tissues, they put in a PBS solution including 1% P/S for 1 hour, 70% ethanol for 10 minutes, and then exposed to UV radiation for 15 minutes on each side.

Scaffolds characterization

Evaluation of DNA content

The measurement of the remaining DNA content in decellularized testis and native tissue was done with the kit of Sinaclon company, Iran. 100 µl of protease buffer and then 5 µl of protease were added to 30 mg of tissues and kept at 55°C for 3 hours. 100 µl of the sample was mixed with 400 µl of lysis solution and 300 µl of precipitation solution and then centrifuged at 12000 g for 10 minutes. Then, after several steps of washing, pour the washing buffer completely. After the pellet is dried, it is suspended in 50 µl of solvent buffer and then centrifuged for 30 seconds at 12,000 g. The DNA concentration of the supernatant was measured by a NanoDrop spectrophotometer (BioTeK, USA) (11).

Evaluation of glycosaminoglycan content

ECM of the testis is rich in GAG, so its measurement in decellularization methods seems necessary. The experiment was performed according to the protocol of Kiazist Iran. The standard solution (chondroitin sulfate) was prepared in serial dilution according to the kit protocol. 20 mg of tissue was homogenized with enzyme solution and incubated at 65°C for 16 hours. Then it was centrifuged at 6000 g for 15 minutes. 50 µl of precipitating protein was added to the supernatant and centrifuged at 6000 g for 15 minutes. We poured 30 µl of the supernatant into each well of 96 plates and added 200 µl of GAG reagent to them and incubated them for 60 seconds at room temperature. Finally, the absorbance of the wells was read with an ELISA Reader (Stat Fax 2100, USA) at a wavelength of 560 nm (12).

Evaluation of collagen content

Hydroxyproline, a collagen components, plays an important role in maintaining the structure of ECM. Therefore, in this study, the measurement of the amount of collagen was performed using the kit of Kiazist Iran. The standard solution was prepared in the dilution series according to the kit protocol. To 20 mg of tissue, 100 µl of deionized water and then 100 µl of 12 M hydrochloric acid were added and incubated for 3 hours at 120°C. Charcoal

was added to each microtube and centrifuged at 12,000 g for 15 minutes. 20 µl of the supernatant was poured into each well of a plate, and oxidation solution (100 µl) and chromogen (100 µl) were added to them and incubated for 60 minutes at of 60°C. In the end, optical absorption was read at a wavelength of 540 nm with an ELISA Reader (Stat Fax 2100, USA) (13).

Histology analysis

The histological evaluation was done after the preparation of the tissues. First, they were fixed, and then extraction and moulding with paraffin were done. Tissues cut with a microtome in diameter of 5 microns. For staining the tissues, each section was deparaffinized with xylene solution, hydrated by ethanol at descending degrees, and then stained with Hematoxylin-Eosin (H-E), Masson's trichrome, Alcian blue and Orcein for identification of nucleus, collagen, GAG, elastin respectively (14).

Evaluation of cell morphology by scanning electron microscope

A scanning electron microscope (SEM, ZEISS Sigma 300 HV, USA) was used to evaluate the morphology of the tissues after decellularization. The samples were fixed in 4% paraformaldehyde for 24 hours. After washing three times with deionized water in ascending ethanol solutions (40, 50, 60, 70, 80, 90, and 100%), dehydration was done, and then it was dehumidified by a freeze dryer (Christ Alpha 2-4 LDplus) for 1 hour. Scaffolds coated with gold and imaged on their cross-section (15).

Biocompatibility evaluation

Tissue biocompatibility was done using an indirect test (standard ISO 10993-5). Briefly, scaffolds in a 24-well plate were placed containing Dulbecco's Modified Eagle's medium (DMEM, Sigma), 7% fetal bovine serum (FBS, Sigma), and 1% P/S (Sigma). They were cultured for 24 hour under suitable incubator conditions (37°C, 5% CO_2, 95% humidity). The supernatant was removed and added to human adipose mesenchymal stem cell 1×104 cells in a 96-well plate, and the MTT assay measured the biocompatibility in periods of 48 and 72 hours. 20 µl of 5 mg/ml MTT was added to each well and incubated for 3 hours at 37°C. The supernatant was withdrawn, and 100 µl of dimethyl sulfoxide (DMSO) was added to the wells. After 30 minutes, it was read with an ELISA Reader (Stat Fax 2100, USA) at a wavelength of 570 nm (16). Biocompatibility was calculated using the formula: cellular biocompatibility (%)=sample OD/ control OD×100

Hemocompatibility evaluation

The samples were placed in 4×4 mm dimensions in 2.5 ml microtubes. 2 ml of PBS was added to them and incubated for 30 minutes at 37°C. Two ml of distilled water, and 2 ml of PBS were added to the positive and negative control microtubes, respectively. Then 40 microliters of fresh blood containing anticoagulants were poured into each tube and incubated for 1 hour at 37°C. The supernatant was removed, and the optical absorbance was read at 540 nm (Stat Fax 2100, USA) (17). The following formula calculated the degree of hemolysis (HD). Dn: sample, D0: negative control, D1: positive control. HD (%)=[(Dn-D0)/(D1-D0)]×100%

Statistical analysis

All the results obtained in this study are based on at least 3 repetitions, which were calculated by taking the average and calculating the standard deviation. The results were analyzed using GraphPad Prism software (version 8, GraphPad Software Inc., USA). The normality of the variable and the homogeneity of the variances were checked, and after confirmation, the data were analyzed by one-way ANOVA and Tukey Post hoc test. $P<0.05$ is considered significant.

Results

DNA, glycosaminoglycan, and collagen content

A significant decrease in DNA levels was attained in the methods of freeze- de freeze (27.11 ng/mg), 1% SD (16.5 ng/mg), 0.1% SDS (27.73 ng/mg), 0.1% SDS-vacuum (25.66), 1% SDS (14.43), 1% SDS-vacuum (12.91), Triton- X100 (26.92) relative to native tissue (583.33 ng/mg) (Fig.1A). A decrease in collagen content was observed in the decellularized tissue compared to the native tissue (0.35 µg/mg), and this significant difference was reported in all groups (***$P<0.001$). The greatest reduction occurred in the 1% SD (0.240 µg/mg) method (Fig.1B). The GAG content in the decellularized tissues was significantly reduced compared to the main tissue (4 µg/mg). The highest decrease was reported in 1% SD (2.48 µg/mg) and the lowest in freeze-de freeze (3.23 µg/mg, Fig.1C). The normality of the variable and the homogeneity of the variances were checked, and after confirmation, the data were analyzed by one-way ANOVA and Tukey Post hoc test. A significant comparison of all groups with each other is shown in Table 1.

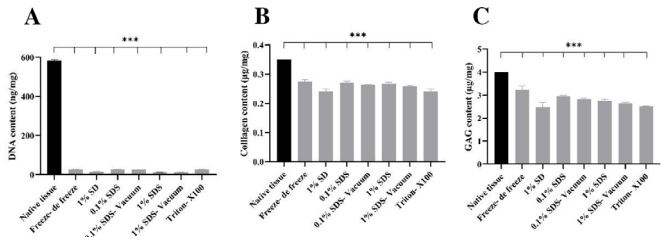

Fig.1: DNA, collagen, and GAG content in testis tissue of native and decellularized. The amounts of **A.** DNA, **B.** Collagen, and **C.** GAG in all decellularized tissues are significantly reduced compared to the native tissue. Findings are presented as means ± SD. Analyzed by ANOVA test. ***; $P<0.001$, GAG; Glycosaminoglycan, SD; Sodium deoxycholate, and SDS; Sodium dodecyl sulfate.

Table 1: Significant comparison in different groups in DNA, collagen, and GAG content

Tukey's multiple comparisons tests	Significant (DNA)	Significant (Collagen)	Significant (GAG)
Freeze-de freeze/1% SD	P<0.001	P<0.001	P<0.05
Freeze-de freeze/0.1% SDS	0.5	0.9	P<0.001
Freeze-de freeze/0.1% SDS-vacuum	0.06	0.2	P<0.001
Freeze-de freeze/1% SDS	P<0.001	0.7	P<0.001
Freeze-de freeze/1% SDS-vacuum	P<0.001	P<0.05	P<0.001
Freeze-de freeze/Triton-X100	0.8	P<0.001	P<0.001
1% SD/0.1% SDS	P<0.001	P<0.001	P<0.001
1% SD/0.1% SDS-vacuum	P<0.001	P<0.01	P<0.01
1% SD/1% SDS	0.8	P<0.001	P<0.05
1% SD/1% SDS-vacuum	0.8	P<0.05	0.5
1% SD/Triton- X100	P<0.001	0.9	0.9
0.1% SDS/0.1% SDS-vacuum	0.9	0.5	0.6
0.1% SDS/1% SDS	P<0.001	0.9	0.2
0.1% SDS/1% SDS-vacuum	P<0.001	0.1	P<0.01
0.1% SDS/Triton-X100	0.8	P<0.001	P<0.001
0.1% SDS-vacuum/1% SDS	P<0.001	0.9	0.9
0.1% SDS-vacuum/1% SDS-vacuum	P<0.001	0.9	0.2
0.1% SDS-vacuum/Triton-X100	0.8	P<0.01	P<0.05
1% SDS/1% SDS-vacuum	0.9	0.4	0.5
1% SDS/Triton-X100	P<0.001	P<0.001	0.6
1% SDS-vacuum/Triton-X100	P<0.001	P<0.05	0.7

GAG; Glycosaminoglycan, SD; Sodium deoxycholate, and SDS; Sodium dodecyl sulfate.

Histology

Staining methods of H-E, Masson's trichrome, Orcein, and Alcian blue were used to study the nucleus, collagen, elastin, and GAG respectively. In the native tissue, the seminiferous tubules and the general structure of the testis tissue can be seen well. In the samples decellularized by the freeze-de freeze, 0.1% SDS, and Triton-X100 methods, the structure of the seminiferous tubules has changed to some extent, and many nuclei can be seen. The structure of collagen, elastin, and GAG are largely preserved. In tissues decellularized with 1% SDS and 1% SD, cells were completely removed. The structure of the seminiferous tubules has changed a lot. Collagen, elastin, and GAG have decreased in tissues. More tissue changes were observed in the samples where a vacuum was used compared to the same groups without a vacuum. These findings were consistent with values obtained from quantitative studies (Fig.2).

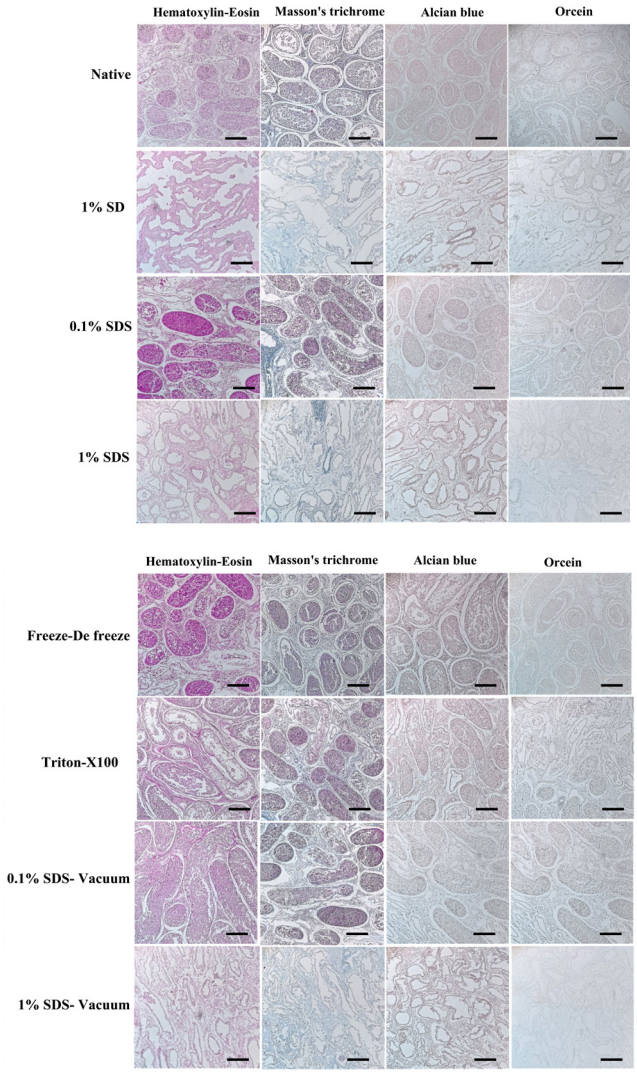

Fig.2: Histological staining (H-E, Masson's trichrome, Orcein, and Alcian blue) in decellularized tissue. In the native tissue, the structure of the tissue and the seminiferous tubules are well preserved, in the freeze-de freeze, 0.1% SDS, and Triton-X100 methods many nuclei can be seen, but 1% SDS and 1% SD the nuclei are completely removed, collagen, elastin, and GAG have decreased in tissues and the texture architecture has changed the most (magnification 200x, scale bar: 200 µm). GAG; Glycosaminoglycan, SD; Sodium deoxycholate, and SDS; Sodium dodecyl sulfate.

Scanning electron microscope

SEM was used to qualitatively assess the morphology structure of the decellularized testis. The cross-section of tissues was utilized to study their three-dimensional structure. In tissue decellularization by freeze-de freeze method, some tissue disintegration was seen. In 0.1% SDS, the collagen fibers are not separated, but in 0.1% SDS-vacuum, tissue disintegration has increased. In the 1% SD method, the collagen strands are separated, and the bundles of the strands are also reduced. In the 1% SDS method, the collagen fibers are separated, but not as much as in the 1% SD method. In the 1% SDS-vacuum method, the break up of these strings is more than 1% SD. In Triton X-100, the cohesion of collagen fibers has decreased, and the strands have separated (Fig.3).

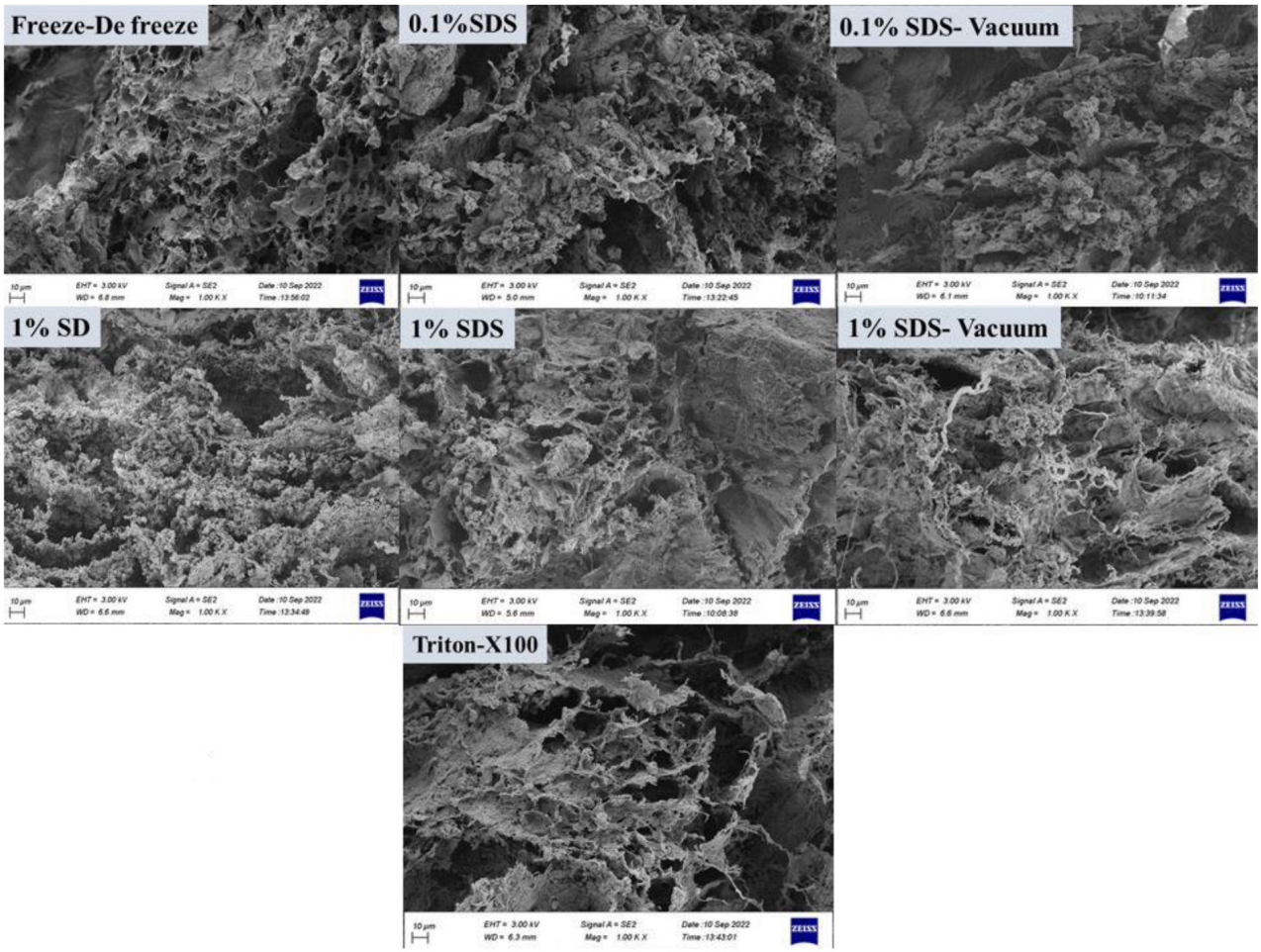

Fig.3: Evaluation of morphology and cell attachment of testis decellularized tissue by SEM. The yellow arrows show that the structure of the scaffold is mostly preserved as a plate, but the red arrows show the separation of collagen fibers (magnification: 500x, crass section, scale bars: 10 µm). SEM; Scanning electron microscopy, SD; Sodium deoxycholate, and SDS; Sodium dodecyl sulfate.

Biocompatibility

The biocompatibility of decellularized testis tissues was assessed using adipose mesenchymal stem cells by indirect MTT assay at 48 and 72 hours. As can be seen in Figure 4, the optical density (OD) in the control group (cells alone) and all decellularized tissues are compared with each other. Cell proliferation increased in different testicular tissue decellularization methods depending on time so that in 72 hours, the cell growth in all groups was higher than in 48 hours. A significant difference in cell survival was reported in 1% SD, 1% SDS, 15 SDS-vacuum, and Triton groups in 48 hours compared to the control group, while this difference was observed in 72 hours only in 1% SDS and Triton groups (**P<0.01, ***P<0.001). It seems that the use of the type and dose of detergents and the decellularization method have a direct effect on cell proliferation. The normality of the variable and the homogeneity of the variances were checked, and after confirmation, the data were analyzed by one-way ANOVA and Tukey Post hoc test.

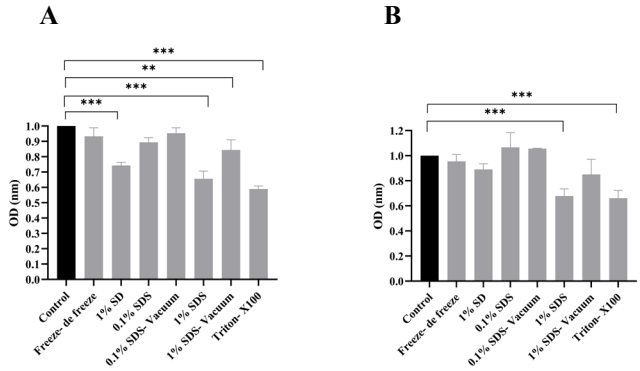

Fig.4: Biocompatibility in testis decellularized tissues by MTT assay. **A.** Within 48 hours, there was no significant difference in Freeze-de freeze, 0.1% SDS, and 0.1% SDS-vacuum groups compared to the control group, but a significant difference in cell proliferation was reported in 1% SD, 1% SDS, 1% SDS-vacuum, and Triton groups than the control group. In none of these groups cell growth inhibition in IC_{50} was seen, **B.** Within 72 hours, cell proliferation in all groups was more than 48 hours. Significant differences were reported in 1% SDS and Triton groups compared to the control group. Findings are presented as means ± SD. Analyzed by ANOVA test. **; P<0.01, ***; P<0.001, SDS; Sodium dodecyl sulfate, SD; Sodium deoxycholate, IC_{50}; The half-maximal inhibitory concentration, and OD; Optical density.

Hemocompatibility

The finding of the hemolytic test is shown in Figure 5. Compatible blood was seen in all tissues decellularized by different methods, which showed a significant difference with the positive control group (***P<0.001). This ratio was reported to be more than 1% in freeze-de freeze (1.23%) and 1% SDS-vacuum (1.1%) methods and less than 1% in other methods. The normality of the variable and the homogeneity of the variances were checked, and after confirmation, the data were analyzed by one-way ANOVA and Tukey Post hoc test.

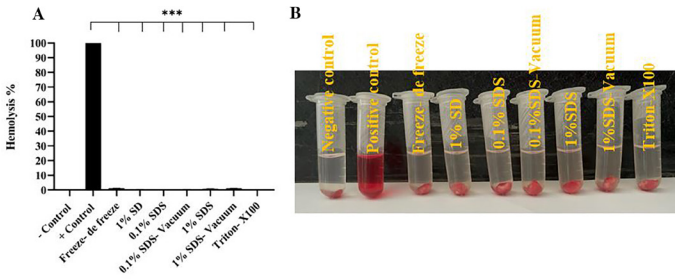

Fig.5: Hemocompatibility in testis decellularized tissues by hemolysis test. **A.** Data were reported as significant in all methods compared to the positive control group. **B.** The image related to the hemolysis test in different testis tissue decellularization methods, the clear supernatant liquid in the tubes shows hemocompatibility. Findings are presented as means ± SD. Analyzed by ANOVA test. ***; P<0.001, SDS; Sodium dodecyl sulfate, and SD; Sodium deoxycholate.

Discussion

This study subjected calf testis to decellularization using combined chemical-physical methods. Decellularization methods included freeze-de freeze, 1% SD, SDS-vacuum (0.1, 1%), and triton-X100. 1% SDS and 1% SD were able to completely remove cells from the tissue. By reducing the percentage of SDS (0.1%) and using the Freeze-de Freeze and triton-X100 methods, the structure of the scaffold remained largely intact, but the removal of cells was not successful. DNA content measurement also quantitatively confirmed the staining findings. Biocompatibility and blood compatibility were observed in all methods. Preservation of collagen and GAG using kits and staining methods showed that these values decrease in 1% SDS (with and without vacuum) and 1% SD methods, more than other methods, and then the tissue architecture changes to some extent. SEM findings also showed the separation of collagen fibers in the above methods. The scaffold was exposed to more porosity in the methods where a vacuum was also present. In our study, the combination of physical and chemical methods caused decellularization of the testicular tissue through the methods of creating turbulence, introducing a vacuum, and using ionic and non-ionic detergents. These methods will work through the mechanism of separation of protein bonds and cause the separation of the cell from the ECM structure. It also results in the separation of many protein bonds such as collagens from the scaffold structure. The simultaneous use of these items will reduce the decellularization time and the percentage of detergents, resulting in better cell removal and preventing the destruction of the scaffold structure.

The final goal of the decellularization process is to remove the tissue cells using detergents and physical methods and preserve the components and proteins of the ECM. These proteins provide structural and biochemical support to cell proliferation, adhesion, cell-to-cell interaction, and migration. Decellularization of sheep testis with 1% SDS at 24 hours reported results similar to our study (18). However, 1% SDS in the ram testis decellularization within 30 minutes, which was much less than the time of our study (24 hours), showed the complete removal of cells (19). Decellularization of the human testis with SD also indicated cell removal, but the amount of this detergent was 4%, which was reported to be more than the amount used by us (1% SDS). The agitation time was same in the both studies (24 hours) (20). Triton alone failed to remove cells from the testis of the calf, but in studies where it was used in combination with SDS, complete decellularization was achieved (19).

Decellularization with our methods in other tissues was also similar to our results. The use of 1% SDS and vacuum decellularized the pericardial tissue well and caused proper porosity in this tissue (12). Decellularization of cow ovaries with 0.1% SDS showed acceptable results in cell removal (21), while in our study with this detergent and the same percentage, cell removal did not happen completely, which may be due to the difference in the nature of the ovarian and testis tissues. are each other or the difference in the size of the pieces of tissues in these two studies. The tissue pieces of the testis in the present study were 1 cm^3 and 500 µm in the cow ovary. The smaller size of the tissue makes the detergent reach the deep areas better and the phenomenon of cell removal occurs.

Less than 50 ng DNA/mg dry weight must remain in the tissue after decellularization to prevent immune reactions (22). In all the methods of current study, the level of DNA was reported lower than this value, but in freeze-de freeze, 0.1% SDS, and Triton methods, DNA removal was less than in other methods. DNA content after pig testis decellularization with different methods was reported in line with our study (23). The DNA content of mouse testis after decellularization with SDS reached 11.37 µg/mg (24), which was largely similar to our decellularization methods with 1% SDS-vacuum method (12.91 µg/mg).

Preservation of the components and structure of the ECM is very important and necessary to create a proper interaction between the cells and the ECM (25). In this study, special dyes and kits were used to evaluate the preservation of ECM components in testicular tissues. Masson's trichrome, Orcein, and Alcian blue staining quantitative data confirmed the preservation of collagen, elastin, and glycosaminoglycans, respectively. GAG is one of the main elements in cell adhesion, proliferation, and differentiation in ECM. Common detergents in tissue

decellularization cause the destruction of this protein structure. However, histological findings (Alcian blue) and quantitative data obtained from this study showed that this protein was not removed, although its amount was reduced compared to the control group. Collagen and elastin fibers are also largely preserved. Other studies in the field of decellularization of testis tissue (19) and other tissues were in line with our study on this topic (26, 27). Collagen decrease in diseases causes testicular atrophy, showing the importance of the ECM in the normal function of the testis because, in case of damage to the ECM of the testicle, the process of spermatogenesis will be disrupted (28).

SEM images represented that the three-dimensional structure of the testis tissue was well preserved in decellularization by freeze-de freeze and 0.1% SDS methods, but as we said, cell removal did not occur completely in these scaffolds. In other methods, the collagen fibers are separated to some extent, which shows their microscopic images. Decellularization of rat testis tissue was also in line with our findings (29).

The biocompatibility of decellularized testis tissues was assessed using human adipose mesenchymal stem cells (cell line) by indirect MTT assay at 48 and 72 hours. Therefore, the scaffolds are biocompatible and can be used for the proliferation and differentiation of spermatogonia cells. Decellularization of rat uterus and bovine ovary tissue with SDS and Triton did not report cytotoxicity similar to our study (30, 31).

Hemocompatibility is a significant property of the scaffold after it is decellularized. The tissue is usually exposed to blood and hurts the erythrocytes to a specific extent. As per the hemolytic index of ASTM F756, scaffolds are believed to be hemolytic when % hemolysis is >5%; slightly hemolytic at % hemolysis is between 2 and 5%, and nonhemolytic for % hemolysis is <2% (32). In our study, all scaffolds were non-hemolytic.

One of the limitations of studies related to testis decellularization is the low filling percentage of seminiferous tubules for injections due to the tubes collapse; the use of the vacuum system, in addition to helping in better decellularization, creates more porosity in the tissue structure and prevents the tubes falling on top of each other. Therefore, options can be suggested by using detergents such as SDS and SD together with the vacuum method for better decellularization of the testis tissue and spermatogenesis studies.

Conclusion

The results showed that the calf testis is successfully decellularized by using 1% SD and 1% SDS (with and without vacuum). The three-dimensional structure and important components of the ECM are preserved to a large extent. The vacuum helps to create more porosity in the tissue and prevents the overlap of seminiferous tubes, therefore suggesting a suitable ECM for spermatogenesis studies.

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Authors' Contributions

M.R.K.; Performed the dissection experiments, data measurement, the statistical analysis, and wrote the first draft of the manuscript. Z.A.; Contributed to conception, design of the study, performed the dissection experiments, and revised the manuscript. M.Kh.; Performed data measurement and the statistical analysis. L.R.; Contributed to conception and design of the study. All authors contributed to manuscript revision, read, and approved the submitted version.

References

1. Faramarzi A, Aghaz F, Golestan Jahromi M, Bakhtiari M, Khazaei M. Does supplementation of sperm freezing/thawing media with Ceratonia siliqua improve detrimental effect of cryopreservation on sperm parameters and chromatin quality in normozoospermic specimens? Cell Tissue Bank. 2019; 20(3): 403-409.
2. Faramarzi A, Khalili MA, Omidi M, Agha-Rahimi A, Taheri F. Pronuclear pattern does not predict morphokinetics behavior in human embryos. Gynecol Endocrinol. 2018; 34(3): 248-251.
3. Neto FT, Bach PV, Najari BB, Li PS, Goldstein M. Spermatogenesis in humans and its affecting factors. Semin Cell Dev Biol. 2016; 59: 10-26.
4. Kubota H, Brinster RL. Spermatogonial stem cells. Biol Reprod. 2018; 99(1): 52-74.
5. Khazaei F, Rezakhani L, Alizadeh M, Mahdavian E, Khazaei M. Exosomes and exosome-loaded scaffolds: Characterization and application in modern regenerative medicine. Tissue Cell. 2023; 80: 102007.
6. Sharifi M, Kheradmandi R, Salehi M, Alizadeh M, Ten Hagen TLM, Falahati M. Criteria, challenges, and opportunities for acellularized allogeneic/xenogeneic bone grafts in bone repairing. ACS Biomater Sci Eng. 2022; 8(8): 3199-3219.
7. Rahmati S, Khazaei M, Nadi A, Alizadeh M, Rezakhani L. Exosome-loaded scaffolds for regenerative medicine in hard tissues. Tissue Cell. 2023; 82: 102102.
8. Khazaei M, Khazaei MR, Alizadeh M, Rahmati S, Rezakhani L. Functional survey of decellularized tissues transplantation for infertile females. Cell Tissue Bank. 2022; 23(3): 407-415.
9. Sun H, Gong TT, Jiang YT, Zhang S, Zhao YH, Wu QJ. Global, regional, and national prevalence and disability-adjusted life-years for infertility in 195 countries and territories, 1990-2017: results from a global burden of disease study, 2017. Aging (Albany NY). 2019; 11(23): 10952-10991.
10. Richer G, Baert Y, Goossens E. In-vitro spermatogenesis through testis modelling: toward the generation of testicular organoids. Andrology. 2020; 8(4): 879-891.
11. Khavarimehr M, Nejati V, Razi M, Najafi G. Ameliorative effect of omega-3 on spermatogenesis, testicular antioxidant status and preimplantation embryo development in streptozotocin-induced diabetes in rats. Int Urol Nephrol. 2017; 49(9): 1545-1560.
12. Alizadeh M, Rezakhani L, Khodaei M, Soleimannejad M, Alizadeh A. Evaluating the effects of vacuum on the microstructure and biocompatibility of bovine decellularized pericardium. J Tissue Eng Regen Med. 2021; 15(2): 116-128.
13. Rahmati S, Jalili A, Dehkordi MB, Przedborski M. An effective method for decellularization of human foreskin: implications for skin regeneration in small wounds. Cell J. 2022; 24(9): 506-514.
14. Asgari F, Asgari HR, Najafi M, Eftekhari BS, Vardiani M,

Gholipourmalekabadi M, et al. Optimization of decellularized human placental macroporous scaffolds for spermatogonial stem cells homing. J Mater Sci Mater Med. 2021; 32(5): 47.
15. Wang J, Zhao L, Zhang A, Huang Y, Tavakoli J, Tang Y. Novel bacterial cellulose/gelatin hydrogels as 3D scaffolds for tumor cell culture. Polymers (Basel). 2018; 10(6): 581.
16. Díaz E, Puerto I, Ribeiro S, Lanceros-Mendez S, Barandiarán JM. The influence of copolymer composition on PLGA/nHA scaffolds' cytotoxicity and in vitro degradation. Nanomaterials (Basel). 2017; 7(7): 173.
17. Sharifi E, Sadati SA, Yousefiasl S, Sartorius R, Zafari M, Rezakhani L, et al. Cell loaded hydrogel containing Ag-doped bioactive glass-ceramic nanoparticles as skin substitute: Antibacterial properties, immune response, and scarless cutaneous wound regeneration. Bioeng Transl Med. 2022; 7(3): e10386.
18. Ashouri Movassagh S, Ashouri Movassagh S, Banitalebi Dehkordi M, Pourmand G, Gholami K, Talebi A, et al. Isolation, identification and differentiation of human spermatogonial cells on three-dimensional decellularized sheep testis. Acta Histochem. 2020; 122(8): 151623.
19. Bashiri Z, Amiri I, Gholipourmalekabadi M, Falak R, Asgari H, Maki CB, et al. Artificial testis: a testicular tissue extracellular matrix as a potential bio-ink for 3D printing. Biomater Sci. 2021; 9(9): 3465-3484.
20. Murdock MH, David S, Swinehart IT, Reing JE, Tran K, Gassei K, et al. Human testis extracellular matrix enhances human spermatogonial stem cell survival in vitro. Tissue Eng Part A. 2019; 25(7-8): 663-676.
21. Laronda MM, Jakus AE, Whelan KA, Wertheim JA, Shah RN, Woodruff TK. Initiation of puberty in mice following decellularized ovary transplant. Biomaterials. 2015; 50: 20-29.
22. Isidan A, Liu S, Li P, Lashmet M, Smith LJ, Hara H, et al. Decellularization methods for developing porcine corneal xenografts and future perspectives. Xenotransplantation. 2019; 26(6): e12564.
23. Vermeulen M, Del Vento F, de Michele F, Poels J, Wyns C. Development of a cytocompatible scaffold from pig immature testicular tissue allowing human sertoli cell attachment, proliferation and functionality. Int J Mol Sci. 2018; 19(1): 227.
24. Yang Y, Lin Q, Zhou C, Li Q, Li Z, Cao Z, et al. A testis-derived hydrogel as an efficient feeder-free culture platform to promote mouse spermatogonial stem cell proliferation and differentiation. Front Cell Dev Biol. 2020; 8: 250.
25. Baert Y, Stukenborg JB, Landreh M, De Kock J, Jörnvall H, Söder O, et al. Derivation and characterization of a cytocompatible scaffold from human testis. Hum Reprod. 2015; 30(2): 256-267.
26. Eivazkhani F, Abtahi NS, Tavana S, Mirzaeian L, Abedi F, Ebrahimi B, et al. Evaluating two ovarian decellularization methods in three species. Mater Sci Eng C Mater Biol Appl. 2019; 102: 670-682.
27. Ventura RD, Padalhin AR, Park CM, Lee BT. Enhanced decellularization technique of porcine dermal ECM for tissue engineering applications. Mater Sci Eng C Mater Biol Appl. 2019; 104: 109841.
28. Cheng CY, Wong EW, Yan HH, Mruk DD. Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: new insights and advances. Mol Cell Endocrinol. 2010; 315(1-2): 49-56.
29. Kiani M, Movahedin M, Halvaei I, Soleimani M. Formation of organoid-like structures in the decellularized rat testis. Iran J Basic Med Sci. 2021; 24(11): 1523-1528.
30. Arezoo N, Mohammad H, Malihezaman M. Tissue engineering of mouse uterus using menstrual blood stem cells (MenSCs) and decellularized uterine scaffold. Stem Cell Res Ther. 2021; 12(1): 475.
31. Nikniaz H, Zandieh Z, Nouri M, Daei-Farshbaf N, Aflatoonian R, Gholipourmalekabadi M, et al. Comparing various protocols of human and bovine ovarian tissue decellularization to prepare extracellular matrix-alginate scaffold for better follicle development in vitro. BMC Biotechnol. 2021; 21(1): 8.
32. Singh H, Purohit SD, Bhaskar R, Yadav I, Bhushan S, Gupta MK, et al. Curcumin in decellularized goat small intestine submucosa for wound healing and skin tissue engineering. J Biomed Mater Res B Appl Biomater. 2022; 110(1): 210-219.

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Hysteroscopic Endometrial Fundal Incision in Oocyte Recipients before Embryo Transfer May Improve Reproductive Outcomes: A Prospective Study

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Abstract

Background: Induced endometrial injury is a technique described that have positive impact on implantation. The aim of this study was to investigate whether hysteroscopic endometrial fundal incision (EFI) in oocyte recipients before embryo transfer increases pregnancy and live birth rates or not.

Materials and Methods: A prospective study was conducted between 2014 and 2019 at an *in vitro* fertilization (IVF) unit in Greece. As part of the protocol, hysteroscopy and EFI were offered to all the egg recipients and the outcomes compared with those from an older cohort from the same Unit not undergoing hysteroscopy.

Results: In total, 332 egg recipients participated in the study; 114 of them underwent EFI prior to embryo transfer. Both groups were similar in terms of age, years of infertility, duration of hormone replacement treatment (HRT) and number of blastocysts transferred. In the EFI group, minor anomalies were detected and treated in 6.1% (n=7) of the participants. Moreover, pregnancy test was positive in 73.7% of the women in the hysteroscopy group compared to 57.8% in the non-hysteroscopy group (P=0.004). Live birth rate was also higher (56.1 vs. 42.2%, P=0.016) in the EFI group compared to the non-hysteroscopy one.

Conclusion: Apart from the obvious benefit of recognizing obscured anomalies, requiring surgical correction, it appears that in oocyte recipients prior to embryo transfer, EFI might improve uterine receptivity and reproductive outcomes.

Keywords: Endometrial Fundal Incision, Endometrial Scratching, Implantation, Oocyte Donation, Recipients

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Introduction

Implantation remains the rate-limiting factor for the success of *in vitro* fertilization (IVF); it comprises a compound process including several cytokines and growth factors, along with a "dialogue" between embryo and endometrium (1). Extended embryo culture, blastocyst selection, assisted hatching and preimplantation genetic screening (PGS) are techniques that mainly focus on the embryo and probably have no impact on implantation itself (2). With regards to the particular mechanism of successful implantation, several aspects still remain unclear (1).

A manipulation suggested to have positive impact on implantation is hysteroscopy alone or combined with induced endometrial injury ("scratching"); scratching has demonstrated favorable effects on implantation rates, mainly in women with recurrent implantation failure (RIF) (3), while no benefit was found in unselected populations of women undergoing IVF (4). The potential mechanisms of positive effect of scratching might be: i. Induction of decidualization, ii. Production of cytokines, growth factors such as leukemia inhibitory growth factor, interleukin-11, heparin-binding endothelial growth factor, macrophages, and dendritic cells and iii. Improvement of synchronization of endometrium and embryo development following endometrial trauma (5, 6).

During hysteroscopy, notable heterogeneity exists with regards to the scratching method, thus the reported improvement in implantation rates differ (7). Moreover, confounding factors related to autologous IVF treatment may complicate the analysis, while the woman's age can adversely affect the embryo quality (8); ovarian

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hyperstimulation is another variable affecting the embryo development and endometrial receptivity (9). To eliminate such bias, ovum donation cycles should be explored, where the quality of the donated blastocysts arisen from young fertile eggs is relatively stable, thus not affecting the probability of pregnancy in the recipients (10).

The aim of the current study was to assess the impact of a novel method described from our group, the endometrial fundal incision (EFI), on recipients of donated oocytes.

Materials and Methods

Population characteristics

This is a prospective study conducted in "Assisting Nature Center Reproduction and Genetics", a private IVF Unit in Thessaloniki, Greece. Patients were recruited from January 2014 to December 2019. In particular, as part of our local protocol, we offered hysteroscopy and scratching to all the egg recipients without extra cost. Then we compared the reproductive outcomes with those from an older cohort of the same IVF Unit not undergoing hysteroscopy. We estimated a ratio of 1:2 in the groups undergoing hysteroscopy or not. Apart from the evaluation of the uterine cavity, women in the hysteroscopy group underwent correction/removal of any underlying pathology (polyps or adhesions or septum or endometritis) and also underwent EFI with endoscopic scissor. The study protocol was approved by the Institutional Review Board of the IVF Unit (0501201404). Additionally, informed consent was obtained from all the patients in the intervention arm.

Inclusion and exclusion criteria

Oocyte recipients were eligible for the study if: i. Their age ranged between 30 and 50 years, ii. Frozen blastocysts were transferred, iii. Absence of submucosal fibromas or polyps in ultrasonography, iv. Endometrial thickness >7 mm and blood progesterone levels <1.5 pg/ml the day before progesterone supplementation during hormone replacement treatment (HRT) preparation and v. EFI was performed with the use of endoscopic scissor only, without use of electrocautery method. Exclusion criteria were: i. Women who had undergone hysteroscopy within 6 months prior to donor oocyte recipient treatment, ii. Women who had undergone any uterine surgery in the past, and iii. Free fluid in endometrial cavity during HRT preparation.

Hormone replacement treatment protocol

All frozen embryo transfers were carried out following the same hormone endometrial preparation protocol; starting on day 2 of the cycle, if ultrasound revealed quiet ovaries and hormone levels were basal [estradiol (E2) <80 pg/ml and progesterone <1.5 ng/ml], the woman could undergo HRT. Estrogen supplementation was administered in the form of 17-b estradiol (estradiol valerate) for 10-20 days before progesterone one. In particular, according to the local protocol, we started (day 2 of the cycle) with 2 mg (1×1), then 4 mg (1×2) until day 5, 6 mg (1×3) for the next 3 days until day 8 and then 8 mg (2×2) onwards until the pregnancy test. Between days 10 and 11 we assessed: i. The endometrial thickness by ultrasound and ii. Blood levels of progesterone, luteinizing hormone (LH) and E2. If endometrial thickness was less than 7 mm, the therapy was continued for 3 more days. Once optimal endometrial thickness was achieved (>7 mm), daily progesterone was offered and embryo transfer scheduled 6 days later. The levels of beta human chorionic gonadotropin (β-hCG) were checked 9 days after embryo transfer or 14 days after the initiation of progesterone supplementation.

Hysteroscopic procedure

All the recipients underwent routine evaluation during their early follicular phase, 1-3 months before the start of a new HRT cycle. Moreover, women planned for hysteroscopy started taking contraceptive pill on day 3 (drospirenone and ethinylestradiol or chlormadinone and ethinylestradiol), in order to achieve better cavity visualization. Following vaginoscopic approach, a hysteroscopy was performed between days 6 and 13 of menstrual cycle. Routine analgesia for sedation was administered. A rigid hysteroscope (4.8 mm hysteroscope; continuous flow; 30° forward oblique view) using 0.9 normal saline was used. After adequate distension of the uterine cavity, systematic inspection was performed. Two senior reproductive medicine consultants (R.N. and E.P.) performed all the hysteroscopic procedures. EFI was performed by using endoscopic scissor 2 mm. The EFI was performed in a single straight line directed from one fallopian ostium to the other; as far as the depth of incision is concerned, incision was continued within the connective tissue until the appearance of the first vessels.

Reproductive outcomes

The primary outcomes were pregnancy and live birth rates; the pregnancy rate was defined as the proportion of women with a positive quantitative serum human chorionic gonadotropin test above 10 mIU/ml, 9 days after blastocyst transfer. First trimester miscarriage rate was defined as the proportion of women with pregnancy loss before 12 weeks of gestation. Live birth was defined as the delivery of a live fetus beyond 24 weeks of gestation.

Statistical analysis

The values of the continuous variables are expressed herein as mean (SD) and absolute (%) frequencies, when applicable. The between-group differences were compared by using the independent samples t test. Categorical variables were statistically analyzed by using Pearson's $\chi 2$ test and Fisher's exact test. Statistical significance was defined as $P<0.05$. SPSS v25.0 (IBM Corp., Armonk, NY, USA) used for data analysis.

Results

Among 342 women initially screened, 10 were excluded from the final analysis as, one become spontaneous

pregnant, one abandoned the treatment and eight women had already known uterine pathology. Therefore, a total of 332 recipients that underwent frozen embryo transfer were included. Moreover, 114 recipients underwent hysteroscopy and 218 were chosen from the older cohort of our Unit.

The age of the women included in the study ranged from 35 to 50 years; no significant differences were found in the age, duration of infertility, duration of HRT, number of blastocysts transferred and peak endometrial thickness between the two groups (Table 1). The mean duration of infertility in the whole sample was 6.4 years. All the women underwent embryo transfer with two blastocysts except from 10 cases that preferred single blastocyst transfer to avoid twins. The mean blastulation rate was 59.4%; 60.4% in EFI-group and 58.3% in control group (P=0.77).

Minor anomalies were detected and treated in 6.1% (n=7) of the participants in the hysteroscopy group; one woman was diagnosed with U1a (T shape uterus), three with U2a (partial septate, arcuate uterus), two with U2b (septate uterus) and one with several adhesions. All these women, including those with minor uterine abnormalities (n=7) and those with normal cavity (n=107) underwent EFI.

Regarding main outcomes, the pregnancy test was positive in 73.7% (n=84) in the hysteroscopy group compared to 57.8% (n=126) in the non-hysteroscopy group (P=0.004). Moreover, live birth rate was significantly higher in the hysteroscopy group (56.1%, n=64), as compared to 42.2% (n=92) in the non-hysteroscopy one (P=0.016, Table 2, Fig.1).

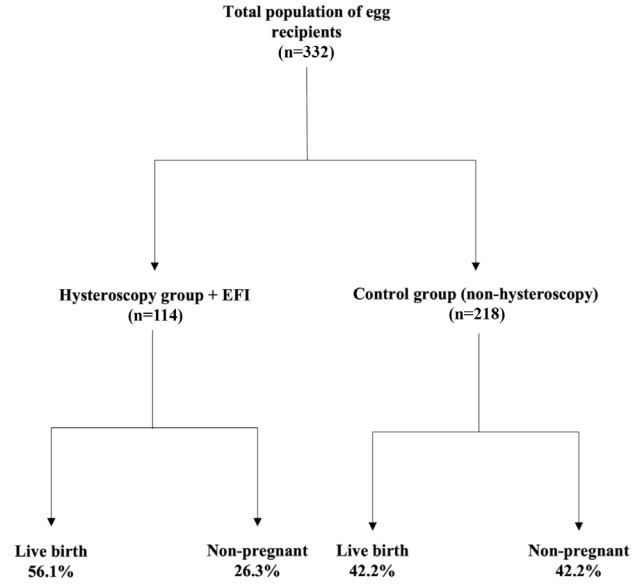

Fig.1: Flow chart of the study. EFI; Endometrial fundal incision.

In a subgroup analysis, we excluded the seven cases diagnosed with uterine abnormalities and found that both pregnancy and live birth rates remained higher in the hysteroscopy group (74.8 vs. 57.8%, P=0.003 and 54.2 vs. 42.2%, P=0.04, respectively).

Of note, there were 2 (1.8%) minor complications related to the hysteroscopy: one during cervical dilatation where false route was taken and corrected under ultrasound guidance and one during operative procedure; moderate bleeding continued after septum resection and patient was offered 6-hours of close monitoring. Both were diagnosed at the time of surgery.

Table 1: Demographic characteristics of the participants

Baseline characteristics of the participants	Hysteroscopy+EFI group (n=114)	Non-hysteroscopy control group (n=218)	P value
Mean age in years	39.7 ± 5.6	40.1 ± 6.1	0.31
Number of patients with history of previous hysteroscopy	3 (2.63)	9 (4.12)	0.34
Mean duration of infertility in years	6.03 ± 1.23	6.86 ± 1.17	0.19
Mean duration of HRT in days	17.48 ± 1.77	17.76 ± 1.16	0.45
Mean number of blastocysts transferred	1.79 ± 0.4	1.79 ± 0.42	0.65
Mean number of blastocysts available for transfer	4.55 ± 1.97	4.56 ± 1.97	0.86
Mean peak endometrial thickness in mm	9.69 ± 1.5	10.13 ± 1.25	0.16

Data are presented as mean ± SD or n (%). For the analyses independent samples t test, Pearson's χ^2 test and Fisher's exact test were employed. EFI; Endometrial fundal incision and HRT; Hormone replacement treatment.

Table 2: Reproductive outcomes in oocyte recipients with or without hysteroscopic endometrial fundal incision

Outcomes	Hysteroscopy+EFI group (n=114)	Non-hysteroscopy control group (n=218)	P value
Positive β-hCG rate per ET	73.7% (n=84)	57.8% (n=126)	0.004
Miscarriage rate <12 weeks	17.5% (n=20)	15.5% (n=34)	0.3
Live birth rate per ET	56.1% (n=64)	42.2% (n=92)	0.016

For the analyses Pearson's χ^2 test and Fisher's exact test were employed. β-hCG; Beta human chorionic gonadotropin, ET; Embryo transfer, and EFI; Endometrial fundal incision.

Discussion

We found that hysteroscopy along with targeted EFI 1-3 months before the embryo transfer in oocyte recipients may improve pregnancy and live birth rates. Moreover, no differences in the miscarriage rates were observed between the two groups.

Hysteroscopy has been proposed as a significant diagnostic tool in the diagnosis of infertility that increases the cost of an IVF cycle (11). Therefore, whether to undergo hysteroscopy or not before the first IVF cycle, still remains an issue, especially in Greece, where assisted reproductive techniques mainly take place in private IVF centers. Of note, in our study the EFI was offered to all the participants free of charge.

Endometrial scratching has been proposed as a simple cost-effective and minimally invasive procedure to improve endometrial receptivity during IVF cycles (12). Nevertheless, data remains inconclusive with regards to reproductive outcomes. The reason that EFI may be beneficial for implantation can be justified by three possible explanations. First, in contrast to the pipelle, where the blinded catheter scratches the posterior or the anterior uterine wall and never the fundus itself, in our technique the injury is directed to the fundus and the surgeon can even control the depth of the injury. Second, in cases with arcuate uterus (type U2a), the scratching is simultaneously therapeutic as it repairs this congenital variation of the uterine fundus considered physiological without impact on implantation in the past. According to data from a retrospective matched-control study, uterine anomalies have a negative impact on both pregnancy and live birth rates and thus, should be treated (13). Third, as we found, up to 6% of minor anomalies can be still identified by hysteroscopy itself, which would have been remained undiagnosed in the non-hysteroscopy group; however, we found that, even after excluding the cases with uterine abnormalities, the pregnancy and live birth rates remained higher in the EFI group.

As already mentioned, the reason that literature remains inconclusive whether scratching is beneficial or not, is that the majority of studies are heterogeneous; most studies investigated the effects of blinded injuries on the uterine cavity. In particular, Jayakrishnan et al. (14) treated the uterine pathology and induced injury with the hysteroscope only. Moreover, in another study, curettage of the fundus and the posterior wall post hysteroscopy was performed (15). Seval et al. (16) performed injury with the use of monopolar needle forcep. Our method is a well described standardized method of endometrial injury of the fundus in the follicular phase.

A Cochrane review concluded that endometrial scratching probably does not affect the pregnancy (OR: 1.08; 95% CI: 0.95-1.23) or the live birth (OR: 1.12; 95% CI: 0.98-1.28) rates, but notes that only evidence of moderate certainty exists (17). Furthermore, the same study found that endometrial scratching does not affect the risk of miscarriage (OR: 0.88; 95% CI: 0.68-1.13), which is in accordance to our findings. Furthermore, according to data from a futility analysis of a double-blind randomized controlled trial, endometrial mechanical stimulation with pipelle in the luteal phase of the cycle before embryo transfer does not improve reproductive outcomes in an unselected subfertile population and may result in lower live birth rates (18). This finding is in contrast with our results; it may be attributed to the technique used for scratching. Of note, we performed the EFI in the proliferative phase and according to published data, there is no significant difference in reproductive outcomes between scratching in the proliferative and the luteal phase (19).

Successful implantation is a complex process requiring a combination of three major physiological events to occur: i. A receptive endometrium, ii. An euploid embryo, and iii. The establishment of a proper dialogue between the semiallotypic embryo and maternal endocrinological/ immune system (1, 20). Although several theories on the association of endometrial injury with improved pregnancy rates have been proposed, our study has strengthened the hypothesis that mechanical injury may enhance uterine receptivity. If this occurs via cytokines released or modification of the immune system or release of vascular growth factors, it needs further investigation; according to published data, the concentrations of interleukin- (IL-) 6, IL-8, IL-12 (p70), IL-13, interferon- (IFN-) γ, monocyte chemotactic protein- (MCP-) 1 and vascular endothelial growth factor (VEGF) are increased in women undergoing endometrial scratching (21). Moreover, according to another study, scratching may affect the expression of genes involved in endometrial preparation for implantation or induce the production of cytokines and growth factors to enhance decidual proliferation (22).

To our knowledge, this is the first study in which a standardizing endometrial injury during hysteroscopy takes place in egg donation cycles. The main strength of our study is that the use of egg donation program can minimize the effect of embryo quality in our results. The embryos transferred are of young women with healthy fertile background, ensuring a limited bias regarding the reproductive outcome between the groups. By standardizing the method, with the use of endoscopic scissor and targeting only the fundus, we propose a method that should be easily applied by any reproductive medicine specialist. On the contrary, the main limitation of the study is the lack of randomization; this may be limited by the fact that no differences in the demographic characteristics were identified between the two groups. Moreover, the complete data set was obtained from a single IVF center and the same two senior doctors performed all the EFI procedures, as this could overcome possible inter-observer discrepancies. Another limitation of the study is that in the hysteroscopy group, uterine pathology was

detected in about 6% of the cases, which is in accordance with previously published data (23). We cannot estimate the proportion of cases with endometrial pathology in the non-hysteroscopy group, but it is probably the same since the two cohorts are from the same IVF Unit. Finally, the time interval between the hysteroscopy and the embryo transfer should be also taken into further consideration.

Conclusion

Our study has provided direct evidence to support the hypothesis that hysteroscopy plus EFI one to three months before embryo transfer is beneficial for the implantation in oocyte recipients. Apart from the obvious benefit of hysteroscopy to detect obscured anomalies, a discrete procedure tested the hypothesis that a site-specific mechanical injury during hysteroscopy could improve reproductive outcomes. More longitudinal trials using EFI in selected populations are encouraged to better understand the mechanism of action and further assess the effect of the procedure. Finally, cost-effectiveness analyses on the routine use of hysteroscopy in all egg recipients should be conducted.

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Authors' Contributions

R.N., N.P.; Conceptualization. N.P., I.T.; Methodology. G.M., E.P.; Validation. E.T.; Investigation. T.C.; Resources. R.N.; Data Curation. N.P; Writing-Original Draft Preparation. I.T.; Writing-Review and Editing. A.A.; Visualization. E.P.; Supervision and Project administration. All authors read and approved the final manuscript.

References

1. Parasar P, Guru N, Nayak NR. Contribution of macrophages to fetomaternal immunological tolerance. Hum Immunol. 2021; 82(5): 325-331.
2. Orvieto R. Preimplantation genetic screening- the required RCT that has not yet been carried out. Reprod Biol Endocrinol. 2016; 14(1): 35.
3. Bar G, Harlev A, Alfayumi-Zeadna S, Zeadna A, Bord I, Har-Vardi I, et al. Recurrent implantation failure: which patients benefit from endometrial scratching prior to IVF? Arch Gynecol Obstet. 2020; 301(3): 817-822.
4. Yeung TW, Chai J, Li RH, Lee VC, Ho PC, Ng EH. The effect of endometrial injury on ongoing pregnancy rate in unselected subfertile women undergoing in vitro fertilization: a randomized controlled trial. Hum Reprod. 2014; 29(11): 2474-2481.
5. Siristatidis C, Vrachnis N, Vogiatzi P, Chrelias C, Retamar AQ, Bettocchi S, et al. Potential pathophysiological mechanisms of the beneficial role of endometrial injury in in vitro fertilization outcome. Reprod Sci. 2014; 21(8): 955-965.
6. Chen T, Shi H, Fang LL, Su YC. The effect of endometrial injury on reproductive outcomes of frozen-thawed embryo transfer cycles in women with one implantation failure. J Int Med Res. 2020; 48(3): 300060520913130.
7. van Hoogenhuijze NE, Kasius JC, Broekmans FJM, Bosteels J, Torrance HL. Endometrial scratching prior to IVF; does it help and for whom? A systematic review and meta-analysis. Hum Reprod Open. 2019; 2019(1): hoy025.
8. Knight AK, Hipp HS, Abhari S, Gerkowicz SA, Katler QS, McKenzie LJ, et al. Markers of ovarian reserve are associated with reproductive age acceleration in granulosa cells from IVF patients. Hum Reprod. 2022; 37(10): 2438-2445.
9. Xu CK, Tang SB. Alteration of endometrial receptivity in rats with ovarian hyperstimulation syndrome. J Obstet Gynaecol. 2014; 34(2): 146-152.
10. Pataia V, Nair S, Wolska M, Linara-Demakakou E, Shah T, Lamanna G, et al. Factors predicting clinical outcomes from 494 vitrified oocyte donation cycles at a UK-regulated egg bank. Reprod Biomed Online. 2021; 43(3): 453-465.
11. Inhorn MC, Patrizio P. Infertility around the globe: new thinking on gender, reproductive technologies and global movements in the 21st century. Hum Reprod Update. 2015; 21(4): 411-426.
12. Lensen S, Osavlyuk D, Armstrong S, Stadelmann C, Hennes A, Napier E, et al. A randomized trial of endometrial scratching before in vitro fertilization. N Engl J Med. 2019; 380(4): 325-334.
13. Tomazevic T, Ban-Frangez H, Virant-Klun I, Verdenik I, Pozlep B, Vrtacnik-Bokal E. Septate, subseptate and arcuate uterus decrease pregnancy and live birth rates in IVF/ICSI. Reprod Biomed Online. 2010; 21(5): 700-705.
14. Jayakrishnan K, Hazra N, Nambiar D. Impact on IVF outcome following pre-IVF hysteroscopy and endometrial scratching. Austin J Reprod Med Infertil. 2015; 2(6): 1029.
15. Shohayeb A, El-Khayat W. Does a single endometrial biopsy regimen (S-EBR) improve ICSI outcome in patients with repeated implantation failure? A randomised controlled trial. Eur J Obstet Gynecol Reprod Biol. 2012; 164(2): 176-179.
16. Seval MM, Sukur YE, Ozmen B, Kan O, Sonmezer M, Berker B, et al. Does adding endometrial scratching to diagnostic hysteroscopy improve pregnancy rates in women with recurrent in-vitro fertilization failure? Gynecol Endocrinol. 2016; 32(12): 957-960.
17. Lensen SF, Armstrong S, Gibreel A, Nastri CO, Raine-Fenning N, Martins WP. Endometrial injury in women undergoing in vitro fertilisation (IVF). Cochrane Database Syst Rev. 2021; 6(6): CD009517.
18. Eskew AM, Reschke LD, Woolfolk C, Schulte MB, Boots CE, Broughton DE, et al. Effect of endometrial mechanical stimulation in an unselected population undergoing in vitro fertilization: futility analysis of a double-blind randomized controlled trial. J Assist Reprod Genet. 2019; 36(2): 299-305.
19. Liu W, Tal R, Chao H, Liu M, Liu Y. Effect of local endometrial injury in proliferative vs. luteal phase on IVF outcomes in unselected subfertile women undergoing in vitro fertilization. Reprod Biol Endocrinol. 2017; 15(1): 75.
20. Skliute G, Bausyte R, Ramasauskaite D, Navakauskiene R. Characterization of epigenetic and molecular factors in endometrium of females with infertility. Biomedicines. 2022; 10(6): 1324.
21. Liang Y, Han J, Jia C, Ma Y, Lan Y, Li Y, et al. Effect of endometrial injury on secretion of endometrial cytokines and IVF outcomes in women with unexplained subfertility. Mediators Inflamm. 2015; 2015: 757184.
22. Gnainsky Y, Granot I, Aldo PB, Barash A, Or Y, Schechtman E, et al. Local injury of the endometrium induces an inflammatory response that promotes successful implantation. Fertil Steril. 2010; 94(6): 2030-2036.
23. Sallee C, Margueritte F, Marquet P, Piver P, Aubard Y, Lavoue V, et al. Uterine factor infertility, a systematic review. J Clin Med. 2022; 11(16): 4907.

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An Experimental Study on The Oxidative Status and Inflammatory Levels of A Rat Model of Polycystic Ovary Syndrome Induced by Letrozole and A New High-Fat Diet

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Abstract

Background: Although there are numerous animal models of polycystic ovary syndrome (PCOS), they often fail to accurately replicate the reproductive and metabolic phenotypes associated with PCOS. The objective of this study is to assess oxidative status and inflammatory levels in a rat model of PCOS subjected to a new high-fat diet (HFD) in combination with letrozole.

Materials and Methods: In this experimental study, mature, six-week-old female Sprague-Dawley rats (n=20) were divided into four groups: control (standard diet); letrozole (letrozole plus a standard diet); HFD; and letrozole+HFD. After 16 weeks, the rats underwent vaginal smear analysis, measurement of hormonal and lipid profiles, and an oral glucose tolerance test (OGTT). Ovarian tissue morphology, oxidative parameters, and inflammatory status were evaluated.

Results: The experimental groups exhibited anoestrus profiles in the vaginal smears and abnormal ovarian morphology, which was not observed in the control group. Steroid hormone levels were significantly higher in the letrozole+HFD group compared to the other groups (P=0.00). The experimental groups also showed abnormal glucose levels and lipid metabolism. The relative expression levels of inflammatory genes were significantly elevated in the experimental groups compared to the control group (P=0.00), and the letrozole+HFD group exhibited the highest expression level (P=0.00). The HFD, letrozole, and letrozole+HFD groups demonstrated significantly increased levels of malondialdehyde (MDA) and reactive oxygen species (ROS), while the levels of enzymatic antioxidants were significantly reduced compared to the control group (P=0.00).

Conclusion: The combination of a new HFD and letrozole treatment induces inflammation and oxidative stress (OS) in a rat model of PCOS. This model accurately exhibits abnormal metabolic phenotypes and disruptions in hormonal profiles associated with PCOS.

Keywords: High-Fat Diet, Inflammation, Oxidative Stress, Polycystic Ovary Syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is an endocrine disorder that affects various stages of a woman's reproductive life (1, 2). The clinical manifestations of women with PCOS are highly variable and often include menstrual dysfunction, hyperandrogenism, and metabolic disorders such as obesity, insulin resistance, type 2 diabetes, hypertension, and dyslipidaemia, with an increased risk of cardiovascular disease (3-6). Additionally, oxidative stress (OS) and low-grade inflammation are factors in the pathogenesis of this syndrome. OS in women with PCOS is associated with metabolic disorders, and an association exists between markers of OS and inflammation with high androgen levels. However, as our understanding of the pathological symptoms of PCOS grows, it is important to examine this disorder from an inflammatory and oxidative perspective. This may lead to a better understanding of the underlying mechanisms of PCOS, which is essential for transitioning from a symptomatic treatment approach to a definitive treatment approach. Recent studies have established the presence of low-grade inflammation and

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OS in women with PCOS. OS is defined as an "oxidant/antioxidant imbalance in favour of the former, which leads to cell damage," while low-grade inflammation is a pathological condition characterised by elevated inflammatory markers (1).

Despite the high prevalence of PCOS, its etiopathogenesis remains unclear (6). Ethical limitations for human studies have led to the development of animal models to further understand PCOS pathogenesis. Among the various animal models that exhibit a wide range of features related to human PCOS, rodent models provide a versatile tool for determining the exact biological mechanisms involved in the development of PCOS (7). PCOS models in rodents have been induced through various methods, including prenatal and postnatal treatment with androgens, oestrogens, aromatase inhibitors, anti-progesterone factors, high-fat diets (HFD), permanent light exposure, and genetic manipulations (8, 9).

PCOS induced by letrozole and HFD are two widely used models that have received special attention regarding morphological, endocrine, and metabolic characteristics; however, investigations of OS and inflammation are insufficient. Letrozole, a cytochrome P450 aromatase inhibitor, blocks the conversion of testosterone to oestradiol (8, 10), leading to hyperandrogenism (7, 8). Letrozole also causes similar changes to those seen in women with PCOS, such as weight gain, increased ovarian size, thickening of the theca inner cell layer, and anovulation (7, 11). However, in the letrozole model, no metabolic abnormalities such as insulin resistance, adiposity, and dyslipidaemia have been observed. On the other hand, HFD-induced PCOS is associated with metabolic disorders similar to those observed in some women with PCOS (12). Previous HFD models did not adequately simulate metabolic factors. Therefore, in this study, we aim to establish a rat model of PCOS using letrozole in combination with a new HFD to investigate hormonal, metabolic, oxidative, and inflammatory status.

Materials and Methods

Animals

In this experimental study, 20 female Sprague-Dawley rats, aged 6 weeks and weighed 180-200 g, were obtained from the Pasteur Institute of Iran. The rats were housed in the animal facility under controlled conditions of a 12/12-hour light/dark cycle, temperature range of 22-24°C, and humidity of 45 ± 2%.

Experimental design

The rats were randomly divided into four groups with five rats per group. i. The control group consumed a standard laboratory diet that consisted of 3.14 kcal/g with an energy supply ratio of 21.5% protein, 65% carbohydrate, and 4% fat. Additionally, they received 5 ml of 0.5% carboxymethyl cellulose (CMC) as a vehicle once per day for four weeks. ii. Letrozole group rats received daily gavages of letrozole (1 mg/kg body weight) dissolved in 0.5 ml of CMC for four weeks. They were also fed the standard laboratory diet. iii. HFD group rats consumed an HFD composed of 5.3 kcal/g, with an energy supply ratio of 20% protein, 36% carbohydrate, and 40% fat. The HFD also contained 1.25% cholesterol, 23.1 g/L d-fructose, and 18.9 g/L d-glucose. Similar to the control group, they received the vehicle (CMC) daily for 16 weeks. iv. The letrozole+HFD group received letrozole dissolved in 0.5 ml of 0.5% CMC daily for four weeks through gavage. They were fed the HFD for 16 weeks.

The rats were measured weekly to assess changes in weight. Oestrous cycle changes were determined by evaluating the cellular composition of vaginal smears ten weeks after initiation of gavage, as described previously (13). At the end of the experiment, all rats were euthanized by decapitation under deep anaesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg).

Oral glucose tolerance test

During the last week of the experiment, an oral glucose tolerance test (OGTT) was performed. The rats fasted for 15 hours, then blood samples were collected from the tail veins (time 0). The rats subsequently received a glucose solution (2 g/kg body weight) through gavage. Additional blood samples were collected from the tail veins at 30, 60, and 120 minutes after glucose administration to measure glucose levels with the Decont Personal Accu-check system. Additionally, the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) was calculated 48 hours after OGTT using the following formula: fasting plasma insulin (mU/l)×fasting plasma glucose (mmol/l)/22.5. Insulin and glucose concentrations were measured by enzyme-linked immunosorbent assay (ELISA; Merck/Merck Millipore, Hungary).

Hormone assay

Serum testosterone, progesterone, and oestradiol levels were measured using an ELISA kit (Demeditec, Germany) following the manufacturer's instructions. In order to accurately assess hormonal changes, blood samples were taken from all of the rats at similar stages of the oestrous cycle.

Lipid profile assay

Serum levels of total cholesterol (TC, mmol/L), triglycerides (TG, mmol/L), low-density lipoprotein (LDL, mmol/L), and high-density lipoprotein (HDL, mmol/L) were assessed using standard colorimetric methods.

C-reactive protein assay

Serum C-reactive protein (CRP) contents were analysed by serological analysis. An ELISA kit (Millipore's MILLIPLEX® MAP Rat/Mouse CRP Single Plex, USA) was used for this purpose.

Histological studies

Ovarian samples were taken from mice at similar phases of the oestrous cycle. The ovaries were serially sectioned at 5 µm from the centre and stained with haematoxylin and eosin (H&E). Ovarian follicles in different growth phases, including preantral, antral, and atretic follicles, as well as the corpus luteum, were evaluated in every fifth section of the largest cross-sectional area. Follicles were classified as previously described (14). Additionally, the thickness of the largest follicular wall, including the theca and granulosa layers, was measured.

Gene expression analysis

Total RNA was extracted from ovaries using TRIzol (Qiagen, USA) following the manufacturer's instructions. The RNA was treated with DNase I (Cinnagen, Iran). cDNA synthesis was performed using a RevertAid kit (Fermentas, MD, USA) according to the manufacturer's protocol. Gene-specific primer sets were designed using AlleleID software version 7.5 (DBA Oligo, Inc., USA). The primer sequences are listed below:

Tnf-α-F: 5′-TCAGCCTCTTCTCATTCC-3′
R: 5′-ACTTCTCCTCCTTGTTGG-3′
Mcp-1-F: 5′-TGGTGGTCTGTGGTGCTAAG-3′
R: 5′-AACTGGAGGCTTGGTAGAATGAG-3′
Il-1β-F: 5′-CACTATTCCTAATGCCTTCC-3′
R: 5′-TCTGAGAGACCTGACTTG-3′
Ef1-F: 5′-AGTCGCCTTGGACGTTCTT-3′
R: 5′-CCGATTACGACGATGTTGATGTG-3′

Real-time polymerase chain reaction (PCR) was performed using an ABI StepOne machine (Applied Biosystems, ABI, USA) and RealQ Plus SYBR Green (Ampliqon, Denmark). The qPCR protocol included one cycle at 95ºC for 15 minutes, followed by 40 cycles of 95ºC for 15 seconds and 60ºC for 45 seconds, following the MIQE guidelines (15). The Ef1 gene was used as the reference gene. Relative mRNA levels were calculated using the $2^{-\Delta\Delta CT}$ method. A no-template control tube for each gene was included in all experiments.

Evaluation of oxidative status

Tissue supernatant was prepared from ovarian tissue to evaluate total antioxidant capacity (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT) activities, and malondialdehyde (MDA) levels as previously described (16, 17). The production of reactive oxygen species (ROS) in ovarian tissue was measured using the 2′,7′- dichlorodihydrofluorescein (DCHF) probe, following a method described previously (17). TAC levels were measured using the ferric reducing/ antioxidant power (FRAP) method as previously described (17) and presented as mol/L. MDA levels were assessed as an index of lipid peroxidation, following a previously described method (16), and presented as nmol/mg protein. GPX, SOD, and CAT activities were measured according to previously described methods (16). GPX activity was defined as the conversion of NADPH to NADP and measured by monitoring absorption changes at 340 nm. One unit of SOD activity was measured by calculating the 50% inhibition of nitro blue tetrazolium reduction. CAT activity was calculated based on absorbance changes in one minute and presented as µMol/min/mg protein. The total protein concentration in the tissue supernatant was measured using the Lowry assay method (18).

Statistical analysis

The data were analysed using SPSS version 16 software package for Windows (SPSS Inc., Chicago, IL, USA). The statistical analysis involved conducting one-way ANOVA followed by Bonferroni post hoc analysis to assess group differences. The Bonferroni adjustment was applied to control for multiple comparisons and maintain the overall significance level. This adjustment helps reduce the likelihood of obtaining false positive results when conducting multiple pairwise comparisons and ensures that the observed differences between groups are not simply due to random chance. A $P<0.05$ was considered statistically significant and indicated meaningful differences between the groups. The results are presented as mean ± SD, providing an indication of the central tendency and variability of the data.

Ethical consideration

All procedures were approved by the Laboratory Animal Ethics Committee of Damghan University, Damghan, Iran (IR.BSDU.REC.1399.14).

Results

Weight changes

There were no significant differences in body weights among the different groups at the start of the experiment. However, after the first four weeks of treatment, the weight of the rats in the HFD-treated and letrozole+HFD-treated groups significantly increased compared to the control and letrozole groups ($P=0.002$, Table 1). Conversely, there was no significant difference in weight between the letrozole and control groups. Interestingly, the letrozole+HFD group showed the highest weight gain compared to the other groups ($P=0.004$, Table 1).

Vaginal smears

The rats treated with letrozole and letrozole+HFD exhibited complete acyclicity. The vaginal smears of the letrozole-treated and letrozole+HFD-treated rats showed a higher presence of leukocytes, which indicated pseudo dioestrus. In contrast, the vaginal smears of the control and HFD rats displayed a regular oestrous cycle (Fig.1).

Table 1: Changes in body weights of rats under different treatment conditions

Weeks/Groups	Control	Letrozole	HFD	Letrozole+HFD
6	199 ± 7.7	202.5 ± 7.0	195.2 ± 6.6	202.5 ± 10.1
7	222.5 ± 7.3	224.7 ± 7.5	219.0 ± 9.2	228.3 ± 11.3
8	236.5 ± 7.3	240.3 ± 12.5	236.5 ± 16.4	240.8 ± 10.1
9	261.5 ± 6.1	264.3 ± 7.0	258.5 ± 9.8	264.5 ± 9.8
10	291.5 ± 5.1[a]	296.2 ± 5.7[a]	301.5 ± 5.1[b]	321.2 ± 5.7[c]
11	315.5 ± 5.2[a]	320.8 ± 5.5[a]	325.5 ± 5.1[b]	345.8 ± 5.5[c]
12	337.5 ± 4.1[a]	346.3 ± 5.0[a]	352.5 ± 5.1[b]	386.3 ± 5.0[c]
13	338.5 ± 5.5[a]	354.8 ± 13.2[a]	353.5 ± 5.1[b]	394.8 ± 13.2[c]
14	363.5 ± 7.1[a]	370.7 ± 7.3[a]	388.5 ± 5.1[b]	425.7 ± 7.3[c]
15	392.5 ± 5.3[a]	399.2 ± 6.4[a]	417.5 ± 5.1[b]	454.2 ± 6.4[c]
16	410.5 ± 5.1[a]	418.3 ± 7.3[a]	448.5 ± 5.1[b]	486.3 ± 7.3[c]
17	435.5 ± 6.4[a]	445.2 ± 17.2[a]	473.5 ± 6.4[b]	513.2 ± 17.2[c]
18	475.8 ± 11.7[a]	485.2 ± 11.9[a]	509.5 ± 4.4[b]	565.2 ± 11.9[c]
19	496.0 ± 13.3[a]	507.5 ± 14.7[a]	549.7 ± 4.3[b]	587.5 ± 14.7[c]
20	515.8 ± 11.7[a]	520.0 ± 11.0[a]	559.5 ± 10.3[b]	610.0 ± 11.0[c]
21	533.0 ± 12.0[a]	547.5 ± 15.6[a]	581.7 ± 4.4[b]	637.5 ± 15.6[c]

Data are presented as mean ± SD. The body weights of the rats were recorded on a weekly basis. Different letters indicate a significant difference among the groups. The statistical analysis involved conducting one-way ANOVA followed by Bonferroni post hoc analysis. HFD; High-fat diet.

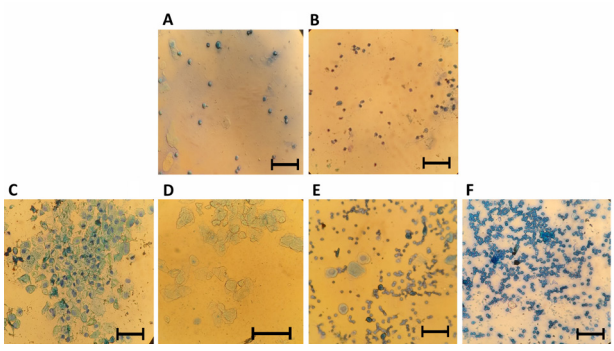

Fig.1: Cellular types in vaginal smears of the experimental groups. **A.** Vaginal smear from the letrozole group. **B.** Vaginal smear from the letrozole+HFD group. Vaginal smears from the HFD and control groups with regular oestrous cycles. **C.** Proestrus. **D.** Estrus. **E.** Metestrus. **F.** Dioestrus (scale bar: 100 µm). HFD; High-fat diet.

Oral glucose tolerance test

There were significant differences in blood glucose levels (BGL) among all the experimental groups at 0, 60, and 120 minutes. The letrozole+HFD group showed significantly higher BGL at 0 minutes, 60 minutes, and 120 minutes compared to the other groups (P=0.00). Additionally, the letrozole and HFD groups exhibited significantly increased BGL at 0, 60, and 120 minutes compared to the control group (P=0.03). BGL of the HFD group at 0 minutes, 60 minutes, and 120 minutes were significantly higher than those of the letrozole group (P=0.00, Table 2).

Homeostasis model assessment of insulin resistance

Homeostasis model assessment of insulin resistance (HOMA-IR) significantly increased in the letrozole+HFD group compared to the other groups (P=0.004). Furthermore, HOMA-IR significantly increased in the letrozole and HFD groups compared to the control group (P=0.03). The HFD group had significantly higher HOMA-IR than the letrozole group (P=0.01, Table 2).

Ovarian morphology

Ovarian sections from the control and HFD groups exhibited several corpus luteous (CL) and follicles at different growth stages, while no CL was observed in the letrozole and letrozole+HFD groups (Fig.2). The ovary weights in both the control and HFD groups were significantly higher than in the letrozole and letrozole+HFD groups (P=0.00). The number of preantral follicles was significantly higher in the letrozole and letrozole+HFD groups compared to the control and HFD groups (P=0.00, Table 2).

There was no significant difference between the control and HFD groups, while the number of preantral follicles in the letrozole+HFD group was significantly higher than in the letrozole group (P=0.00).

No significant difference was observed in the number of antral follicles between the control and HFD groups. However, the number of antral follicles in the letrozole+HFD and letrozole groups was significantly higher than in the control and HFD groups (P=0.009). The CL count in both the control and HFD groups was significantly higher than in the letrozole and letrozole+HFD groups (P=0.00). Additionally, there were significantly less atretic follicles in the control group compared to the other groups (P=0.00). The letrozole+HFD group

exhibited the highest number of atretic follicles (P=0.001), and the number of atretic follicles in the letrozole+HFD group was significantly higher than in the letrozole group (P=0.01).

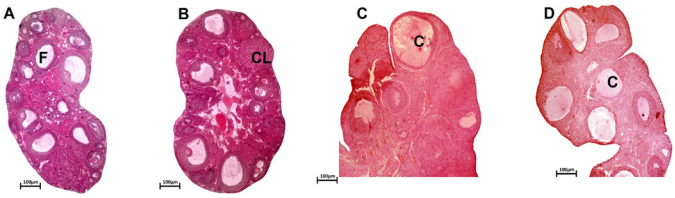

Fig.2: Ovarian histology. Micrographs correspond to the largest section of the haematoxylin-eosin (H&E) stained ovary. **A.** Ovary from the control group. **B.** Ovary from the HFD group. **C.** Ovary from the control letrozole group. **D.** Ovary from the control letrozole+HFD group (scale bar: 100 µm). HFD; High-fat diet, F; Follicle, CL; Corpus Loteum, and C; Cyst.

The mean number of total cystic follicles showed a significant difference among all groups. The control group had a significantly lower number of cystic follicles compared to the other groups. In the letrozole+HFD group, the mean number of total cystic follicles was significantly higher compared to the HFD and letrozole groups (P=0.002). Additionally, the thickness of the follicle wall in the letrozole and letrozole+HFD groups was significantly increased compared to the control and HFD groups. However, the thickness of the follicle wall in the letrozole+HFD group showed a significant decrease compared to the letrozole group (P=0.013).

Hormone assay

Oestrogen levels were significantly lower in the letrozole and letrozole+HFD groups compared to the control and HFD groups (P=0.004). There was no significant difference between the letrozole and letrozole+HFD groups (P=0.001). Additionally, there was no significant difference in oestrogen levels between the control and HFD groups. Progesterone levels were significantly decreased in the letrozole and letrozole+HFD groups compared to the control and HFD groups (P=0.009). No significant difference was observed in progesterone levels between the control and HFD groups. The same pattern was observed for the letrozole and letrozole+HFD groups. Serum testosterone was significantly higher in the letrozole+HFD group compared to the other groups (P=0.001). Testosterone levels did not significantly change in the HFD group compared to the control group. However, testosterone levels were significantly increased in the letrozole group compared to the control group (P=0.03, Table 2).

Lipid profile assay

TC and LDL levels in the letrozole+HFD group were significantly higher than in the other groups (P=0.001). There was no significant difference between the letrozole and HFD groups in terms of TC and LDL levels. The TC and LDL levels in the control group were significantly lower than in the other groups (P=0.006). Additionally, there was no significant difference among HDL levels (P=0.004, Table 2).

C-reactive protein assay

CRP levels were significantly increased in the letrozole+HFD group compared to the other groups (P=0.014). Furthermore, CRP levels were significantly higher in the letrozole and HFD groups compared to the control group (P=0.001). However, CRP levels in the HFD group were significantly lower than in the letrozole group (P=0.006, Table 2).

Table 2: Ovarian morphological parameters, lipid and hormonal profile, and CRP of the experimental groups

Groups	Control	Letrozole	HFD	Letrozole+HFD
Ovary weight (Kg)	0.15 ± 0.02^{a}	0.27 ± 0.05^{b}	0.16 ± 0.04^{a}	0.25 ± 0.02^{b}
Preantral follicle	4.50 ± 1.05^{a}	9.50 ± 1.05^{b}	5.17 ± 0.98^{a}	14.00 ± 2.00^{b}
Antral follicle	3.00 ± 0.63^{a}	0.67 ± 0.52^{b}	3.00 ± 1.10^{a}	0.50 ± 0.55^{b}
Corpus luteum	4.17 ± 1.17^{a}	0.00 ± 0.00^{b}	1.33 ± 0.52^{c}	0.00 ± 0.00^{b}
Atretic follicles	0.83 ± 0.75^{a}	6.83 ± 1.17^{b}	2.17 ± 0.75^{c}	11.67 ± 1.63^{d}
Cystic follicles	0.50 ± 0.55^{a}	5.67 ± 1.033^{b}	3.33 ± 1.03^{c}	11.83 ± 1.06^{d}
Thickness of follicle wall (µm)	61.5 ± 7.31^{a}	121.83 ± 7.65^{b}	68.00 ± 3.63^{a}	135.83 ± 14.80^{b}
Oestrogens (pg/ml)	13.5 ± 3.08^{a}	4.67 ± 1.86^{b}	15.17 ± 4.58^{a}	5.50 ± 2.07^{b}
Testosterone (pg/ml)	0.19 ± 0.04^{a}	0.33 ± 0.05^{b}	0.19 ± 0.04^{a}	0.46 ± 0.05^{c}
Progesterone (ng/ml)	53.33 ±7.20^{a}	23.50 ± 6.57^{b}	54.33 ± 8.94^{a}	22.33 ± 5.47^{b}
TC (mmol/ml)	2.28 ± 0.35^{a}	2.95 ± 0.32^{b}	3.27 ± 0.27^{b}	3.89 ± 0.25^{c}
HDL (mmol/ml)	1.18 ± 0.19	1.03 ± 0.35	1.10 ± 0.34	1.05 ± 0.50
LDL (mmol/ml)	0.30 ± 0.04^{a}	0.43 ± 0.030^{b}	0.44 ± 0.03^{b}	0.50 ± 0.03^{c}
CRP (pg/ml)	14.02 ± 1.28^{a}	21.68 ± 1.03^{b}	17.35 ± 0.82^{c}	30.36 ± 1.44^{d}
HOMA-IR	5.09 ± 0.17	7.85 ± 0.40	12.48 ± 1.28	14.64 ± 1.17
BGL at 0 minute	5.93 ± 0.26	6.77 ± 0.25	7.43 ± 0.17	7.82 ± 0.12
BGL at 60 minutes	6.93 ± 0.25	8.22 ± 0.24	9.37 ± 0.25	10.92 ± 0.21
BGL at 120 minutes	6.13 ± 0.26	7.27 ± 0.25	8.23 ± 0.2	8.82 ± 0.12

Values are presented as mean ± SD. One-way ANOVA was performed for data analysis. A P<0.05 indicates statistical significance. Different letters are used to indicate significant differences within the same row. HFD; High-fat diet, HOMA-IR; Homeostasis model assessment of insulin resistance, TC; Total cholesterol, LDL; Low-density lipoprotein, HDL; High-density lipoprotein, BGL; Blood glucose levels, and CRP; C-reactive protein.

Gene expression analysis

We evaluated the mRNA transcripts of *Tnf-α*, *Wnt4*, *Il-1β*, and *Mcp-1* as they are involved in ovarian tissue inflammation (Fig.3). The relative mRNA levels of *Tnf-α* showed significant differences among the experimental groups (P=0.003). *Tnf-α* mRNA levels significantly increased in the letrozole+HFD group compared to the other groups (P=0.012), while there was no significant difference between the letrozole and HFD groups. Additionally, the expression level of *Tnf-α* mRNA showed a significant increase in the experimental groups compared to the control group (P=0.009). Relative expression levels of *Il-1β* increased significantly in the letrozole+HFD group compared to the other groups (P=0.009). However, there was no significant difference between the letrozole and HFD groups. Furthermore, the expression levels of *Mcp-1* mRNA in the letrozole+HFD group significantly increased compared to the other groups (P=0.012), while there was no significant difference between the letrozole and HFD groups. The expression level of *Mcp-1* mRNA in the control group was significantly lower compared to the other groups (P=0.001).

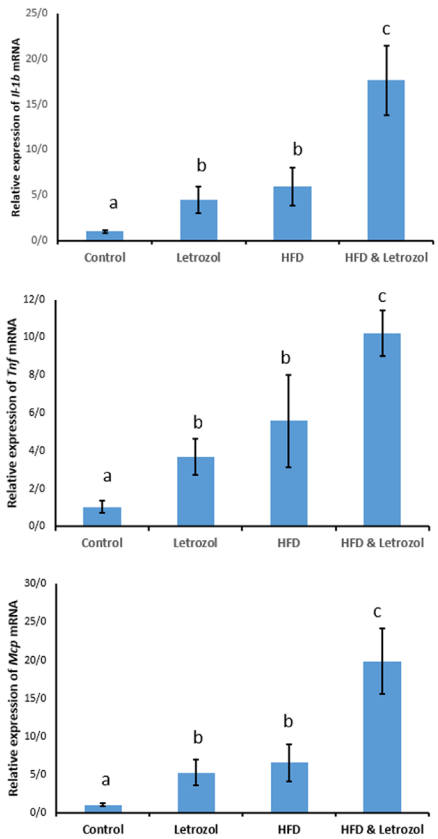

Fig.3: Relative mRNA expression levels of *Il-1β*, tumour necrosis factor-alpha (*Tnf-α*), and *Mcp-1*. The data are presented as mean ± SD, obtained from three independent experiments. P<0.05 indicate statistical significance. Different letters indicate significant differences among the groups. HFD; High-fat diet.

Evaluation of oxidative status

Figure 4 shows the OS parameters in the experimental groups. ROS significantly increased in the letrozole+HFD group compared to the other groups (P=0.002). In the letrozole group, there was a significant increase in ROS levels compared to the HFD group (P=0.006), while both groups had significantly higher ROS levels than the control group (P=0.003). TAC levels significantly decreased in the experimental groups compared to the control group (P=0.009). Furthermore, TAC levels were significantly lower in the letrozole+HFD group compared to the HFD and letrozole groups (P=0.002). Additionally, TAC levels were significantly lower in the letrozole groups compared to the HFD group (P=0.001).

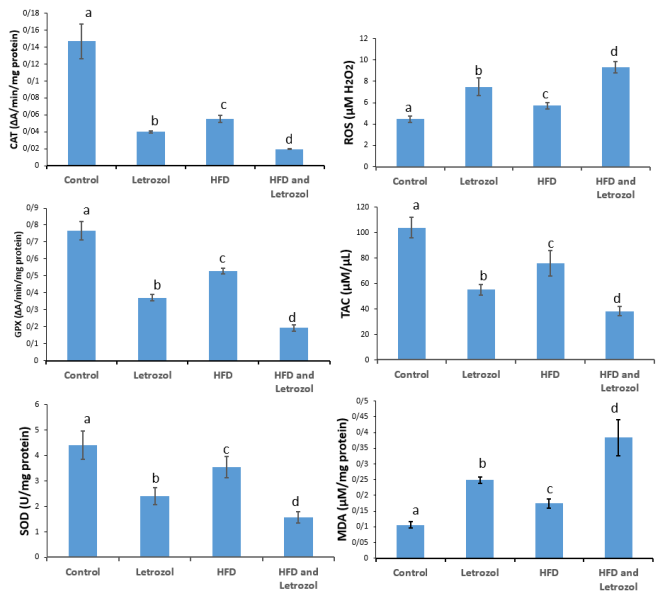

Fig.4: Oxidative stress (OS) parameters in ovarian tissue of the experimental groups. The results are presented as mean ± SD. Different letters indicate significant differences between groups (P<0.05). CAT; Catalase, ROS; Reactive oxygen species, GPX; Glutathione peroxidase, TAC; Total antioxidant capacity, SOD; Superoxide dismutase, and MDA; Malondialdehyde.

The concentrations of MDA in the letrozole+HFD group were significantly higher than in the other groups (P=0.006). Similarly, the MDA concentration was significantly lower in the HFD group compared to the letrozole groups, while both groups had significantly higher MDA levels than the control group (P=0.003).

CAT, GPX, and SOD were significantly decreased in the letrozole+HFD group compared to the other groups (P=0.003). Conversely, the activities of CAT, GPX, and SOD were significantly higher in the control group compared to the other groups (P=0.005). CAT, GPX, and SOD in the letrozole and HFD groups were significantly lower compared to the control group (P=0.006), while the activities of CAT, GPX, and SOD were significantly higher in the HFD group compared to the letrozole group (P=0.001).

Discussion

The results of the present study align with previous studies conducted in rats regarding the animal model characteristics and ovarian and endocrine changes associated with PCOS (8, 19). However, the novelty of

this study lies in the utilisation of a new diet regimen combined with letrozole in the rat model, as well as the investigation of inflammatory and oxidative profiles. This study successfully established a rat PCOS model that used a new HFD in conjunction with letrozole, which resulted in the manifestation of PCOS characteristics observed in humans.

Several studies have examined the reproductive and metabolic characteristics of rodent models of PCOS. HFD induce various metabolic changes in animals. The combination of androgens with HFD can induce a PCOS model that exhibits both ovarian and metabolic characteristics of PCOS (9). Vaginal smear analysis is widely accepted as a key indicator of ovarian physiological function (13). In the PCOS rat model, acyclic vaginal smears were observed, which indicated disrupted oestrous cycles in the letrozole+HFD group. Hormonal profiles were also disrupted and led to morphological changes in the ovarian tissues. Histopathological assessment revealed a high number of cystic follicles with thickened walls, attributed to the thickened theca cell layer, as well as a decrease in the corpus luteum, which was consistent with findings from other studies (9).

The PCOS rats exhibited a significant increase in body weight. In recent years, the role of nutrition in PCOS has been recognized in reproductive research, particularly the strong association between obesity and PCOS. Obese individuals are more susceptible to menstrual irregularities, hyperandrogenism, and hirsutism (12). Therefore, diet plays a crucial role in the incidence and severity of PCOS (20). HFD can exacerbate the effects of androgens, which leads to glucose intolerance and insulin resistance (8). The results of the present study support previous findings where HFD and letrozole significantly contributed to weight gain (19). Overweight individuals are widely known to experience hyperandrogenism, anovulation, and are at increased risk for metabolic syndrome (8, 9), which aligns with the results of the present study. Hyperandrogenism is a major factor responsible for abnormal ovarian physiology and a prominent characteristic of PCOS, often leading to irregular reproductive cycles. In this study, letrozole+HFD-treated rats exhibited anovulation and hyperandrogenism. Therefore, the new HFD diet combined with letrozole shows promise in inducing a PCOS model by impairing glucose intolerance, insulin resistance, and hormonal profiles.

Letrozole inhibits the conversion of androgens to oestrogens in the ovary (8, 10, 19), which results in decreased oestrogen and progesterone levels and increased testosterone levels (7). The hormonal changes observed in the letrozole and letrozole+HFD-treated rats in this study support these findings. The hormonal alterations in HFD and letrozole+HFD-treated rats could be attributed to insulin resistance and hyperinsulinemia. Insulin activates the inositol phosphoglycan pathway in theca cells by reducing hepatic sex hormone-binding globulin production, enhancing the effect of LH on theca cells, and ultimately causing hyperandrogenism (19). Moreover, hyperinsulinemia disrupts gonadotropin secretion and increases LH receptor expression, which stimulates the ovaries to produce androgens, and leads to impaired follicular growth and anovulation. The findings of our study are in line with previous research that support the role of insulin resistance, hyperandrogenism, and abnormal gonadotropin secretion as key factors in the pathophysiology of PCOS. However, there is an ongoing debate of whether insulin resistance precedes the other criteria. The insulin-gonadotropin-like activity affects ovarian steroidogenesis, disrupts insulin signalling in the brain, and ultimately impacts ovulation and body weight. Therefore, an assessment of glucose intolerance using the OGTT method is considered more useful than measuring fasting glucose levels alone when evaluating PCOS patients (21). In our study, the results indicate that the OGTT test can effectively reflect insulin resistance, which is consistent with previous findings (10). Feeding adult female rats with an HFD for 120 days can induce insulin resistance and infertility. Our results suggest that letrozole+HFD treatment may have a more direct effect on insulin signalling pathways compared to letrozole alone. However, describing the underlying mechanisms will be a challenge for future studies.

Another aspect examined in our study was the lipid profile. Both HFD and letrozole exhibited abnormal lipoprotein profiles. It is estimated that 70% of PCOS patients have abnormal serum lipid levels. Insulin resistance is believed to be responsible for reducing the activity of lipoprotein lipase and consequently causing dyslipidaemia (19). In our study, both HFD and letrozole, either alone or in combination, increased LDL and TG levels. Elevated LDL and TG levels have also been reported in HFD-treated C57BL/6 mice (22). In letrozole-treated rats, higher TG and LDL levels were observed, and this supported the results of a study where letrozole increased both TG and TC levels (10). In contrast, another study reported no effect on the lipid profile (7, 11).

Objectives of the present study included the evaluation of OS and inflammatory markers in a rat model of PCOS and an investigation of the role of nutrition and obesity in the pathogenesis of PCOS. Growing evidence suggests that inflammation plays a significant role in the development of PCOS. Persistent moderately elevated levels of CRP are characteristic of low-grade chronic inflammation, which is a systemic and chronic condition. PCOS is considered to be part of this inflammatory group. Based on this, our study aimed to examine whether gene expression of inflammatory markers and CRP levels increased in the PCOS rat model. Low-grade chronic inflammation has been associated with insulin resistance syndrome. Our study showed a significant increase in CRP levels in the HFD, letrozole, and letrozole+HFD groups compared to the control group that had normal oestrous cycles. This finding supported with previous data that demonstrated a correlation between CRP levels, obesity, and insulin sensitivity (23). Adipose tissue-

derived cytokine expression is believed to play a key role in low-grade chronic inflammation (1), which aligns with our results of increased gene expressions of inflammatory markers in the HFD, letrozole, and letrozole+HFD groups compared to the control group. In other words, visceral adipose tissue may contribute to the features of low-grade chronic inflammation (1). These observations also support the correlation between insulin sensitivity and CRP reported by Festa et al. (23).

The involvement of OS in PCOS is well-established. Studies show that diet-induced OS, documented by increased ROS production and NFkB activation, leads to an inflammatory response (24). Glucose absorption can affect the production and secretion of Tnf-α and IL-6 from circulating monocytes in PCOS. These findings are consistent with the results of our study. Furthermore, markers of OS are associated with the insulin resistance index (2, 24), which aligns with our observation of changes in the oxidative profile and insulin resistance index in the PCOS rat model. Additionally, hyperandrogenaemia during PCOS can trigger an inflammatory response induced by diet. Administration of oral androgens has been shown to activate mononuclear cells, leading to ROS production, NF/kB activation, and increased Tnf-α mRNA. Therefore, OS may contribute to the induction and/or exacerbation of PCOS (6).

Conclusion

The present study results demonstrate that the combination of a new HFD and letrozole is suitable for studying both the ovarian and metabolic features of PCOS and may be useful for evaluating new treatments. In addition to ovarian changes, this regimen closely mimics clinical PCOS by inducing OS, inflammation, and metabolic disorders in rats.

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Authors' Contributions

S.F.M.; Performed the experiments, meticulously executing the planned procedures and ensuring the accurate collection of data. S.Z.; Played in the inception and design of the experiments, demonstrating a profound understanding of techniques, drafted the paper, crafting a comprehensive and insightful manuscript that presented the research findings and their implications coherently. M.N.; Diligently analyzed the data, employing sophisticated statistical methods to draw meaningful conclusions from the experimental results. Their expertise in data analysis provided valuable insights into the observed trends and correlations. H.H.-M.; Contributed by providing essential reagents, materials, and analysis tools that were crucial to the successful execution of the experiments. Their expertise and support significantly enhanced the experimental process. All authors read and approved the final manuscript.

References

1. Mancini A, Bruno C, Vergani E, d'Abate C, Giacchi E, Silvestrini A. Oxidative stress and low-grade inflammation in polycystic ovary syndrome: controversies and new insights. Int J Mol Sci. 2021; 22(4): 1667.
2. Hernández-Jiménez JL, Barrera D, Espinoza-Simón E, González J, Ortíz-Hernández R, Escobar L, et al. Polycystic ovarian syndrome: signs and feedback effects of hyperandrogenism and insulin resistance. Gynecol Endocrinol. 2022; 38(1): 2-9.
3. Rizk MG, Thackray VG. Intersection of polycystic ovary syndrome and the gut microbiome. J Endocr Soc. 2020; 5(2): bvaa177.
4. Chaudhary H, Patel J, Jain NK, Joshi R. The role of polymorphism in various potential genes on polycystic ovary syndrome susceptibility and pathogenesis. J Ovarian Res. 2021; 14(1): 125.
5. Carvalho LML, Dos Reis FM, Candido AL, Nunes FFC, Ferreira CN, Gomes KB. Polycystic ovary syndrome as a systemic disease with multiple molecular pathways: a narrative review. Endocr Regul. 2018; 52(4): 208-221.
6. Nehir Aytan A, Bastu E, Demiral I, Bulut H, Dogan M, Buyru F. Relationship between hyperandrogenism, obesity, inflammation and polycystic ovary syndrome. Gynecol Endocrinol. 2016; 32(9): 709-713.
7. Osuka S, Nakanishi N, Murase T, Nakamura T, Goto M, Iwase A, et al. Animal models of polycystic ovary syndrome: A review of hormone-induced rodent models focused on hypothalamus-pituitary-ovary axis and neuropeptides. Reprod Med Biol. 2018; 18(2): 151-160.
8. Wang MX, Yin Q, Xu X. A rat model of polycystic ovary syndrome with insulin resistance induced by letrozole combined with high fat diet. Med Sci Monit. 2020; 26: e922136.
9. Zhang H, Yi M, Zhang Y, Jin H, Zhang W, Yang J, et al. High-fat diets exaggerate endocrine and metabolic phenotypes in a rat model of DHEA-induced PCOS. Reproduction. 2016; 151(4): 431-441.
10. Reddy P, Begum N, Mutha S, Bakshi V. Beneficial effect of curcumin in letrozole induced polycystic ovary syndrome. Asian Pac J Reprod. 2016; 5(2): 116-122.
11. Ryu Y, Kim SW, Kim YY, Ku SY. Animal models for human polycystic ovary syndrome (PCOS) focused on the use of indirect hormonal perturbations: a review of the literature. Int J Mol Sci. 2019; 20(11): 2720.
12. Wu S, Divall S, Nwaopara A, Radovick S, Wondisford F, Ko C, et al. Obesity-induced infertility and hyperandrogenism are corrected by deletion of the insulin receptor in the ovarian theca cell. Diabetes. 2014; 63(4): 1270-1282.
13. Bagheripour N, Zavareh S, Ghorbanian MT, Paylakhi SH, Mohebbi SR. Changes in the expression of OCT4 in mouse ovary during estrous cycle. Vet Res Forum. 2017; 8(1): 43-48.
14. Myers M, Britt KL, Wreford NG, Ebling FJ, Kerr JB. Methods for quantifying follicular numbers within the mouse ovary. Reproduction. 2004; 127(5): 569-580.
15. Nasiri M, Saadat M, Karimi MH, Azarpira N, Saadat I. Evaluating mRNA Expression Levels of the TLR4/IRF5 Signaling Axis During Hepatic Ischemia-Reperfusion Injuries. Exp Clin Transplant. 2019; 17(5): 648-652.
16. Hosseinzadeh E, Zavareh S, Lashkarbolouki T. Antioxidant properties of coenzyme Q10-pretreated mouse pre-antral follicles derived from vitrified ovaries. J Obstet Gynaecol Res. 2017; 43(1): 140-148.
17. Talebi A, Zavareh S, Kashani MH, Lashgarbluki T, Karimi I. The effect of alpha lipoic acid on the developmental competence of mouse isolated preantral follicles. J Assist Reprod Genet. 2012; 29(2): 175-183.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951; 193(1): 265-275.
19. Xu J, Dun J, Yang J, Zhang J, Lin Q, Huang M, et al. Letrozole rat model mimics human polycystic ovarian syndrome and changes in insulin signal pathways. Med Sci Monit. 2020; 26: e923073.
20. Ressler IB, Grayson BE, Ulrich-Lai YM, Seeley RJ. Diet-induced obesity exacerbates metabolic and behavioral effects of polycystic ovary syndrome in a rodent model. Am J Physiol Endocrinol Metab.

2015; 308(12): E1076-E1084.
21. Patel R, Shah G. High-fat diet exposure from pre-pubertal age induces polycystic ovary syndrome (PCOS) in rats. Reproduction. 2018; 155(2): 141-151.
22. Lai H, Jia X, Yu Q, Zhang C, Qiao J, Guan Y, et al. High-fat diet induces significant metabolic disorders in a mouse model of polycystic ovary syndrome. Biol Reprod. 2014; 91(5): 127.
23. Festa A, D'Agostino R Jr, Howard G, Mykkänen L, Tracy RP, Haffner SM. Chronic subclinical inflammation as part of the insulin resistance syndrome: the Insulin Resistance Atherosclerosis Study (IRAS). Circulation. 2000; 102(1): 42-47.
24. González F, Rote NS, Minium J, Kirwan JP. Increased activation of nuclear factor kappaB triggers inflammation and insulin resistance in polycystic ovary syndrome. J Clin Endocrinol Metab. 2006; 91(4): 1508-1512.

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Sex-Specific Total Testosterone and Dehydroepiandrosterone Sulfate Status in Noncritically Ill Hospitalized Patients with Coronavirus Disease 2019: A Cross-Sectional Study

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Abstract

Background: In individuals with coronavirus disease 2019 (COVID-19), male subjects have consistently been linked to poor severity and prognosis. Data on sex hormones in non-critical COVID-19-infected patients are scarce. The aim of this study was to assess the status of total testosterone (TT) and dehydroepiandrosterone sulfate (DHEAS) among noncritical patients with COVID-19 according to sex and their associations with clinical and biochemical features.

Materials and Methods: This cross-sectional observational study was done in the COVID-19 unit of a University hospital during the period of September 2021 to February 2022 among 91 adults (18-65 years) with reverse transcriptase-polymerase chain reaction confirmed noncritical COVID-19 patients. Blood was drawn by venipuncture before receiving steroids between 07:00 to 09:00 a.m. in a fasting state to measure serum TT and DHEAS by chemiluminescent microparticle immunoassay. Diagnosis and classification of COVID-19 were done according to World Health Organization's interim guidance. Age- and sex-specific laboratory reference values were used to classify the TT and DHEAS status of the patients.

Results: Only three males (8.1%) had low TT and the rest had normal TT. On the other hand, 15 (27.8%) of the females had high TT with normal levels in the rest. Similarly, 11 (29.7%) males had low DHEAS. Females had low, normal, and high DHEAS in four (7.4%), 48 (88.9%), and two (3.7%) cases respectively. Males with moderate severity of COVID-19 had significantly lower DHEAS (post hoc P=0.038) than the mild group. Both TT (P=0.008) and DHEAS (P=0.023) significantly correlated with neutrophils/lymphocytes ratio and only DHEAS with platelets/lymphocytes ratio (P=0.044) in males. In females, TT significantly correlated with serum sodium (P=0.034).

Conclusion: In noncritical COVID-19 patients, substantial gender variations in TT and DHEAS were detected and correlated with severity markers in males.

Keywords: Androgen, Coronavirus Disease 2019, Dehydroepiandrosterone Sulfate, Noncritical, Testosterone

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Introduction

The global pandemic of coronavirus disease 2019 (COVID-19) has affected people all over the world. Several plausible causes, including immune system abnormalities, behavioral factors, and changes in sex hormones may be associated with an increased predilection for male sex for this respiratory virus (1). Evidence showed a higher prevalence of androgenic alopecia in hospitalized males with COVID-19 but a lower prevalence of the disease in prostate cancer patients on antiandrogen therapy. Similarly, women with polycystic ovary syndrome (a mild hyperandrogenic condition) are at increased risk of COVID-19 (2). These indicate a potential role of male sex hormones in the

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susceptibility to COVID-19. Sex hormones regulate different receptors and proteins such as androgen receptors, angiotensin-converting enzyme 2 (ACE2), and transmembrane serine protease 2 (TMPRSS2) that are related to the pathogenesis of COVID-19 (3). Targeting these sites may have therapeutic implications in COVID-19 that are currently being studied in antiandrogen like spironolactone (4). On the other hand, dehydroepiandrosterone (DHEA) may increase the susceptibility to COVID-19 by inhibiting glucose-6-phosphate dehydrogenase (5).

The causative organism, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes inflammatory and vascular changes in testes via ACE2 leading to Sertoli and Leydig cells' dysfunction with impaired hormonal function and fertility (6, 7). Also, Similar adrenal tropism was observed in autopsy cases (8).

Testosterone is the main androgen that is exclusively secreted from male testes with little contribution from the adrenals. However, in addition to synthesis from the ovaries, adrenal DHEA is a significant source of androgen in females. DHEA sulfate (DHEAS) is specific for adrenal glands (9). The levels of these hormones are expected to decrease in COVID-19 patients as the disease is more prevalent in older people who usually have many co-morbidities. However, controversial levels of TT levels are found in recovered patients. Whether low testosterone is a marker of disease severity or a permanent change of COVID-19 is still controversial. TT levels further deteriorated in hypogonadism patients with COVID-19 at admission and even after recovery from COVID-19 (10, 11). Moreover, most of the studies were done among critical patients ignoring the noncritical ones who are the main bulk of the patients and disease survivors. The aim of this study was to see the status of TT and DHEAS among noncritical patients with COVID-19 according to sex and their associations with clinical and biochemical features.

Materials and Methods

This cross-sectional study was done in the COVID-19 unit of a University Hospital during the period of September 2021 to February 2022. The institutional review board of the University approved the study protocol Bangabandhu Sheikh Mujib Medical University (No.BSMMU/2021/557). Informed written consent was taken from each participant.

Adults (18-65 years) with reverse transcriptase-polymerase chain reaction (RT-PCR) confirmed noncritical COVID-19 patients exact test was done as appropriate. Spearman's correlation test was done to see the correlation of TT and DHEAS with different clinical and biochemical that were included after excluding the following criteria: critical COVID-19, known chronic disorders affecting androgen levels (hypogonadism, chronic liver disease, chronic kidney disease, malignancy, heart failure), history of COVID-19 vaccination, history of taking a steroid, testosterone within last three months and serum albumin <2.0 g/dL. For females-pregnancy and lactation, history of taking any hormonal contraceptives within the last three months or having significant hirsutism (modified Ferriman-Gallwey score ≥6), or known cases of any hyperandrogenic disorders were also excluded. History (socio-demographic and symptoms) and relevant physical examinations (height, weight, pulse, respiratory rate, oxygen saturation, and blood pressure) were taken. Initial investigations (complete blood count, electrolytes, C-reactive protein, and D-dimer) at admission were checked and all recorded in a semi-structured questionnaire. The median duration of COVID symptoms was 7 days (4-10 days). Blood was drawn by venipuncture before receiving steroids between 07:00 to 09:00 a.m. in a fasting state within 48 hours of admission to measure serum total testosterone (TT) and DHEAS by chemiluminescent microparticle immunoassay (Siemens, USA).

Diagnosis and classification of noncritical COVID-19 (mild, moderate, and severe) were done according to World Health Organization's interim guidance at admission (12). Age- and sex-specific laboratory reference values were used to classify the TT and DHEAS status of the patients (13).

Data were analyzed by SPSS software version 22.0 (Armonk, NY, IBM Corp). They were expressed in median (inter-quartile range, IQR) or frequency (%). There were some missing data and the available numbers were mentioned within third brackets. Associations between two groups were analyzed by the Mann-Whitney U test and more than two groups by Kruskal-Wallis one-way ANOVA with post hoc Dunn's test for quantitative values. For qualitative variables, Pearson's chi-square or Fisher's variables. Statistical significance was considered with a $P<0.05$.

Results

This study included 91 noncritical COVID-19 patients of whom 37 (40.7%) were males and 54 (59.3%) were females. Both study groups were statistically similar in clinical features and investigation profile except for body mass index (BMI, P=0.026) and neutrophil/lymphocyte ratio (NLR, P=0.046) which were significantly higher in males than females (Table 1).

Considering laboratory reference values, only three males (8.1%) had low TT with the rest of them having normal TT. On the other hand, 15 (27.8%) of the females had high TT with normal levels in the rest of them. Similarly, 11 (29.7%) males had low DHEAS. Females had low, normal, and high DHEAS in four (7.4%), 48 (88.9%), and two (3.7%) cases respectively (Table 2).

Comparison of different clinical and biochemical variables between the different TT and DHEAS statuses showed that males with low DHEAS were significantly younger than those with normal DHEAS (P=0.013). There were no statistically significant differences in any clinical and biochemical variables in females with normal and high TT (Table 3).

Table 1: Characteristics of the study population with respect to sex (n=91)

Variables	Males	Females	P value
Age (Y)	55.0 (31.50-63.0) [37]	50.0 (37.25-60.0) [54]	0.535
Co-morbidities			
Hypertension	15 (40.5)	26 (48.1)	0.525
Diabetes mellitus	16 (43.2)	21 (38.9)	0.828
OLD	6 (16.2)	7 (13.0)	0.763
Symptoms			
Fatigue	25 (67.6)	46 (85.2)	0.070
Cough	27 (73.0)	41 (75.9)	0.809
Fever	25 (67.6)	36 (66.7)	1.00
Dyspnea	21 (56.8)	33 (61.1)	0.828
Headache	10 (27.0)	26 (48.1)	0.052
Signs			
Pulse (bpm)	88.0 (77.0-101.0) [37]	88.0 (80.75-102.0) [54]	0.639
Resp. rate (pm)	14.0 (12.0-18.0) [36]	14.0 [12.0-15.0) [53]	0.527
Systolic BP (mm-Hg)	120.0 (110.0-132.75) [34]	120.0 (108.50-139.50) [49]	0.636
Diastolic BP	80.0 (70.0-84.25) [34]	79.0 (70.0-90.0) [49]	0.752
BMI (kg/m^2)	21.64 (20.32-25.44) [36]	24.44 (20.90-28.30) [52]	0.026
Severity of disease			
Mild	21 (56.8)	33 (61.1)	0.828
Moderate-severe	16 (43.2)	21 (38.9)	
Investigations			
TWBC ($\times10^3/\mu L$)	7.99 (6.50-12.09) [32]	9.65 (7.0-12.38) [45]	0.321
NLR	4.59 (2.82-9.80) [32]	3.0 (1.89-5.86) [45]	0.046
PLR	131.08 (94.84-226.22) [32]	127.57 (77.73-185.45) [45]	0.247
Na^+ (mmol/L)	135.0 (130.0-137.0) [23]	135.0 (131.25-136.75) [36]	0.851
K^+ (mmol/L)	4.0 (3.50-4.54) [23]	4.0 (3.80-4.42) [36]	0.613
CRP (mg/L)	33.67 (12.0-91.04) [26]	23.30 (7.1-71.74) [23]	0.203
D-dimer (mg/L)	0.52 (0.11-1.60) [23]	0.68 (0.28-3.98) [34]	0.187

Data were expressed in median (IQR) or frequency (%). Available no. of participants. Mann-Whitney U test or Pearson's chi-square test was done as appropriate. OLD; Obstructive lung disease, BP; Blood pressure, BMI; Body mass index, TWBC; Total white blood cell count, NLR; Neutrophil/lymphocyte ratio, PLR; Platelet/lymphocyte ratio, and CRP; C-reactive protein.

Table 2: Total testosterone and DHEAS status of the study population (n=91)

Age group (Y)	Males (n=37)					Females (n=54)				
	n (%)	Reference value (ng/dL)	Low	Normal	High	n (%)	Reference value (ng/dL)	Low	Normal	High
Total testosterone										
18-49 Y	15 (40.54)	270.0-1734.0	1 (2.7)	14 (37.84)	0 (0.0)	26 (48.15)	13.84-53.36	0 (0.0)	20 (37.04)	6 (11.11)
≥50 Y	22 (59.46)	212.0-755.0	2 (5.4)	20 (54.05)	0 (0.0)	28 (51.85)	12.40-35.76	0 (0.0)	19 (35.18)	9 (16.67)
Total	37		3 (8.1)	34 (91.89)	0 (0.0)	54		0 (0.0)	39 (72.22)	15 (27.78)
DHEAS		(µg/dL)					(µg/dL)			
18-20	3 (8.11)	24.0-537.0	0 (0.0)	3 (8.12)	0 (0.0)	2 (3.70)	51.0-321	1 (1.85)	1 (1.85)	0 (0.0)
21-30	5 (13.51)	85.0-690.0	3 (8.12)	2 (5.40)	0 (0.0)	9 (16.67)	18.0-391.0	0 (0.0)	9 (16.67)	0 (0.0)
31-40	4 (10.81)	106.0-464.0	3 (8.12)	1 (2.70)	0 (0.0)	6 (11.11)	23.0-266.0	1 (1.85)	5 (9.26)	0 (0.0)
41-50	4 (10.81)	70.0-495.0	2 (5.40)	2 (5.40)	0 (0.0)	12 (22.22)	19.0-231.0	2 (3.70)	10 (18.52)	0 (0.0)
51-60	11 (29.73)	38.0-313.0	3 (8.12)	8 (21.62)	0 (0.0)	15 (27.78)	8.0-188.0	0 (0.0)	13 (24.07)	2 (3.70)
61-70	10 (27.03)	24.0-244.0	0 (0.0)	10 (27.03)	0 (0.0)	10 (18.52)	12.0-133.0	0 (0.0)	10 (18.52)	0 (0.0)
Total	37		11 (29.73)	26 (70.27)	0 (0.0)	54		4 (7.41)	48 (88.89)	2 (3.70)

DHEAS; Dehydroepiandrosterone sulfate.

Table 3: Characteristics of COVID-19 male patients with DHEA-S status and female patients with TT status

Variables	Male			Female		
	Low DHEAS (n=11)	Normal DHEAS (n=26)	P value	Normal TT (n=39)	High TT (n=15)	P value
Age (Y)	38.0 (28.0-51.0) [11]	60.0 (42.50-64.0) [26]	0.013	48.0 (38.0-58.0) [39]	55.0 (27.0-60.0) [15]	0.923
Systolic BP (mm-Hg)	122.0 (110.0-126.25) [10]	120.0 (111.50 -143.25) [24]	0.642	120.0 (109.25-140.0) [34]	121.0 (101.0-136.0) [15]	0.803
Diastolic BP (mm-Hg)	81.0 (70.0-85.75) [10]	79.0 (70.0-84.75) [24]	0.381	79.50 (68.0-90.0) [34]	78.0 (71.0-85.0) [15]	0.939
BMI (kg/m^2)	21.77 (20.70-27.55) [11]	21.51 (18.89-24.25) [25]	0.161	24.97 (22.23-28.56) [39]	23.81 (20.16-26.44) [13]	0.228
Disease severity						
Mild	7 (63.6)	14 (53.8)	0.723	26 (66.7)	7 (46.7)	0.220
Moderate-severe	4 (36.4)	12 (46.2)		13 (33.3)	8 (53.3)	
Investigations						
TWBC	7.11 (4.98-9.0) [10]	9.06 (6.94-14.14) [22]	0.077	9.45 (6.63-13.53) [32]	9.65 (8.17-11.13) [13]	0.980
NLR	5.45 (2.09-7.81) [10]	3.46 (2.94-1047) [22]	0.920	3.56 (1.90-8.35) [32]	2.41 (1.66-3.26) [13]	0.157
PLR	146.65 (95.39-243.34) [10]	124.90 (93.04-235.40) [22]	0.675	133.59 (91.19-206.59) [32]	111.96 (64.48-130.54) [13]	0.130
Na^+ (mmol/L)	136.0 (130.0-136.0) [7]	134.0 (130.63-139.0) [16]	0.769	134.0 (131.0-136.25) [30]	136.0 (133.50-138.25) [6]	0.268
K^+ (mmol/L)	3.50 (3.10-4.0) [7]	4.0 (3.69-4.54) [16]	0.089	4.0 (3.80-4.53) [30]	3.95 (3.10-4.19) [6]	0.467
CRP (mg/L)	33.74 (15.45-157.18) [9]	28.0 (12.0-80.0) [17]	0.491	22.20 (7.09-59.68) [16]	24.0 (7.75-123.95) [7]	0.452
D-dimer (mg/L)	0.41 (0.10-3.41) [9]	0.66 (0.11-1.59) [14]	0.926	1.47 (0.39-4.67) [25]	0.33 (0.12-1.84) [9]	0.086

Data were expressed in median (IQR) or frequency (%). Available no. of participants. Mann-Whitney U test or Fisher's exact test was done as appropriate. TT; Total testosterone, BP; Blood pressure, TWBC; Total white blood cell count, BMI; Body mass index, NLR; Neutrophil/lymphocyte ratio, C-reactive protein, and PLR; Platelet/lymphocyte ratio.

Comparison of TT and DHEAS among the severity of illness showed that males with moderate severity of COVID-19 had significantly lower DHEAS (post hoc P=0.038) than the mild group. TT in both sexes and DHEAS in females were statistically similar across the spectrum of noncritical illness of COVID-19 (Table 4).

TT had a moderate negative correlation with age in both sexes (males: r=-0.50, P=0.001; females: r=-0.42, P=0.002). DHEAS had a significant negative correlation with age only in females (r=-0.31, P=0.025). DHEAS had a significant negative correlation with platelet/lymphocyte ratio (PLR) in males (r=-0.36, P=0.044) and TT had a positive significant correlation with serum sodium (Na^+) in females (r=0.36, P=0.034). TT had a significantly negative correlation with NLR (r=-0.46, P=0.008) but DHEAS had a significantly positive correlation with NLR (r=0.40, P=0.023) only in males (Table 5). TT significantly correlated with DHEAS in females (r=0.33, P=0.014).

Table 4: TT and DHEAS in COVID-19 patients with different severity (n= 91)

	Mild	Moderate	Severe	P value
Males (n= 37)	(n=21)	(n=11)	(n=5)	
TT (ng/dL)	391.0 (302.50-472.50)	299.0 (270.0-374.0)	279.50 (301.0-425.0)	0.132
DHEAS (µg/dL)	80.20 (50.85-90.50)	41.0 (34.0-65.0)	67.40 (40.25-97.50)	**0.045**
Females (n= 54)	(n=33)	(n=15)	(n=6)	
TT (ng/dL)	33.0 (25.50-49.55)	32.0 (24.0-48.20)	27.45 (22.05-36.25)	0.430
DHEAS (µg/dL)	49.0 (34.50-83.50)	51.50 (34.20-67.0)	41.75 (19.35-97.0)	0.764

Data were expressed in median (IQR). Kruskal Wallis one-way ANOVA with post hoc Dunn's test was done. TT; Total testosterone and DHEAS; Dehydroepiandrosterone sulfate. Bold indicates significant P.

Table 5: Correlations of TT and DHEA-S with different clinical and biochemical variables

Determinants of 'r'	Males					Females				
	Available no.	TT		DHEAS		Available no.	TT		DHEAS	
		r	P	r	P		r	P	r	P
Age (Y)	37	-0.504	**0.001**	-0.266	0.112	54	-0.419	**0.002**	-0.305	**0.025**
BMI (kg/m^2)	36	0.228	0.181	-0.139	0.419	52	-0.146	0.302	-0.016	0.910
Systolic BP	34	-0.201	0.255	-0.083	0.640	49	-0.231	0.110	-0.063	0.668
Diastolic BP		-0.075	0.674	-0.063	0.723		-0.090	0.538	0.126	0.388
TWBC	32	-0.095	0.604	0.117	0.522	45	-0.107	0.484	-0.134	0.381
NLR		-0.459	**0.008**	0.401	**0.023**		-0.216	0154	-0.175	0.250
PLR		-0.221	0.224	-0.358	**0.044**		-0.129	0.399	-0.095	0.534
Na^+ (mmol/L)	23	0.107	0.626	0.335	0.118	36	0.355	**0.034**	0.120	0.484
K^+ (mmol/L)		0.078	0.724	0.350	0.102		-0.267	0.115	-0.186	0.278
CRP (mg/L)	26	-0.102	0.620	-0.254	0.211	23	0.031	0.888	0.030	0.891
D-dimer (mg/L)	23	-0.393	0.063	-0.407	0.054	34	-0.105	0.553	-0.138	0.435

Bold indicates significant P. BMI; Body mass index, BP; Blood pressure, TWBC; Total white blood cell count, NLR; Neutrophil/lymphocyte ratio, and PLR; Platelet/lymphocyte ratio.

Discussion

In this study, we found that most of the noncritical COVID-19 patients had normal TT and DHEAS. While a few males had low TT, around 30% of females had high TT. About 30% of males had low DHEAS and 10% of females had abnormal (low/high) DHEAS. Males with moderate COVID-19 had lower DHEAS than mild COVID-19. TT had a negative correlation with age in both sexes; with NLR in males and serum Na+ in females. With DHEAS, females had a negative correlation with age but males had a significant correlation with NLR and PLR. TT positively correlated with DHEAS in females.

Most of the noncritical COVID-19 patients had normal TT irrespective of sex. We found only 8.1% of males had low TT. Salonia et al. (11) found 90% of cases of hypogonadism among 286 males, and 85% of patients with hypogonadism were secondary (hypothalamic-pituitary). However, most of their patients had severe to critical illness (~80%) that they used a lower cut-off of TT levels to define hypogonadism (<265 ng/dL). Among 89 COVID-19 patients with 53% of mild cases, Kadihasanoglu et al. (14) found 74.2% of males with hypogonadism (TT <300 ng/dL). So, the prevalence of hypogonadism depends on the severity of COVID-19 as well as the cut-off of TT. Similarly, we found a lower prevalence of low TT in males due to the inclusion of higher percentages of mild cases (~57%) as well as using a lower cut-off (<212 ng/dL). Involvement of the hypothalamic-pituitary-testicular axis at each level may be responsible for this low level of TT in males (15). In contrast to males, we found nearly 26% of females had high TT levels. Di Stasi et al. (16) also found higher TT levels in females with a positive association with inflammatory markers, as opposed to that found among males. So, low TT in males has a similar effect to high TT in females. However, estrogen plays a primary role in females providing better immunity than males. Due to an extra X chromosome as well as the anti-inflammatory effects of estrogen, females get advantages over males for the infectivity rate, severity, and mortality from COVID-19 (17). This benefit is lost after menopause. However, despite lower levels of TT older males suffer more than young ones because of less estrogen from aromatization as well as the inflammatory effects of different comorbidities (1, 17).

We observed a trend of lower TT with increasing severity of COVID-19 in both sexes without significant associations. Cinislioglu et al. (18) also found lower TT in moderate to severe cases than in mild cases of COVID-19. Beltrame et al. (13) found significantly lower TT levels in severe cases than in non -severe ones. An inverse association between severity and TT levels has been found in most of the studies (19, 20). TT levels were similar between asymptomatic and mild-moderate hospitalized symptomatic patients (21). Camici et al. (22) found lower TT in severe cases than in mild ones and similar levels of androstenedione, 5α-dihydroxy testosterone, and sex hormone-binding globulin between them. Thus, lower TT is a response to acute illness and may serve as a marker of the severity and prognosis of COVID-19. However, we did not find a significant association because of less severe cases of COVID-19 as well as a small number of participants in the severe group.

DHEAS levels were found significantly lower in males with moderate cases than in mild cases of noncritical illness of COVID-19. About 30% of males had low and 11% of females had abnormal DHEAS. Vaez Mahdavi et al. (23) found lower DHEAS in severe/critical patients than in moderate cases of COVID-19. Alzahrani et al. (24) found 75% of non-severe cases of COVID-19 with normal DHEAS (1.81 - 8.3 μmol/L) with only two cases with high and four cases with low DHEAS. Therefore, it seems that levels of DHEAS may decline with the severity of the illness.

A negative correlation between TT and age was found in both sexes and between DHEAS and age was found only in females. These indicate age-related as well as the

co-morbidity-accumulated decline of androgens along with the minor role of DHEAS in males (25).

We found a negative correlation between TT with NLR in males. Other authors found a negative correlation between TT with neutrophils and a positive correlation between lymphocytes in men with TT (20, 22). So, TT may be a reciprocal inflammatory marker in noncritical COVID-19 patients. A positive correlation between TT with serum Na+ in females may be explained by the role of testosterone in increasing renal reabsorption (26).

There were several limitations of our study. The small sample size especially the small number of severe patients was the main drawback. Besides, there were many missing data. We also could not measure luteinizing hormone, follicle-stimulating hormone, oestradiol, and sex-hormone binding globulin to further clarify the reproductive hormone status of COVID-19 patients.

Conclusion

There is considerable sex-specific differences in TT and DHEAS status in noncritical patients with COVID-19. Moreover, serum DHEAS is associated with the disease severity that both are correlated with inflammatory markers in males.

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Authors' Contributions

H.B., M.S.M., N.S., A.A.S., S.M.A.; Conceptualization, Methodology, and Software. M.A.H.; Validation. H.B., T.A.; Investigation and Data curation. H.B., M.S.M., N.S.; Formal analysis, Writing, Original draft preparation, and Visualization. M.A.H., A.A.S., S.M.A.; Supervision, Writing, Review, Editing, Project administration, and Funding acquisition. All authors read and approved the final manuscript.

References

1. Galbadage T, Peterson BM, Awada J, Buck AS, Ramirez DA, Wilson J, et al. Systematic review and meta-analysis of sex-specific COVID-19 clinical outcomes. Front Med (Lausanne). 2020; 7: 348.
2. Nyce J. Alert to US physicians: DHEA, widely used as an OTC androgen supplement, may exacerbate COVID-19. Endocr Relat Cancer. 2021; 28(2): R47-R53.
3. Galbadage T, Peterson BM, Wang JS, Jayasekara A, Ramirez DA, Awada J, et al. Molecular mechanisms lead to sex-specific COVID-19 prognosis and targeted therapies. Front Med (Lausanne). 2020; 7: 589060.
4. Cadegiani FA, Goren A, Wambier CG. Spironolactone may provide protection from SARS-CoV-2: Targeting androgens, angiotensin converting enzyme 2 (ACE2), and renin-angiotensin-aldosterone system (RAAS). Med Hypotheses. 2020; 143: 110112.
5. Wu YH, Tseng CP, Cheng ML, Ho HY, Shih SR, Chiu DT. Glucose-6-phosphate dehydrogenase deficiency enhances human coronavirus 229E infection. J Infect Dis. 2008; 197(6): 812-816.
6. Duarte-Neto AN, Teixeira TA, Caldini EG, Kanamura CT, Gomes-Gouvêa MS, Dos Santos ABG, et al. Testicular pathology in fatal COVID-19: a descriptive autopsy study. Andrology. 2022; 10(1): 13-23.
7. Tiwari S, Kc N, Thapa S, Ghimire A, Bijukchhe S, Sah GS, et al. Semen parameters in men recovered from COVID-19: a systematic review and meta-analysis. Middle East Fertil Soc J. 2021; 26(1): 44.
8. Paul T, Ledderose S, Bartsch H, Sun N, Soliman S, Märkl B, et al. Adrenal tropism of SARS-CoV-2 and adrenal findings in a post-mortem case series of patients with severe fatal COVID-19. Nat Commun. 2022; 13(1): 1589.
9. Yen SSC, Jaffe RB. Yen & Jaffe's reproductive endocrinology: physiology, pathophysiology, and clinical management. 8th ed. Elesevier; 2017; 74-114.
10. Xu H, Wang Z, Feng C, Yu W, Chen Y, Zeng X, et al. Effects of SARS-CoV-2 infection on male sex-related hormones in recovering patients. Andrology. 2021; 9(1): 107-114.
11. Salonia A, Pontillo M, Capogrosso P, Gregori S, Carenzi C, Ferrara AM, et al. Testosterone in males with COVID-19: a 7-month cohort study. Andrology. 2022; 10(1): 34-41.
12. World Health Organization. Clinical management of COVID-19: interim guidance, 27 May 2020. August 2, 2020. Available from: https://apps.who.int/iris/handle/10665/332196 (2 Aug 2021).
13. Beltrame A, Salguero P, Rossi E, Conesa A, Moro L, Bettini LR, et al. association between sex hormone levels and clinical outcomes in patients with COVID-19 admitted to hospital: an observational, retrospective, cohort study. Front Immunol. 2022; 13: 834851
14. Kadihasanoglu M, Aktas S, Yardimci E, Aral H, Kadioglu A. SARS-CoV-2 Pneumonia affects male reproductive hormone levels: a prospective, cohort study. J Sex Med. 2021; 18(2): 256-264.
15. Clarke SA, Abbara A, Dhillo WS. Impact of COVID-19 on the endocrine system: a mini-review. endocrinology. 2022; 163(1): bqab203.
16. Di Stasi V, Rastrelli G, Inglese F, Beccaria M, Garuti M, Di Costanzo D, et al. Higher testosterone is associated with increased inflammatory markers in women with SARS-CoV-2 pneumonia: preliminary results from an observational study. J Endocrinol Invest. 2022; 45(3): 639-648.
17. Al-Lami RA, Urban RJ, Volpi E, Algburi AMA, Baillargeon J. Sex hormones and novel corona virus infectious disease (COVID-19). Mayo Clin Proc. 2020; 95(8): 1710-1714.
18. Cinislioglu AE, Cinislioglu N, Demirdogen SO, Sam E, Akkas F, Altay MS, et al. The relationship of serum testosterone levels with the clinical course and prognosis of COVID-19 disease in male patients: a prospective study. Andrology. 2022; 10(1): 24-33.
19. Dhindsa S, Zhang N, McPhaul MJ, Wu Z, Ghoshal AK, Erlich EC, et al. Association of circulating sex hormones with inflammation and disease severity in patients with COVID-19. JAMA Netw Open. 2021; 4(5): e2111398.
20. Rastrelli G, Di Stasi V, Inglese F, Beccaria M, Garuti M, Di Costanzo D, et al. Low testosterone levels predict clinical adverse outcomes in SARS-CoV-2 pneumonia patients. Andrology. 2021; 9(1): 88-98.
21. Çayan S, Uğuz M, Saylam B, Akbay E. Effect of serum total testosterone and its relationship with other laboratory parameters on the prognosis of coronavirus disease 2019 (COVID-19) in SARS-CoV-2 infected male patients: a cohort study. Aging Male. 2020; 23(5): 1493-1503.
22. Camici M, Zuppi P, Lorenzini P, Scarnecchia L, Pinnetti C, Cicalini S, et al. Role of testosterone in SARS-CoV-2 infection: a key pathogenic factor and a biomarker for severe pneumonia. Int J Infect Dis. 2021; 108: 244-251.
23. Vaez Mahdavi MR, Kaboudanian Ardestani S, Rezaei A, Mohammadi S, Chenary MR, Gharegozlou B, et al. CO-VID-19 patients suffer from DHEA-S sufficiency. Immunoregulation. 2020; 3(2): 135-144.
24. Alzahrani AS, Mukhtar N, Aljomaiah A, Aljamei H, Bakhsh A, Alsudani N, et al. The impact of COVID-19 viral infection on the hypothalamic-pituitary-adrenal axis. Endocr Pract. 2021; 27(2): 83-89.
25. Younis JS, Skorecki K, Abassi Z. The double edge sword of testosterone's role in the COVID-19 pandemic. Front Endocrinol (Lausanne). 2021; 12: 607179.
26. Liu B, Ely D. Testosterone increases: sodium reabsorption, blood pressure, and renal pathology in female spontaneously hypertensive rats on a high sodium diet. Adv Pharmacol Sci. 2011; 2011: 817835.

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Validity and Reliability of The Persian Version of Uterine Fibroid Symptom and Health-Related Quality of Life Questionnaire: A Psychometric Study

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Abstract

Background: Women with uterine fibroids (UFs) experience many clinical manifestations that affect their quality of life (QOL). The Uterine Fibroid Symptom and Health-related Quality of Life (UFS-QOL) questionnaire is an English instrument specifically designed to assess fibroid-related symptoms and their impact on QOL. This study aims to investigate the reliability and validity of the Persian version of the UFS-QOL questionnaire in Iranian women with UF.

Materials and Methods: In this psychometric study, women with UFs who presented to Imam Hossein Hospital (Tehran, Iran) between August 2022 and January 2023 were enrolled in this study. A forward-backward approach was applied to translate the UFS-QOL questionnaire into Persian. The reliability of the UFS-QOL questionnaire was assessed by internal consistency and test-retest correlation. Confirmatory factor analysis (CFA) was used to assess convergent validity between items and subscales of the UFS-QOL questionnaire. Pearson's correlation coefficient was used to assess convergence validity between subscales of the UFS-QOL and the World Health Organization Quality of Life Brief Version 26 questionnaire (WHOQOL-BREF-26).

Results: Overall, we assessed 226 women with UFs. All subscales of the UFS-QOL questionnaire had acceptable internal consistency (Cronbach's alpha>0.7). Test-retest analysis indicated significant positive correlations between two measurements of all subscales of the UFS-QOL questionnaire: symptom severity (P<0.001), concern (P<0.001), activities (P<0.001), energy/mood (P<0.001), control (P<0.001), self-consciousness (P=0.002), and sexual function (P<0.001). The Kaiser-Meyer-Olkin (KMO) measure value was 0.920, and the result of Bartlett's test of sphericity was significant (P<0.001). CFA identified six factors for the health-related QOL (HRQL) questionnaire, which explained 73.827% of the total variation. Most subscales of the UFS-QOL questionnaire correlated with domains of the WHOQOL-BREF-26 questionnaire (P<0.05).

Conclusion: The Persian version of the UFS-QOL questionnaire is a valid and reliable instrument to evaluate UF-related symptoms and QOL among Iranian women.

Keywords: Leiomyoma, Quality of life, Psychometrics, Surveys and Questionnaires

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Introduction

Uterine fibroids (UFs), also called leiomyomas, are the most common benign uterine tumours that arise from the myometrium in reproductive-age women. They are the most common diagnosis associated with hysterectomy in the United States (1, 2). According to a systematic review, the prevalence of UFs varies from 4.5 to 68.6% (3). Many women with UFs suffer from heavy menstrual bleeding (a leading cause of anaemia in reproductive-age women), abdominopelvic pain, urinary frequency, and urinary incontinency. Clinical manifestations of UFs restrict the physical and social activities of patients and impact their quality of life (QOL) (4). Furthermore, UFs may cause gynaecological dysfunctions such as infertility, recurrent miscarriage, and preterm labour (5).

Treatment of UFs is based on size, location, and

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symptoms. This imposes direct (hospitalisation, outpatient visits, medication, and other medical services) and indirect (work loss and costs associated with incapacity for household work) expenses. In the United States, it is estimated that approximately 34.4 billion USD per year is spent on UFs treatment, which is more than the costs spent on breast, colon, and ovarian cancer treatments (6, 7). Around a third of hysterectomies performed in the USA are due to fibroids, with costs for their management estimated to be over two billion USD per year. Non-surgical treatments may be more beneficial for women with UFs, especially for those who want to preserve fertility (8). Clinicians need an instrument to compare the efficacy of different therapeutic options for women with UFs by assessing the impact of patients' symptoms on their QOL before and after treatment.

The Uterine Fibroid Symptom and Health-related Quality of Life (UFS-QOL) is an English instrument specially designed to assess the broad spectrum of fibroid-related symptoms and their impact on QOL. It has been translated into Brazilian, Portuguese, Spanish, and Chinese. Measurement of UF-related symptoms and QOL can provide an efficient practice and comprehensive management for patients from different cultures (9). Thus, this study aims to investigate the reliability and validity of the Persian version of the UFS-QOL questionnaire in Iranian women with UFs and proffer an instrument with acceptable psychometric properties to assess the impact of UF symptoms on QOL.

Materials and Methods

Study design and participants

This was a psychometric study conducted at Imam Hossein Hospital (Tehran, Iran) between August 2022 and January 2023. The inclusion criteria consisted of: women with UF diagnosed with ultrasound by an expert radiologist; largest diameter of UF between 2 and 10 cm; age between 18 and 45 years; and the ability to read and write. Patients with the following characteristics were excluded: use of oral contraceptive pills in the last three months; history of surgery due to gynaecological diseases; underlying diseases such as malignancy, chronic kidney disease, liver failure, metabolic diseases (diabetes mellitus, hyperthyroidism, hypothyroidism, adrenal disorders), hypertension; mental disorders; the presence of other pathologies visualised by ultrasound (e.g., adenomyosis or gynaecological malignancies); and pregnancy or breastfeeding.

Sample size

The sample size was calculated based on factor analysis (five patients per item) (10). The UFS-QOL questionnaire contains 37 items and we took into consideration a 20% drop out rate to derive a sample size of 222. We used consecutive sampling in this study.

Data collection

Initially, eligible patients signed an informed consent form for study participation. A research team member interviewed the participants about their demographics and clinical characteristics. Ultrasound findings of the patients were obtained after reviewing patients' medical records. All patients completed the study questionnaires.

Study instruments

The World Health Organization Quality of Life Brief Version 26 questionnaire (WHOQOL-BREF-26) is an abbreviated English version of the WHOQOL-100 developed by the World Health Organization. It is a 26-item questionnaire that assesses QOL of a person during the previous two weeks. Each item is scored on a Likert scale that ranges from 1 (very dissatisfied) to 5 (very satisfied) with the exception of three questions (3, 4, and 26) that are scored inversely. This questionnaire consists of four domains (physical health, psychological, social relationships, and environment) and an overall QOL and general health score. In this questionnaire, higher scores indicate better QOL. Jahanlou and Karami (11) assessed the psychometric properties of the Persian version of the WHOQOL-BREF-26.

The UFS-QOL is a 37-item questionnaire that assesses symptoms and health-related QOL (HRQL) in women with UF over the previous three months. Each item is scored on a Likert scale that ranges from 1 (none of the time/not at all) to 5 (a very great deal/all of the time). This questionnaire consists of seven subscales: symptom severity, concern, activities, energy/mood, control, self-consciousness, and sexual function. In this questionnaire, higher scores indicate better QOL. The original English version of the UFS-QOL questionnaire was developed by Spies et al. (12), which had acceptable validity and reliability.

Translation

A forward-backward approach was applied to translate the UFS-QOL questionnaire into Persian. The English version of the UFS-QOL questionnaire was independently translated into Persian by two translators. These translated drafts were assessed by a committee of two gynaecologists, a methodologist, and a general practitioner. The questionnaire was translated back into English by another translator who was proficient in Persian and English to ensure that the back-translated version of the questionnaire was similar to the original version. After solving any problems, the Persian version of the UFS-QOL questionnaire was approved, and the study entered the subsequent steps.

Reliability

The reliability of the UFS-QOL questionnaire was assessed by internal consistency and test-retest correlation. Internal consistency investigates the association between different items within a subscale. We considered a Cronbach's alpha of >0.7 to have acceptable internal consistency. A total of 20 patients completed

the Persian version of the UFS-QOL questionnaire for the second time, with an interval of four weeks from the first completion. Test-retest reliability was assessed using Pearson's correlation test. We interpreted the correlation coefficient as follows: low <0.20, 0.21<fair<0.40, 0.41<moderate<0.60, 0.61<substantial<0.80, and 0.80<almost perfect<1.00 (13).

Validity

Confirmatory factor analysis (CFA) was conducted to assess convergent validity between items and subscales of the HRQL (items 9 to 37). First, sampling adequacy and data appropriateness for CFA were examined using the Kaiser-Meyer-Olkin (KMO) measure and Bartlett's test of sphericity, respectively. A KMO value of >0.6 with significant Bartlett's test of sphericity (P<0.05) was considered suitable for CFA. Then, we determined CFA by using the principal components method with varimax rotation (Eigenvalue>1.00). Items with weak loading (value<0.40) were excluded from the analysis (1). Pearson's correlation coefficient was used to assess convergence validity between subscales of the UFS-QOL and WHOQOL-BREF-26 questionnaires. In order to interpret the correlation coefficient, we undertook the same procedure as for test-retest reliability.

Statistical analysis

Data were processed using the IBM® Statistical Package for Social Sciences (SPSS) ® software version 23.0 (IBM®, USA). Variables were described as frequency, percentage, mean and standard deviation. Pearson's correlation test was used to assess test-retest reliability and convergence validity between instruments. In this study, a P<0.05 was considered statistically significant.

Ethical considerations

The Ethics Committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran approved this study (IR.SBMU.RETECH.REC.1401.219). All steps of the study were performed in accordance with the Declaration of Helsinki 2000.

Results

Patients' baseline characteristics

Initially, 232 patients were included in the study and six patients were excluded after application of the exclusion criteria (adenomyosis [4], gynaecologic cancer [1], and pregnancy [1]). The mean age of the 226 included patients was 42.10 ± 6.02 years (range: 18 to 45). Most had a normal body mass index (42.9%), while others were overweight (37.6%) and obese (19.5%). Patients mainly complained of abnormal uterine bleeding (49.1%), while others came because of routine check-ups (43.4%) and abdominopelvic pain (7.5%). Table 1 shows the baseline characteristics of the patients. On ultrasound examination, 57.5% had one UF, 20.8% had two UFs, and the remaining (21.7%) had three or more UFs. According to the International Federation of Gynaecology and Obstetrics (FIGO) classification system, the most common location of the UFs was 4 or intramural (49.1%), followed by 6 (19.5%), 5 (15.9%), 7 (5.3%), 2 (3.5%), 0 (2.7%), 1 (2.7%), and 8 (1.3%).

Table 1: Baseline characteristics of the patients (n=226)

Variables	Values
Demographic characteristics	
Age (Y)	42.10 ± 6.02
Body mass index (kg/m^2)	
Normal (<25)	97 (42.9)
Overweight (25-30)	85 (37.6)
Obese (≥30)	44 (19.5)
Marital status	
Married	194 (85.8)
Single	32 (14.2)
Employment status	
Unemployed	133 (58.8)
Employed	93 (41.2)
Educational status	
Elementary	42 (18.6)
Diploma	88 (39.0)
Bachelor or higher degree	96 (42.4)
Clinical characteristics	
Chief complaint*	
Abnormal uterine bleeding	111 (49.1)
Abdominopelvic pain	17 (7.5)
Menstrual volume	
Decreased	17 (7.5)
Normal	114 (50.5)
Increased	95 (42.0)
Menstrual duration	
Decreased	17 (7.5)
Normal	169 (74.8)
Increased	40 (17.7)
Menstrual regularity	
Regular	175 (77.4)
Irregular	51 (22.6)
Family history of UFs	79 (35.0)
Ultrasound findings	
Number of UFs	
One	130 (57.5)
Two	47 (20.8)
Three or more	49 (21.7)
Size of the largest UF (mm)	44.15 ± 34.64
Endometrial thickness (mm)	7.26 ± 3.03

Data are reported as frequency (%) or mean ± SD. *; Others (43.4%) came for a routine check-up and UFs; Uterine fibroid.

Reliability

Table 2 presents the internal consistency of the UFS-QOL questionnaire. All the subscales had acceptable internal consistency: symptom severity (α=0.812), concern (α=0.856), activities (α=0.902), energy/mood (α=0.919), control (α=0.861), self-consciousness (α=0.839), and sexual function (α=0.949). Additionally, total HRQL (all subscales, except for symptom severity) had acceptable internal consistency (α=0.956).

Table 2: Internal consistency of the UFS-QOL questionnaire

Subscales	Number of items	Cronbach's alpha*
Symptom severity	8 (Q1-Q8)	0.812
Concern	5 (Q9, Q15, Q22, Q28, Q35)	0.856
Activities	7 (Q10, Q11, Q13, Q19, Q20, Q27, Q29)	0.902
Energy/mood	7 (Q12, Q17, Q23, Q24, Q25, Q31, Q34)	0.919
Control	5 (Q14, Q16, Q26, Q30, Q33)	0.861
Self-consciousness	3 (Q18, Q21, Q32)	0.839
Sexual function	2 (Q36, Q37)	0.949
HRQL total	29 (sum of subscales except symptom severity)	0.956

HRQL; Health-related quality of life, UFS-QOL; Uterine Fibroid Symptom and Health-related Quality of Life, and *; α>0.70 is considered acceptable internal consistency.

Table 3 depicts test-retest reliability of the UFS-QOL subscales. Test-retest analysis indicated significant positive correlations between two measurements of all subscales: symptom severity (r=0.757, P<0.001), concern (r=0.822, P<0.001), activities (r=0.806, P<0.001), energy/mood (r=0.709, P<0.001), control (r=0.827, P<0.001), self-consciousness (r=0.652, P=0.002), and sexual function (r=0.723, P<0.001).

Validity

In our study, the KMO measure value was 0.920, which indicated sampling adequacy. Bartlett's test of sphericity was significant (chi-square value=5653.929, df =406, P<0.001), which indicated that the CFA was suitable for data. Figure 1 depicts a scree plot of Eigenvalues for each item before applying the rotation. After application of the varimax rotation, the test variables explained 73.827% of the total variance. The Eigenvalues and percentages of variance were as follows: factor one: 4.692 (16.178%), factor two: 4.628 (15.959%), factor three: 3.771 (13.003%), factor four: 3.180 (10.966%), factor five: 2.984 (10.291%), and factor six: 2.155 (7.429%). Table 4 illustrates the rotated component matrix to assess convergent validity between the items and subscales of HRQL. By comparing the factors obtained from CFA and subscales of the HRQL, we noted that all items belonged to the same subscales, with the exception of items 9, 14, 19, 27, and 29. Table 5 shows the convergent validity of subscales of the UFS-QOL questionnaire. The symptom severity subscale of the UFS-QOL questionnaire had a negative correlation with all domains of the WHOQOL-BREF-26 questionnaire. The concern (r=0.098, P=0.141), self-conscious (r=0.111, P=0.095), and sexual function (r=0.057, P=0.396) subscales of the UFS-QOL questionnaire did not significantly correlate with the environment domain of the WHOQOL questionnaire. Other subscales of UFS-QOL had a positive correlation with domains of the WHOQOL questionnaire.

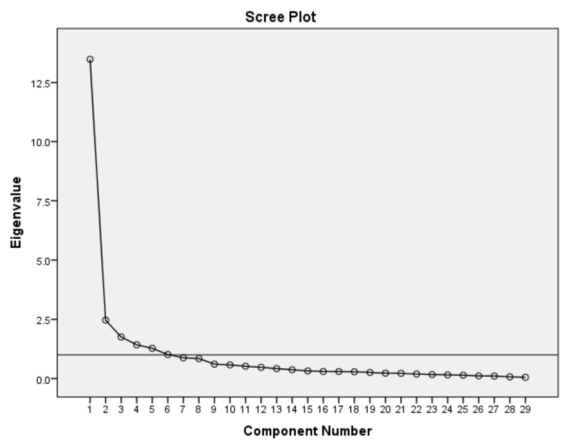

Fig.1: Scree plot of Eigenvalues for each item before applying rotation.

Table 3: Test-retest reliability of UFS-QOL subscales

Subscales	Symptom severity	Concern	Activities	Energy/ mood	Control	Self-con-sciousness	Sexual function
Correlation coefficient (r)	0.757	0.822	0.806	0.709	0.827	0.652	0.723
P value	<0.001	<0.001	<0.001	<0.001	<0.001	0.002	<0.001

UFS-QOL; Uterine Fibroid Symptom and Health-related Quality of Life.

Table 4: Rotated component matrix to assess convergent validity between items and subscales of the HRQL

Items	Factor 1 (Energy/ mood)	Factor 2 (Activity)	Factor 3 (Concern)	Factor 4 (Self-consciousness)	Factor 5 (Control)	Factor 6 (Sexual dysfunction)
Q23	**0.787**					
Q24	**0.784**					
Q25	**0.750**					
Q17	**0.644**	0.473				
Q12	**0.611**	0.611				
Q34	**0.579**					
Q31	**0.573**					
Q11		**0.800**				
Q13		**0.760**				
Q10		**0.624**			0.403	
Q16		**0.584**				
Q20		**0.533**		0.454		
Q14	0.411	0.513				
Q35			**0.854**			
Q22			**0.839**			
Q15			**0.826**			
Q28			**0.482**			
Q29			0.464			
Q18				**0.854**		
Q21				**0.825**		
Q32				**0.781**		
Q19		0.524		0.547		
Q27					0.681	
Q26					**0.658**	
Q30	0.447				**0.647**	
Q33	0.443				**0.580**	
Q9		0.436			0.488	
Q36						**0.909**
Q37						**0.894**

Extraction method; Principal component analysis, Rotation method; Varimax with Kaiser normalization, HRQL; Health-related quality of life. Values in bold indicate that they belong to the factors named in the column. Rotation converged in ten iterations.

Table 5: Convergent validity: pearson's correlation between subscales of UFS-QOL and WHOQOL-BREF-26

Subscales		Overall QOL	Physical health	Psychological	Social relationships	Environment
Symptom severity	r	-0.262	-0.307	-0.283	-0.269	-0.146
	P value	<0.001	<0.001	<0.001	<0.001	0.029
Concern	r	0.200	0.228	0.296	0.222	0.098
	P value	0.003	0.001	<0.001	0.001	0.141
Activities	r	0.215	0.274	0.289	0.262	0.144
	P value	0.001	<0.001	<0.001	<0.001	0.03
Energy/mood	r	0.372	0.405	0.473	0.377	0.296
	P value	<0.001	<0.001	<0.001	<0.001	<0.001
Control	r	0.357	0.327	0.446	0.306	0.307
	P value	<0.001	<0.001	<0.001	<0.001	<0.001
Self-consciousness	r	0.117	0.324	0.238	0.206	0.111
	P value	0.079	<0.001	<0.001	0.002	0.095
Sexual function	r	0.180	0.178	0.267	0.242	0.057
	P value	0.007	0.007	<0.001	<0.001	0.396

UFS-QOL; Uterine Fibroid Symptom and Health-related Quality of Life, WHOQOL-BREF-26; World Health Organization Quality of Life Brief Version 26 questionnaire.

Discussion

This study investigated the reliability and validity of the Persian version of the UFS-QOL questionnaire in Iranian women with UFs. Our assessed Persian version of the UFS-QOL questionnaire has acceptable psychometric properties and it can help researchers and clinicians to evaluate UF-related symptoms and HRQL in Iranian women.

The USF-QOL questionnaire is an efficient instrument to investigate the impact of UFs on patients' perspectives of their QOL. The original questionnaire was developed by Spies et al. (12) in English in 2002, and further validation was conducted by them and Coyne et al. (14). The USF-QOL questionnaire is an international instrument that has been translated and validated in Chinese (2), Spanish (15), Dutch (10), Brazilian Portuguese (16), Bengali (1), and Sinhala (17).

In our study, all subscales of the UFS-QOL questionnaire had acceptable internal consistency. In other words, three subscales (activities, energy/mood, and sexual function) had excellent internal consistency (Cronbach's alpha >0.9), while others (symptom severity, concern, control, and self-consciousness) had high internal consistency (Cronbach's alpha 0.70-0.90) (18). The Chinese version of the UFS-QOL indicated almost perfect Cronbach's alpha scores in the activities, energy/mood, and sexual function subscales (2). A study by Keizer et al. (10) illustrated that the sexual function and control subscales had the same Cronbach's alpha score and the concern subscale had more internal consistency than the sexual function subscale in the Dutch version of UFS-QOL.

Test-retest was performed to ensure that the measurements remained constant. Three subscales of the UFS-QOL questionnaire (concern, activities, and control) had almost perfect reliability (Pearson's correlation coefficient 0.81-1.00), while others (symptom severity, energy/mood, self-consciousness, and sexual function) had substantial reliability (Pearson's correlation coefficient 0.61-0.80) (13). In the Dutch version, concern (0.93), activities (0.9), and energy/mood (0.9) had the highest intraclass correlation coefficient in test-retest results (10), which is in line with our findings.

Based on the CFA, most items of the Persian version of the UFS-QOL questionnaire belonged to the same subscales of the English questionnaire. Inconsistencies in other items may be attributed to cultural context and language differences (9).

Convergent validity is frequently used in psychological and behavioural sciences and indicates the relationship between the new scale and other scales with the same construct (19). The symptom severity subscale of the UFS-QOL questionnaire had a negative correlation with all domains of the WHOQOL-BREF-26 questionnaire, which was due to the scoring method of this subscale. The higher scores indicate more severe symptoms. Symptom severity is associated with decreased QOL. Furthermore, most subscales of the UFS-QOL questionnaire did not correlate with the environment domain of the WHOQOL-BREF-26 questionnaire. This shows that our questionnaire does not probably cover the "environment" domain. A study by Oliveira Brito et al. (20) demonstrated that scores obtained from the Brazilian Portuguese version of UFS-QOL and the subscales of the Short Form-36 questionnaire were negatively correlated, which was consistent with our findings.

Our study had some limitations. We did not evaluate responsiveness as the psychometric properties of the UFS-QOL questionnaire. Based on factor analysis, 5 to 15 individuals should be considered for each item to calculate the sample size. However, due to the limited number of patients, we considered five patients per item.

Conclusion

The Persian version of the UFS-QOL questionnaire is a reliable and valid instrument to evaluate UF symptoms and HRQL in Iranian women with UFs. It could be helpful for clinicians and researchers to assess the severity of symptoms from the patient's perspective.

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Authors' Contributions

H.N.; Conceptualisation, Study design, Data collection, Data analysis, and Wrote the primary draft. A.K.; Study design, Data analysis, Critical thinking, and Edited the manuscript. F.R.M.; Data collection and Wrote the primary draft. H.D.; Conceptualisation and Wrote the primary draft. Sh.H.Ch.; Participated in statistical analysis and drafting. Sh.R.F.; Participated in study design, Resources, and Data curation. Z.B.; Data collection, Resources, and Data curation. M.S.H.; Study design, Critical thinking, and Edited the manuscript. F.F.; Conceptualisation, Supervision, Critical thinking, and Edited the manuscript. All authors read and approved the final manuscript.

References

1. Luthra J, Halder P, Nahar L, Sultana N, Banerjee A, Kumar A, et al. Evaluation of psychometric properties of the uterine fibroids symptoms and health-related quality of life (ufsqol) questionnaire: The translated Bengali version. Trop J Obstet Gynaecol. 2019; 36(3): 338-347.
2. Yeung SY, Kwok JWK, Law SM, Chung JPW, Chan SSC. Uterine fibroid symptom and health-related quality of life questionnaire: a chinese translation and validation study. Hong Kong Med J. 2019; 25(6): 453-459.
3. Stewart EA, Cookson CL, Gandolfo RA, Schulze-Rath R. Epidemiology of uterine fibroids: a systematic review. BJOG. 2017; 124(10): 1501-1512.
4. Marsh EE, Al-Hendy A, Kappus D, Galitsky A, Stewart EA, Kerolous M. Burden, prevalence, and treatment of uterine fibroids: a survey of U.S. women. J Womens Health (Larchmt). 2018; 27(11): 1359-1367.
5. Yang Q, Ciebiera M, Bariani MV, Ali M, Elkafas H, Boyer TG, et al. Comprehensive review of uterine fibroids: developmental origin,

pathogenesis, and treatment. Endocr Rev. 2022; 43(4): 678-719.
6. Al-Hendy A, Myers ER, Stewart E. Uterine fibroids: burden and unmet medical need. Semin Reprod Med. 2017; 35(6): 473-480.
7. Soliman AM, Yang H, Du EX, Kelkar SS, Winkel C. The direct and indirect costs of uterine fibroid tumors: a systematic review of the literature between 2000 and 2013. Am J Obstet Gynecol. 2015; 213(2): 141-160.
8. Dolmans MM, Donnez J, Fellah L. Uterine fibroid management: today and tomorrow. J Obstet Gynaecol Res. 2019; 45(7): 1222-1229.
9. Xu W, Chen W, Chen J, Hu L, Su X, Nie Y, et al. Adaptability and clinical applicability of UFS-QoL in Chinese women with uterine fibroid. BMC Womens Health. 2022; 22(1): 372.
10. Keizer AL, van Kesteren PJM, Terwee C, de Lange ME, Hehenkamp WJK, Kok HS. Uterine fibroid symptom and quality of life questionnaire (UFS-QOL NL) in the Dutch population: a validation study. BMJ Open. 2021; 11(11): e052664.
11. Jahanlou AS, Karami NA. WHO quality of life-BREF 26 questionnaire: reliability and validity of the Persian version and compare it with Iranian diabetics quality of life questionnaire in diabetic patients. Prim Care Diabetes. 2011; 5(2): 103-107.
12. Spies JB, Coyne K, Guaou Guaou N, Boyle D, Skyrnarz-Murphy K, Gonzalves SM. The UFS-QOL, a new disease-specific symptom and health-related quality of life questionnaire for leiomyomata. Obstet Gynecol. 2002; 99(2): 290-300.
13. Cohen J. Statistical power analysis for the behavioral sciences. 2nd ed. New York: Routledge; 2013; 20-66.
14. Coyne KS, Margolis MK, Bradley LD, Guido R, Maxwell GL, Spies JB. Further validation of the uterine fibroid symptom and quality-of-life questionnaire. Value Health. 2012; 15(1): 135-142.
15. Calaf J, Palacios S, Cristóbal I, Cañete ML, Monleón J, Fernández J, et al. Validation of the Spanish version of the uterine fibroid symptom and quality of life (UFS-QoL) questionnaire in women with uterine myomatosis. Med Clin (Barc). 2020; 154(6): 207-213.
16. Silva RO, Gomes MT, Castro RA, Bonduki CE, Girão MJ. Uterine fibroid symptom - quality of life questionnaire translation and validation into Brazilian Portuguese. Rev Bras Ginecol Obstet. 2016; 38(10): 518-523.
17. Karunagoda K, Perera PK, Senanayake H. Uterine fibroid symptoms and quality-of-life questionnaire (UFS-QOL): translation and validation study of the Sinhala version. Ceylon J Sci. 2020; 49(3): 311-315.
18. Taherdoost H. Validity and reliability of the research instrument; how to test the validation of a questionnaire/survey in a research. IJARM. 2016; 5(3): 28-36.
19. Hofman CS, Lutomski JE, Boter H, Buurman BM, de Craen AJ, Donders R, et al. Examining the construct and known-group validity of a composite endpoint for The Older Persons and Informal Caregivers Survey Minimum Data Set (TOPICS-MDS); A large-scale data sharing initiative. PLoS One. 2017; 12(3): e0173081.
20. Oliveira Brito LG, Malzone-Lott DA, Sandoval Fagundes MF, Magnani PS, Fernandes Arouca MA, Poli-Neto OB, et al. Translation and validation of the Uterine Fibroid Symptom and Quality of Life (UFS-QOL) questionnaire for the Brazilian Portuguese language. Sao Paulo Med J. 2017; 135(2): 107-115.

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Embryo Condition Media Collected from Polycystic Ovary Syndrome Patients with Abdominal Obesity Can Increase The Decidualization Potential of Healthy Endometrial Stromal Cells

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Abstract

Background: Polycystic ovary syndrome (PCOS) is a common endocrinological disorder associated with abdominal obesity (AO) and some reproductive complications including low pregnancy rate. Embryo-endometrium cross-talk has a key role in successful embryo implantation and subsequent normal pregnancy rate. The primary objective of this study is to evaluate the decidualization potential of endometrial stromal cells (ESCs) using the embryo condition media (ECM) collected from PCOS patients with AO, compared to ECM of those patients without AO.

Materials and Methods: In this experimental study, we measured the capacity of ECM collected from PCOS patients with or without AO for decidualization induction in healthy ESCs after coculture. A total number of 53 embryos from 40 couples belonging to PCOS with AO, PCOS without AO, nonPCOS with AO, and nonPCOS without AO patients, were included in our study. The embryosof four groups were single-cultured up to the blastocyst stage. Their ECM (45λ/well) were pooled and added to healthy ESCs monolayer culture media to investigate their effects on decidualization potential via gene (*PRL*, *IGFBP1*, *IL1-β*, *HOXA10*, *IL-6* and *TNF-α*) and protein (PRL, IGFBP1, IL1-β) expression analysis and ESCs migration assay.

Results: The morphological analysis, migration assay (P≤0.0321), protein (P≤0.0139) and gene expression analysis showed PCOS with AO accounted for the highest gene (*PRL*, *IGFBP1*, *IL1-β*, *HOXA10*, *IL-6*, *TNF-α*) and protein markers (PRL, IGFBP1, IL1-β) (P≤0.05). NonPCOS individuals without AO had the lowest level of both gene and protein decidualization markers (P≤0.05).

Conclusion: Considering decidualization as an inflammatory process, a higher level of decidualization markers was associated with a higher inflammatory status created by AO and PCOS, separately. Inflammation may disrupt the process of inflammatory to anti-inflammatory phase required for prevention of pregnancy loss, this could explain the high rate of abortion in these cases.

Keywords: Abdominal Obesity, Decidualization, Polycystic Ovary Syndrome, Stromal Cells, Supernatant

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Introduction

In recent years, polycystic ovary syndrome (PCOS) diverted the attention to the reproductive-age women, who suffer of this disorder with a 4-21% prevalence rate (1). PCOS has been considered a heterogenic endocrine and metabolic disorder with pathological characteristics includes hyperandrogenemia (HA), anovulation, polycystic ovary disease, obesity, insulin resistance (IR), and infertility. This syndrome has been recognized as a low-grade chronic inflammatory disease, but the exact mechanism of this association has not been fully understood. It has been demonstrated that both natural and assisted reproductive technology (ART) cycles in PCOS patients are challenging due to low pregnancy rate, reduced live birth rate and high miscarriage rate even after a high-quality embryos transfer in ART plans (2). It seems that other pathological complications, including endometrial

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insufficiency, may contribute to the pathogenesis of infertility disorders (3).

Recent evidence shows that IR, HA and obesity play an essential role in the PCOS pathogenesis and its consequences (4). Obesity contributes to one of the most critical complications among PCOS women, in terms of their reproduction (5). Obesity (body mass index, BMI>30) affects 61-76% of these patients (6). A cohort study in more than 9500 ART cycles indicates that obesity may lead to embryonic implantation failure, which is caused by an impaired endometrial receptivity (7). In PCOS patients, the presence of metabolic disorders, inflammation and endocrine abnormalities, the same features that we observe in obesity, play a significant role in ovulation function, oocyte quality, endometrial receptivity and miscarriage rate (8). The implantation disorder and abortion occurrence in these patients indicate that the cross-talk between the embryo and the endometrium is probably disturbed.

Naturally, successful embryo implantation depends on a receptive endometrium, the presence of a good-quality blastocyst, and synchronization of embryo-endometrium cross-talk. In addition, before the embryo's implantation, there is a communication between the uterus and the blastocyst that occurs during a specific period of time in which implantation is possible (9).

There is an evidence that the embryo-endometrium cross-talk is carried out through the secretion of various factors by an embryo (10). Previous studies showed that interleukins, immunosuppressive factors and growth factors can be isolated from the embryo condition media (ECM) (11). In order to induce the expression of integrins, endometrial leukemia inhibitory factor, and other inflammatory factors that improve the rate of implantation via facilitating embryo-endometrium cross-talk (12).

Endometrial receptivity, a physiological changes complex, provides an appropriate environment for the different phases of an embryo implantation, including the opposition, attachment, invasion, and decidualization. In humans, decidualization occurs routinely during the latter half of each menstrual cycle and disappears in the absence of an embryo in the endometrium (13). Decidualization is a morphological and functional change of stromal cells under ovarian steroidal hormones (estrogen; E2 and progesterone; P4) secretion to prepare these cells to accept the embryo (14). Prolactin (PRL) and insulin like growth factor binding protein 1 (IGFBP-1) are two specific markers of decidualization process (15). In addition, homeboxA10 (HOXA10) is an essential inducer factor for promoting decidualization (16). Induction of decidualization requires the expression of inflammatory cytokines and low-grade inflammation such as interleukin 1-beta (IL-1β) (17), tumor necrosis factor alpha (TNF-α) (17), and IL-6 (18). It has been shown that an excessive inflammation in the implantation process can enhance the decidualization process (19).

Considering that in ART, the embryo is cultured until the blastocyst stage on day 5, the cross-talk between the uterus and the embryo may be lost. For this purpose, Goto et al. (20) evaluated a new method called stimulation of endometrium embryo transfer (SEET). In their experiment, ECM from a fresh cycle was frozen and then injected into the uterus of patients before blastocyst transfer, that led to an increase in the pregnancy rate. Therefore, this approach may help us to define the embryo capacity for decidualization induction in endometrial stromal cells (ESCs) which required for embryo implantation.

The implantation rate following a blastocyst transfer has been around 4-55% worldwide, and it has remained constant over the last decades (21). A higher rate of live birth in a blastocyst transfer procedure in comparison with the cleavage stage transfer, placed it in the first line of ART. Accordingly, the embryo- endometrial cross talk lack can be accounted for main reason of a low implantation rate after *in vitro* embryo transfer (22). An impaired decidualization creates a series of reproductive disorders e.g., miscarriage, implantation and various pregnancy complications (13).

The exact mechanism by which the PCOS and abdominal obesity (AO) may affect the outcome of both natural and ART cycles is not yet well understood. Therefore, in the present study, we intend to investigate the independent roles of PCOS or/and obesity on the implantation rate of embryos which is characterized by ESCs decidualization process.

Materials and Methods

This experimental study was approved by the Ethical Committee of Royan Institute, Tehran, Iran (IR.ACECR.ROYAN.REC.1400.004). All volunteer participants submitted their written informed consent. All volunteer participants were invited among aged 25-35 infertile couples who had referred to the Royan Institute Tehran, Iran, between 1st May until 1st December in 2021.

For isolating ESCs, we had to invite healthy married fertile women who were referred to the Royan Clinic, Tehran, Iran, for a diagnostic laparoscopy for pelvic pain without any uterine abnormality. After describing the aim and procedure of our study, of those who were volunteering to take part in this trial expressed their willingness and provided their self-signed consent for participation.

Study population and group design

Totally, 53 infertile couples, including PCOS affected and normal oogenesis women participated in this experimental study. According to the Rotterdam

criteria, 25 women diagnosis a PCOS affected. The normal group included 28 normal oogenesis women who were placed in the ART indication due to their husbands' infertility. Different plans, including *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and frozen embryo transfer (FET) were considered for them. The participants were divided into four subgroups: the PCOS patients with AO (waist/hip ratio ≥0.80) named as $PCOS^{+}$- AO^{+} group. The PCOS patients without AO named as $PCOS^{+}$-AO^{-} group. Non-PCOS patients with AO considered as $PCOS^{-}$-AO^{+} group, and non-PCOS patients without AO were as $PCOS^{-}$-AO^{-} group. We designed two supplementary groups, ESCs treatment neither ECM nor induction medium [control induction (CI)] and ESCs which were treated with only induction medium [control supernatant (CS)]. The participants who suffered an obvious sign of systemic inflammatory diseases, including diabetes, hypertension, hypothyroidism, hyperthyroidism, ankylosing spondylitis (AS), gout, rheumatoid arthritis, scleroderma, and systemic lupus erythematosus were excluded of this study. Also, patients without at least three months before the start of the present study anti-inflammatory drug consumption history were included Ibuprofen, Diclofenac and Mefenamic acid made this dug box.

Embryo condition media preparation

Ovarian hyperstimulation and embryo freeze transfer protocol

Our patients underwent standard ovarian stimulation protocol, according to age ≤36 years (23). For this aim, during the first 6 days of their menstrual cycle, 150 IU of the recombinant follicle-stimulating hormone (follitropin-b, MSD, Ballerup, Denmark) was injected daily. On day 2 or 3, controlled ovarian stimulation (COS) was performed until ovulation induction. Through the serial vaginal ultrasonography monitoring, on day 6, 0.25 mg dosage of GnRH antagonist (Ganirelix-Orgalutranw; MSD, Ballerup, Denmark) was used in a daily injection. When the patients had at least 2-3 retrievable follicles with an average diameter of ≥13 mm, 6500IU dosage of the human chorionic gonadotrophin (hCG, Ovitrelle; Merk Serono, Hellerup, Denmark) was used to induce final oocyte maturation. The "Freeze all strategy" was performed for the high-risk ovarian hyper stimulation syndrome (OHSS) patients who had more than 15 follicles. Our patients who had <15 follicles and/or estradiol level <3000, received a 10000 IU hCG. Oocytes retrieved was performed 36-38 hours after hCG injection using a standard ultrasonically guided follicular puncture. Subsequently, these oocytes underwent an IVF/ICSI process.

The vitrification and warming were performed according to the previously protocols (24). For this aim the 3rd of embryos culture, high quality ones (≥8 cells and ≤25% fragmentation) were incubated for 5-15 minutes at the room temperature (RT) in the equilibrium solution. Then, the embryos were placed 1 minutes at RT in the vitrification solution.

For warming, the Cryotop was taken out of liquid nitrogen and the embryos were exposed for 50-60 seconds at 37.0°C to the thawing solution. Then, the embryos were transferred into the dilution solution (0.5 mol/L sucrose) for 3 minutes at RT. After which, the embryos were converted to another solution (0.25 mol/L sucrose) for 3 minutes at RT. The warmed embryos were washed four or five times in washing solution (VitROwash medium supplemented with 20% Albuminal-5) and then cultured in SAGE-1 stepTM (ORIGIO® CULTURE MEDIA, Denmark) until day 5. After 48 hours culture, the embryos were transferred to mother's uterus in the FET cycle and the ECM residual were collected and stored at -080°C until use. Our study participants included 53 embryos from 40 infertile couples (25 PCOS and 28 normal oogenesis) who were subdivided into patients with or without AO. It is as follows: $PCOS^{+}$-AO^{+} (n=12), $PCOS^{+}$-AO^{-} (n=13), $PCOS^{-}$-AO^{+} (n=13) and $PCOS^{-}$-AO^{-} (n=15).

Endometrial stromal cells isolation and *in vitro* culture

The tissue biopsies of healthy participants were obtained during the secretory phase of the menstrual cycle. The collected biopsies were transferred to a medium for transfer containing phosphate-buffered saline (PBS, Gibco, USA) (0.8 g NaCl, 1.44 g Na_3HPO_4, 0.2 g KCl, and 0.24 g KH_2PO_4), 2% pen/strep (Gibco, USA) and 1% anti-fungal (Gibco, USA). Then, tissue biopsies were washed in the PBS three to four times under the sterile condition in a petry dish (Falcon, USA). After mechanical digestion, the collected tissue biopsies (≈1 mm^3) were transferred to an enzymatic digestion medium (0.0125 g of collagenase type IV, Gibco, USA), dissolved in 5 ml of Dulbecco's modified eagle medium nutrient mixture F-12 (DMEM-F12, Gibco, USA), then incubated at 37°C in a water bath. The digested samples were filtered through 100, and 40 µm mesh filter, respectively. The final filtered cells were centrifuged at 1200 rpm for 20 minutes. The sediment was suspended in 1 ml of DMEM-F12 medium (1.2 g DMEM-F12, 0.24 g $NaHCO_3$, 0.005 g penicillin, and 0.006 g streptomycin) containing 15% fetal bovine serum (FBS, Gibco, USA). Primary ESCs were cultured up to first passage, due to long term *in vitro* culture can alter their decidualization potential (25). After first passage, 70000 cells/150λ were transferred to each 24-well dish (Falcon).

Decidualization induction of endometrial stromal cells

To induce *in vitro* decidualization conditions in the ESCs, in each well of 24 wells, 200 µL of induction medium including DMEM-F12+FBS 2%+8-Br-cyclic adenosine monophosphate (cAMP, 0.5 mM, Sigma) and medroxyprogesterone acetate (MPA, 1 µM; Sigma) were added and incubated at 37ºC, 5% CO_2 for 48

hours. The ESCs morphological change during 48 hours after decidualization induction was recorded by light microscopy and was including appearance of rounded, epithelioid-like cells.

Culture of induced endometrial stromal cells with the embryo condition media

The primary induced ESCs were cultured with the ECM collected from different experimental groups [$PCOS^{+}$-AO^{+} (n=12), $PCOS^{+}$-AO^{-} (n=13), $PCOS^{-}$-AO^{+} (n=13) and $PCOS^{-}$-AO^{-} (n=15)]. For this aim, 150 µL/well of pooled ECM (30% dilution: 30% ECM+70% DMEM-F12 with 10% FBS) was added per well in 24 wells and incubated for 12 hours (26).

Evaluation of decidualization capacity in cultured induced cells with the embryo condition media

For all groups, the ECM was removed after a 12 hours exposure of the ESCs with the ECM and replaced by the media culture (DMEM-F12 supplemented with 10% FBS). Then, the collected condition media were stored at -80°C until evaluation by Enzyme linked immunosorbent assay (ELISA). The concentration of two decidualization biomarkers PRL (M06L1H1, ng/ml, Monokit Prolactin ELISA Kit inc., Iran), and IGFBP-1 (ZB-OEH543211103-21, ng/ml, Zellbio assay kit Inc., Germany), as well as pro-inflammatory cytokine, IL1-β (HL1-0222002, pg/ml, Biotech assay kit Inc., Iran) was evaluated using commercial ELISA kits, according to the manufacturer's protocol.

Assessment of migratory capacity in endometrial stromal cells cultured with the embryo condition media

The wound healing assay was performed according to our previous study (23) with some modification due to ECM limitation in this study for this aim, we used 24 well plate for performing a modified wound healing assay to investigate the migration capacity of ESCs. Then 70.000 cells were seeded into each well of 24 well plate. A wound was created by a sterile christal pipette tip on the centre of each well. Subsequently, the ESCs were treated with 0.1 mg/ml of mitomycin C (Sigma) to prevent cell proliferation for 2 hours, followed by washing samples twice with 1% PBS. Then, 200 µL DMEM-F12+10% FBS were added to each well. The ESCs migration capacity was evaluated at 0, 6, 12, 18, and 24 hours after wounding. The grove width was measured at each time point, using Image J software following the below formula:

Measuring of narrowing furrow width in each time point=The width of the grove at 0 time-The width of grove width at each time point

Evaluation of decidualization gene markers expression in endometrial stromal cells treated with embryo condition media

ESCs were stored in RNA protect solution (Cat. No. 76526, Qiagen, Germany) at -80°C. After thawing, ESCs were centrifuged and total RNA was extracted using RNeasy kit (Qiagen, Germany) according to manufacturer's instructions. RNA quantity was assessed using "Nanodrop One" spectrophotometer (Thermo Scientific™, Germany) Subsequently, 11 µg RNA was converted into cDNA per manufacturer's Reverse Transcription kit instruction (SMO-BIO, Taiwan).

All primers were designed by using Primer-BLAST software (NCBI Primer-BLAST website, Table 1). Primers were assessed for specificity, dimers and splice variant detection if applicable. Quantitative polymerase chain reactions (PCRs) were conducted using SYBR Green (Thermo Scientific™) on StepOnePlus™ Real-Time PCR System (Thermo Scientific™), according to the manufacturer's instructions in strips. Samples reactions were done in duplicate. For each reaction, 2 µl cDNA and 8 µl master mix was used.

Table 1: Primer sequence for decidualization markers

Gene	Primer sequence (5′-3′)	PCR product (bp)
PRL	F: TCTGTATCATCTGGTCAC R: ATGAACCTGGCTGACTAT	133
IGFBP1	F: GCCCTGCCGAATAGAACTC R: GTCTCACACTGTCTGCTG	136
IL-1β	F: CTGTCCTGCGTGTTGAAAGA R: TTCTGCTTGAGAGGTGCTGA	180
HOXA10	F: ACAAGCACACCACAATTCTC R: AATCCAAACAATGTCTCCCTTC	161
IL-6	F: AGGAGACTTGCCTGGTGAAA R: CAGGGGTGGTTATTGCATCT	180
TNF-α	F: CCTCTCTCTAATCAGCCCTCTG R: GAGGACCTGGGAGTAGATGAG	212

PCR; Polymerase chain reaction.

Analyse data comparative cycle threshold (CT) method as a fold change and normalize with GAPDH as a reference gene.

RNA was extracted from ESCs which were cultured for 2 days in induction medium supplemented with cAMP and MPA and next 12 hours with ECM. followed by culture in ECM from four patients and two control groups, qRT-PCR was conducted to evaluate *PRL*, *IGFBP1*, *IL1-β*, *HOXA10*, *IL-6*, and *TNF-α* expression among the studied groups.

Statistical analysis

In this research, SPSS version 26 Software (IBM, Germany) was used for statistical analysis. Continuous variables were expressed as mean ± standard deviation and categorical variables were expressed as number (%). The normality of the investigated variables was checked with the Shapiro-Wilk test. In case of normality, the one-way ANOVA test and Tukey's post hoc test, and in case of non-normality, Kruskal-Varis and Conover's post hoc test were used, and the significance level of $P<0.05$ was considered as statistically significant. All qPCR results were analysed using the $2^{-\Delta\Delta CT}$, as a relative expression method. Chi-square was used to test the possible differences

in pregnancy rate among the studied groups. All graphs were drawn using the GraphPad Prism version 9 Software (GraphPad, San Diego, CA).

Results

Our study participants included 25 PCOS and 28 normal oogenesis subdivided into patients with or without AO. Analysis of BMI showed no significant differences between the studied groups (Table 2).

Decidualization induction in endometrial stromal cells cultured

After decidualization induction, the morphological changes of ESCs cells were recorded by light microscopy and visualized (Fig.1).

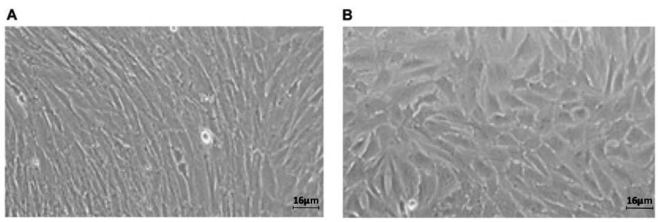

Fig.1: Morphological change of ESCs from 24 hours to 48 hours after induction with MPA and cAMP. **A.** Before decidualization and **B.** After decidualization (scale bar: 16 µm). ESC; Endometrial stromal cells, cAMP; Cyclic adenosine monophosphate, and MPA; Medroxyprogesterone acetate.

Evaluation of decidualization protein markers

The concentration of secreted IL1-β, PRL and IGFBP-1 proteins in condition media of ESCs cultured by ECM for 12 hours was measured using ELISA kit and is illustrated in Figure 2. Results showed considerably higher secretion level of PRL (P≤0.0139) in $PCOS^{+}$-AO^{+} group (2.6 ± 3.8) in comparison with the $PCOS^{+}$-AO^{-} (0.20 ± 0.19), $PCOS^{-}$-AO^{+} (0.33 ± 0.32), $PCOS^{-}$-AO^{-} (0.19 ± 0.16), CS (0.10 ± 0.00), and CI (0.23 ± 0.23) groups. However, levels of IL1-β, and IGFBP-1 were not significantly different among our groups (Fig.2).

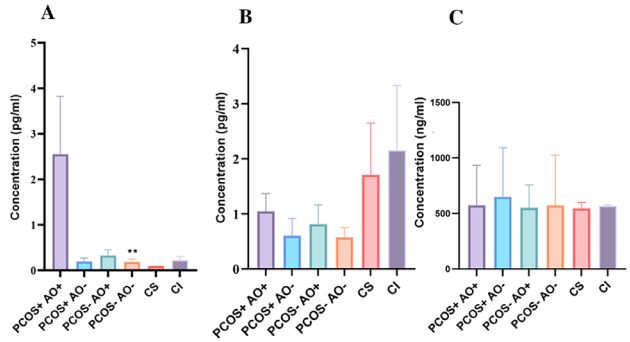

Fig.2: Results of ELISA for quantification of secreted **A.** PRL, **B.** IL1-β, and C. IGFBP1 proteins in the ECM from different studied groups by One-Way ANOVA statistical test. PRL; Prolactin, IL1- β; Interluekin 1 beta, IGFBP1; Insulin like growth factor binding protein 1, ECM; Embryo condition media, PCOS; Polycystic ovary syndrome, AO; Abdominal obesity, $^{+}$; with (PCOS or AO), -; without (PCOS or AO), CI; Control induction, CS; Control supernatant. Stars show a statistically significant difference with $PCOS^{+}$-AO^{+} group (**; P<0.01).

Calculating the migration capacity of endometrial stromal cells cocultured with embryo supernatant in different groups

The result of cell migration assay through scratch assay demonstrated that the highest migration capacity was belonged to the $PCOS^{+}$-AO^{+} group (1600 µm ± 83.3), and the lowest migration capacity belonged to the $PCOS^{-}$- AO^{+} group (532.2 µm ± 132.8). The calculated migration in other groups were $PCOS^{+}$-AO^{-} group (1017 µm ± 205.3), $PCOS^{-}$-AO^{-} group (1237 µm ± 216.9), CI group (590.2 µm ± 164.7), and CS group (982.2 µm ± 88.3) (P≤0.0321, Fig.3).

Table 2: Comparison of the patient's characteristics among four studied groups

Characteristics	$PCOS^{+}$-AO^{+} (n=12)	$PCOS^{+}$-AO^{-} (n=13)	$PCOS^{-}$-AO^{+} (n=13)	$PCOS^{-}$-AO^{-} (n=15)	P value of ANOVA
Age (Y)	27.4 ± 3.2^{B}	30.1 ± 2.3^{AB}	30.8 ± 3.1^{A}	28.3 ± 4.1^{AB}	0.025
BMI (kg/m^2)	28.2 ± 3.7	28.1 ± 3.9	27.3 ± 2.8	25.2 ± 3.5	0.066
WHR (cm/cm)	0.85 ± 0.05^{A}	0.75 ± 0.04^{B}	0.82 ± 0.05^{A}	0.74 ± 0.05^{B}	<0.001
Neck circumstance (cm)	35.9 ± 2.7^{A}	34.1 ± 1.8^{AB}	34.4 ± 2.0^{A}	32.2 ± 1.0^{B}	<0.001
Wiest/Neck (cm/cm)	2.54 ± 0.17	2.35 ± 0.14	2.47 ± 0.18	2.33 ± 0.17	0.002
Arm circumstance (cm)	30.1 ± 2.9	30.5 ± 2.6	30.4 ± 3.0	28.6 ± 3.2	0.270
Wiest/Arm (cm/cm)	3.03 ± 0.20	2.64 ± 0.20	2.81 ± 0.20	2.65 ± 0.19	<0.001
TSH (µl/ml)	2.5 (1.2-3.9)	2.2 (1.3-3.2)	2.6 (1.3-3.7)	1.8 (1.5-2.3)	
Previews ART cycles	1	0	0	0	
Pregnancy rate	12 (42)	13 (33)	13 (54)	15 (44)	0.769
Abortion rate	12 (17)	13 (15)	13 (0)	15 (13)	0.517

Data is represented as mean ± SD or n (%) or mean (minimum-maximum). PCOS; Polycystic ovary syndrome, AO; Abdominal obesity, BMI; Body mass index, WHR; Waist/hip ratio, TSH; Thyroid stimulating hormone, and ART; Assisted reproductive technology. Similar superscripts indicate a statistically significant difference (P≤0.001).

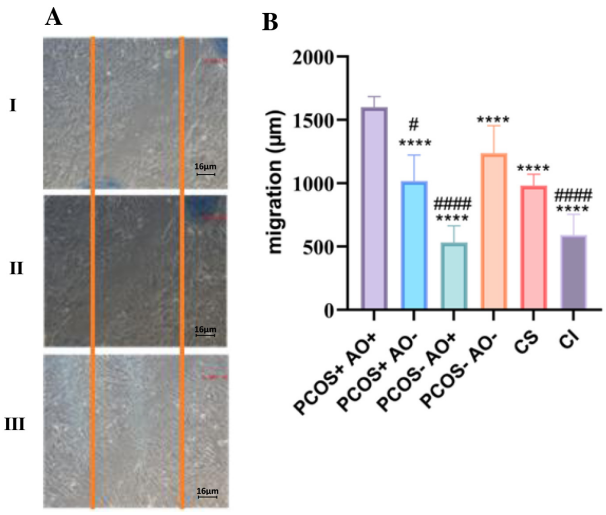

Fig.3: Cell migration assay for ESCs treated with ECM collected from the conditioned media of blastocysts culture from study groups by One-Way ANOVA statistical test. **A.** Migration of endometrial stromal cells treated with ECM at 6 hours (I), 12 hours (II) and 18 hours (III). **B.** Migration results of decidualization endometrial stromal cells 18 hours after scratching. PCOS; Polycystic ovary syndrome, AO; Abdominal obesity, +; With (PCOS or AO), -; Without (PCOS or AO), ESC; Endometrial stromal cells, ECM; Embryo condition media, CI; Control induction, CS; Control supernatant. Stars show a statistically significant difference with PCOS^{+}-AO^{+} group (****; P<0.0001). Hashtags show a statistically significant difference with PCOS--AO- group (#; P=0.0001 and ####; P=0.0001). Bar graphs show the calculated migration of ESCs.

Gene expression analysis

The qRT-PCR results were illustrated in Figure 4. Analysis of decidualization markers showed a significant highest expression of *PRL* in the PCOS^{+}-AO^{+} group among our groups (P≤0.009). Moreover, the PCOS^{-}-AO^{+} group showed significantly higher *PRL* expression level than the PCOS^{-}-AO^{-} group (P=0.0128). The same trend was observed for *IGFBP-1* gene, but the differences were not statistically significant due to high variation among the individuals in the PCOS^{+}-AO^{+} group. Moreover, the PCOS^{+}-AO^{+} group showed significantly highest expression of level *HOXA10* in comparison with among our groups (P≤0.0248). These results show higher decidualization properties in the PCOS^{+}-AO^{+} group.

Analysis of decidualization related genes showed highest expression level of *IL-1β* in the PCOS^{+}-AO^{+} group among our groups, while this difference was significant in comparison with the CS (P=0.013), and CI groups (P=0.0095). The expression level of *TNF-α* was significantly highest in the PCOS^{+}-AO^{+} group among our groups, including PCOS^{-}-AO^{+} group (P=0.0039), PCOS^{-}-AO^{-} group (P=0.0005), CS group (P=0.0004), and CI group (P=0.0004). The lowest expression level of *IL-6* was belonged to the PCOS^{+}-AO^{+} group (0.0029 ± 0.0025) in comparison with other groups. The CI group (409.4 ± 354.5) showed a significant highest expression level of *IL-6* among our groups (P<0.05, Table 2).

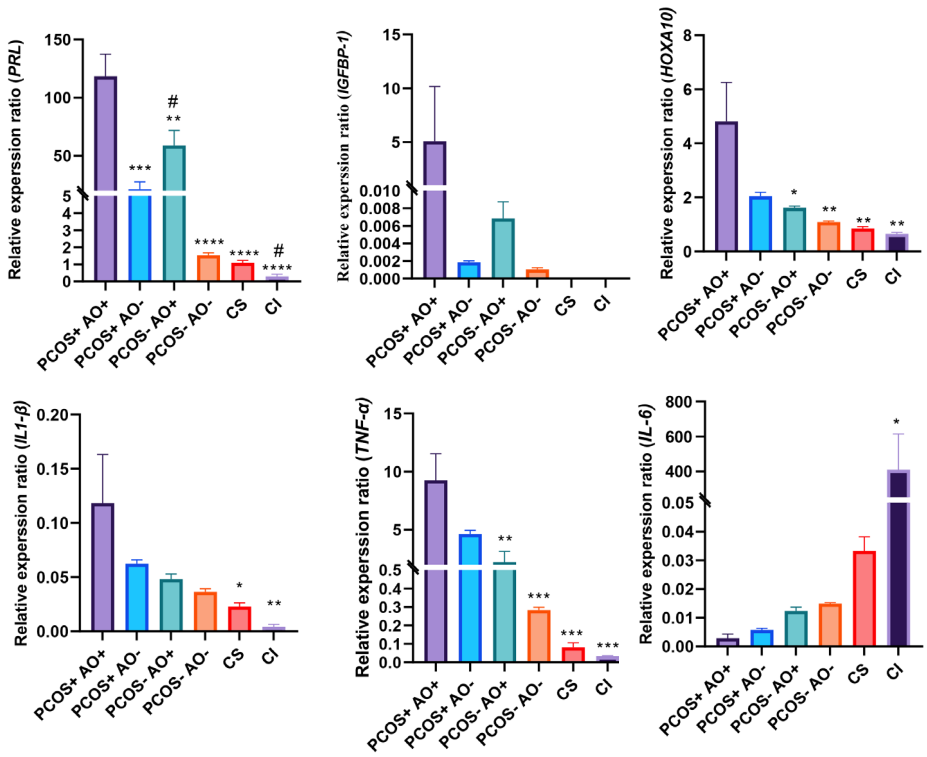

Fig.4: Results of qRT-PCR of genes related to decidualization and inflammation in the studied groups by One-Way ANOVA statistical test. qRT-PCR; Quantitative reverse transcription polymerase chain reaction, PCOS; Polycystic ovary syndrome, AO; Abdominal obesity, +; With (PCOS or AO), -; Without (PCOS or AO), CI; Control induction, and SC; Control supernatant. Stars (*) show a statistically significant difference with the PCOS^{+}-AO^{+} group (*; P<0.05, **; P<0.01, ***; P<0.001, and ****; P<0.0001). Hashtags (#) show a statistically significant difference with the PCOS^{-}-AO^{-} group (#; P<0.05).

Discussion

In this study, the role of AO in the PCOS patients was investigated. Recently, it was suggested that the ECM can be used to evaluate the capacity of embryo to induce decidualization representing the *in vitro* embryo implantation potential (27). Accordingly, ECM samples were obtained of PCOS patients, with and without of AO, and also healthy fertile women to evaluate the potential of decidualization induction in the primary ESCs.

In the present study, we showed that the markers of ECM-induced decidualization in $PCOS^{+}$-AO^{+} were significantly increased compared to other groups. Accordingly, decidualization induction by the ECM from the $PCOS^{+}$-AO^{+} group caused a change in the morphology of ESCs, as well as a significant increase in the concentration of secreted PRL, although there is no significant difference between IGFBP-1, and IL-1β proteins secretion between groups. Moreover, we observed higher expression of *PRL*, *IGFBP-1*, *IL-1β*, *TNF-α*, and *HOXA10* genes in ESCs from different groups. Nonetheless, the expression of IL-6, as an acute phase response stimulator, was decreased in the $PCOS^{+}$-AO^{+} group, along with the highest rate of migration capacity which was observed in cultured decidualized ESCs with their ECM.

Notably, it has been demonstrated that PCOS patients have a larger number of retrieved oocytes in comparison with normal oogenesis individuals. The PCOS patients experience a lower live birth rate and also, a higher spontaneous abortion rates (28). It was suggested that, disrupted decidualization process, endometrial receptivity and subsequent blastocyst implantation may be involved in these outcomes.

Based on the contribution of proinflammatory cytokines in the etiology of PCOS, it was suggested that this disease can be considered as an inflammatory disease (29). Evidences show that HA and IR, two main complications of syndrome, may play a role in inflammation induction in women with PCOS (30). Moreover, obesity is a known condition that activates inflammatory pathways in cells and tissue. It has been shown that the triglyceride storage process is disturbed in the PCOS patients with increase of free fatty acids which could lead to a state of IR. Inflammation cytokines can lead to IR by disrupting the insulin post receptor signalling pathway. The relationship between HA, IR, and chronic inflammation is still not fully understood (31).

While a chronic inflammation may disrupt fertility related process in healthy women, a low level acute endometrial inflammation is necessary for successful embryo implantation (19). It has been showed that an inflammatory response in the uterine mucosa is required prior to blastocyst invasion (32). Therefore, Pro-inflammatory cytokines can produce by multiple source including embryo secretory profile, maternal immune cells such as natural killer cells (NKc), macrophages, dendritic cells, and ESCs (33).

Therefore, the ESCs can produce pro-inflammatory cytokines including TNF-α and IL-1 at this critical stage. Similar studies have been observed that PCOS patients have higher level of TNF-α and IL-1 in comparison with non-PCOS patients (34). It has been suggested that the early detection of such cytokines in the endometrium may be considered as markers of an implantation competency (19). In the present study, we observed that IL-6 level decreased significantly in the $PCOS^{+}$-AO^{+} group. Scheller et al. (35) reported that IL-6 seems to possess both pro inflammatory and anti-inflammatory properties. The association of IL-6 levels with PCOS susceptibilities has been investigated in several studies (36). However, the results were controversial, failing to find a clear role of IL-6 in pathogenesis of PCOS.

In this study, the $PCOS^{+}$-AO^{+} group showed the highest expression of decidualization markers. It has been explained that, inflammatory process is required for improvement of decidualization and implantation within days of conception (19). Accordingly, all the decidualization markers investigated in this study also has been considered as inflammatory markers in previous study. It seems that pro inflammatory cytokines are enriched in a PCOS associated AO conditions which can lead to higher induction of decidualization in the $PCOS^{+}$-AO^{+} group in comparison with other groups.

The association of increasing decidualization markers and endometrial receptivity has been demonstrated in other studies (37). Our result show that embryos collected from PCOS patients have higher potency for a decidualization induction in healthy endometrial receptivity. Zhao et al. (38) reported that IR and hyperinsulinemia (HI), two main complication of PCOS, may exacerbate the endometrial receptivity. Our results including a higher expression of level decidualization markers in the ESCs co-cultured with ECM was consistent with this report. Therefore, we may conclude that the ECM collected from the $PCOS^{+}$-AO^{+} group, may increase the endometrial decidualization and subsequent endometrial receptivity. Due to an increased endometrium receptivity in these patients, even suboptimal embryos may likely implant, which subsequently may result a higher abortion rate (39). In the other hand, It has been shown that a pre-inflammatory response followed by an anti-inflammatory phase is necessary for successful in mammalian embryo implantation (40). We suggest that the uterine microenvironment may switch disruptively from an inflammatory to an anti-inflammatory phase in the $PCOS^{+}$-AO^{+} women. We can propose that higher abortion rate in PCOS patients may arise from these two possible hypotheses. In the present study, abortion rate was evaluated but it was not significant that it seems because of our participants number limitation or due to freeze embryo transfer indication. However, several studies shown that abortion rate was higher in these patients (39).

Controversially, several studies showed that endometrial receptivity potential can be decreased in PCOS patients due to metabolic alterations, inflammatory events, and

some abnormally expressed endometrial molecular markers (38). In these studies, the decidualization capacity of endometrial cells which obtained from PCOS patients was not intended. Also, we did not examine the decidualization phenotype of ESCs collected from our PCOS patients then, we don't have a clear view of the ESCs of these PCOS patients. Further studies with large population are needed to clarify the decidualization capacity of ESCs collected from the PCOS patients under treatment of their ECM.

Conclusion

Our results showed that the collected embryos from PCOS patients with AO have elevated level of inflammatory mediators leading to the higher potential for decidualization induction in the healthy ESCs. This result may be employed for future therapeutic approach in which the increased decidualization capacity and receptivity of ESCs are intended.

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Authors' Contribution

Z.Sh., S.T.; Conducted the experiments, Analyzed and Illustrated the results, and Wrote the first draft of the manuscript. P.E.Y.; Designed the study, Verified the analytical methods, Supervised the findings, and Critically revised the manuscript. N.N.; Helped design the study, Verify the analytical methods, Supervise the findings, and Critically revised the manuscript. M.H., T.M.; Designed the project and provided clinical advices. All authors read and approved the final manuscript.

References

1. Deswal R, Narwal V, Dang A, Pundir CS. The prevalence of polycystic ovary syndrome: a brief systematic review. J Hum Reprod Sci. 2020; 13(4): 261-271.
2. Xue Z, Li J, Feng J, Han H, Zhao J, Zhang J, et al. Research progress on the mechanism between polycystic ovary syndrome and abnormal endometrium. Front Physiol. 2021; 12: 788772.
3. Cooney LG, Dokras A. Beyond fertility: polycystic ovary syndrome and long-term health. Fertil Steril. 2018; 110(5): 794-809.
4. Wang C, Wen YX, Mai QY. Impact of metabolic disorders on endometrial receptivity in patients with polycystic ovary syndrome. Exp Ther Med. 2022; 23(3): 221.
5. Shah D, Patil M. Infertility management in lean versus obese PCOS. In: Genazzani AR, Ibáñez L, Milewicz A, Shah D, editors. Impact of polycystic ovary, metabolic syndrome and obesity on women health. Springer: 2021; 105-127.
6. Barrea L, Frias-Toral E, Verde L, Ceriani F, Cucalón G, Garcia-Velasquez E, et al. PCOS and nutritional approaches: differences between lean and obese phenotype. Metabol Open. 2021 3; 12: 100123.
7. Bellver J, Rossal LP, Bosch E, Zúñiga A, Corona JT, Meléndez F, et al. Obesity and the risk of spontaneous abortion after oocyte donation. Fertil Steril. 2003; 79(5): 1136-1140.
8. Singh R, Kaur S, Yadav S, Bhatia S. Gonadotropins as pharmacological agents in assisted reproductive technology and polycystic ovary syndrome. Trends Endocrinol Metab. 2023; 34(4): 194-215.
9. Ochoa-Bernal MA, Fazleabas AT. Physiologic events of embryo implantation and decidualization in human and non-human primates. Int J Mol Sci. 2020; 21(6): 1973.
10. Sehring J, Beltsos A, Jeelani R. Human implantation: the complex interplay between endometrial receptivity, inflammation, and the microbiome. Placenta. 2022; 117: 179-186.
11. Badri Seighaldehi O, Mohseni Kouchesfahani H, Nasiri N, Shahverdi M, Hezavehei M, Shahverdi A. New findings in non-invasive assessment of embryo quality. Journal of Cell and Tissue (JCT). 2021; 12(3): 206-219.
12. Diedrich K, Fauser B, Devroey P, Griesinger G. The role of the endometrium and embryo in human implantation. Hum Reprod Update. 2007; 13(4): 365-377.
13. Brosens JJ, Bennett PR, Abrahams VM, Ramhorst R, Coomarasamy A, Quenby S, et al. Maternal selection of human embryos in early gestation: insights from recurrent miscarriage. Semin Cell Dev Biol. 2022; 131: 14-24.
14. Large MJ, DeMayo FJ. The regulation of embryo implantation and endometrial decidualization by progesterone receptor signaling. Mol Cell Endocrinol. 2012; 358(2): 155-165.
15. Tamura I, Asada H, Maekawa R, Tanabe M, Lee L, Taketani T, et al. Induction of IGFBP-1 expression by cAMP is associated with histone acetylation status of the promoter region in human endometrial stromal cells. Endocrinology. 2012; 153(11): 5612-5621.
16. Xu Y, Lu J, Wu J, Jiang R, Guo C, Tang Y, et al. HOXA10 co-factor MEIS1 is required for the decidualization in human endometrial stromal cell. J Mol Endocrinol. 2020; 64(4): 249-258.
17. Snegovskikh V, Foyouzi N, Schatz F, Buhimschi C, Buhimschi I, Funai E, et al. IL-1 stimulates TNF production by human first trimester decidual stromal cells: Implications for implantation. Am J Obstet Gynecol. 2005; 193 Suppl 6: S175.
18. Pathare ADS, Hinduja I, Mahadik RC. Basic aspects of endometrial receptivity in PCOS patients. Mol Biol Rep. 2022; 49(2): 1519-1528.
19. Sieg W, Kiewisz J, Podolak A, Jakiel G, Woclawek-Potocka I, Lukaszuk J, et al. Inflammation-related molecules at the maternal-fetal interface during pregnancy and in pathologically altered endometrium. Curr Issues Mol Biol. 2022; 44(9): 3792-3808.
20. Goto S, Kadowaki T, Hashimoto H, Kokeguchi S, Shiotani M. Stimulation of endometrium embryo transfer (SEET): injection of embryo culture supernatant into the uterine cavity before blastocyst transfer can improve implantation and pregnancy rates. Fertil Steril. 2007; 88(5): 1339-1343.
21. Li Y, Liu S, Lv Q. Single blastocyst stage versus single cleavage stage embryo transfer following fresh transfer: A systematic review and meta-analysis. Eur J Obstet Gynecol Reprod Biol. 2021; 267: 11-17.
22. Franasiak JM, Alecsandru D, Forman EJ, Gemmell LC, Goldberg JM, Llarena N, et al. A review of the pathophysiology of recurrent implantation failure. Fertil Steril. 2021; 116(6): 1436-1448.
23. Nasiri N, Babaei S, Moini A, Eftekhari-Yazdi P. Controlling semi-invasive activity of human endometrial stromal cells by inhibiting NF-kB signaling pathway using aloe-emodin and aspirin. J Reprod Infertil. 2021; 22(4): 227-240.
24. Fathi R, Valojerdi MR, Yazdi PE, Ebrahimi B, Alipour H, Hassani F. Development of 4-cell mouse embryos after re-vitrification. Cryobiology. 2012; 64(1): 23-26.
25. Jividen K, Movassagh MJ, Jazaeri A, Li H. Two methods for establishing primary human endometrial sromal cells from hysterectomy specimens. J Vis Exp. 2014; (87): 51513.
26. Brosens JJ, Salker MS, Teklenburg G, Nautiyal J, Salter S, Lucas ES, et al. Uterine selection of human embryos at implantation. Sci Rep. 2014 Feb 6;4:3894.
27. Siristatidis CS, Sertedaki E, Karageorgiou V, Vaidakis D. Endometrial injection of embryo culture supernatant for subfertile women in assisted reproduction. Cochrane Database Syst Rev. 2020; 8(8): CD013063.
28. Tan IF, Lim AJ, Indran IR, Kramer MS, Yong EL. Reproductive outcomes of women with polycystic ovarian syndrome following in-vitro fertilization—a meta-analysis and systematic review. Fertility & Reproduction. 2019; 1(4): 193-201.
29. Regidor PA, Mueller A, Sailer M, Gonzalez Santos F, Rizo JM, Egea FM. Chronic inflammation in PCOS: the potential benefits of specialized pro-resolving lipid mediators (SPMs) in the improvement of the resolutive response. Int J Mol Sci. 2020; 22(1): 384.
30. Dabravolski SA, Nikiforov NG, Eid AH, Nedosugova LV,

Starodubova AV, Popkova TV, et al. Mitochondrial dysfunction and chronic inflammation in polycystic ovary syndrome. Int J Mol Sci. 2021; 22(8): 3923.
31. O'Rourke RW. Inflammation in obesity-related diseases. Surgery. 2009; 145(3): 255-259.
32. Robertson SA, Moldenhauer LM, Green ES, Care AS, Hull ML. Immune determinants of endometrial receptivity: a biological perspective. Fertil Steril. 2022; 117(6): 1107-1120.
33. Zhang T, Shen HH, Qin XY, Li MQ. The metabolic characteristic of decidual immune cells and their unique properties in pregnancy loss. Immunol Rev. 2022; 308(1): 168-186.
34. Blumenfeld Z. The possible practical implication of high CRP levels in PCOS. Clin Med Insights Reprod Health. 2019; 13: 1179558119861936.
35. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The pro- and anti-inflammatory properties of the cytokine interleukin-6. Biochim Biophys Acta. 2011; 1813(5): 878-888.
36. Eser B, Islimye Taskin M, Hismiogullari AA, Aksit H, Bodur AS. The effects of IL-1A and IL-6 genes polymorphisms on gene expressions, hormonal and biochemical parameters in polycystic ovary syndrome. J Obstet Gynaecol. 2017; 37(3): 358-362.
37. Liu H, Huang X, Mor G, Liao A. Epigenetic modifications working in the decidualization and endometrial receptivity. Cell Mol Life Sci. 2020; 77(11): 2091-2101.
38. Zhao J, Chen Q, Xue X. An Update on the progress of endometrial receptivity in women with polycystic ovary syndrome. Reprod Sci. 2022; 29(8): 2136-2144.
39. Sun YF, Zhang J, Xu YM, Cao ZY, Wang YZ, Hao GM, et al. High BMI and Insulin resistance are risk factors for spontaneous abortion in patients with polycystic ovary syndrome undergoing assisted reproductive treatment: a systematic review and meta-analysis. Front Endocrinol (Lausanne). 2020; 11: 592495.
40. Zhang T, Zhu W, Zhao Y, Cheung WC, Liu Y, Chen X, et al. Early transient suppression of immune checkpoint proteins T-cell immunoglobulin mucin-3 and programmed cell death-1 in peripheral blood lymphocytes after blastocyst transfer is associated with successful implantation. Fertil Steril. 2020; 114(2): 426-435.

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Carbon Monoxide Exposure Does Not Improve The *In Vitro* Fertilization Rate of Oocytes Obtained from Heterozygous *Hmox1* Knockout Mice

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Abstract

In our experimental study we explored the impact of maternal reduced heme oxygenase-1 (HO-1) gene (*Hmox1*) expression on the *in vitro* fertilization (IVF) rate through the use of heterozygous *Hmox1* knockout mice models (*HET/Hmox1*$^{+/-}$). Also, we hypothesized a beneficial role of gametes exposure during fertilization to carbon monoxide (CO), one of HO-1 by-products, that might be relevant for the improvement of IVF rates. IVF technique was performed by using oocytes obtained from wild-type (WT) or *Hmox1*$^{+/-}$ dams fertilized with WT, *Hmox1*$^{+/-}$ or *Hmox1*$^{-/-}$ mice-derived sperm. The fertilization step occurred either in a conventional incubator (37°C, 5% CO_2) or in an incubator implemented with CO (500 ppm). The superovulation yield of WT and *Hmox1*$^{+/-}$ mice and the number of fertilized oocytes was assessed using an optical microscope. The dams' *Hmox1* heterozygous knockout neither impact the superovulation yield, nor did influence the fertilization success rate. Moreover, CO exposure during fertilization could not significantly improve the outcome. Our study showed that the maternal *Hmox1*$^{+/-}$ condition is not affecting the IVF rate in mice. Furthermore, we discovered that CO exposure cannot be exploited to ameliorate this critical step of the IVF protocol.

Keywords: Carbon Monoxide, Gene Knockout, HO-1 Protein, *In Vitro* Fertilization, Pregnancy

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Fertilization failure is still a major concern of the *in vitro* fertilization (IVF) procedure (1). In particular, total fertilization failure (TFF), which refers to the complete absence of fertilized oocytes after the incubation step with the donor sperm, occurs in 5-10% of IVF cycles (2). The intracytoplasmic sperm injection (ICSI) technique was introduced to overcome the possible obstacles related to the sperm ability to fuse with the egg. Nevertheless, TFF after ICSI occurs in the 2-3% of the cases, and generally up to 30% of the oocytes are not fertilized (3). This suggests that not only the paternal side, but the maternal as well is determinant for a successful fertilization, since sperm penetration is only one of the several events which need to occur efficiently. The age of the maternal donor is one of the main limitations to a successful outcome, however fertilization problems might occur even if the female donor is in the recommended age range to undergo IVF (< 38 years old), and when oocytes of at least apparent good quality and sperm of proven fertility are utilized (4). For this reason, it is crucial to gain a better understanding of possible genetic aspects and specific modulators that might be involved in the fertilization stages to support the choice of the right IVF approach. Besides, this additional knowledge could be relevant to optimize the current available methodologies.

Recent studies enlightened the involvement of heme oxygenase-1 (HO-1), the ubiquitous stress-induced isoform of HO, in diverse pregnancy-related processes, including fertilization (5, 6). HO-1 is encoded by the *HMOX1* gene, which was found to be expressed in both placenta and fetus starting from early gestational stage (7). HO is primarily known for catalysing the initial and rate-limiting reaction of heme degradation, and together with its by-products carbon monoxide (CO), biliverdin, and ferrous iron (Fe^{2+}), exhibit important cytoprotective, immunomodulatory, antioxidant, and anti-inflammatory properties (8). Remarkably, HO-1 deficiency in mice is correlated to diverse pregnancy complications, such as growth restriction,

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fetal loss, defective spiral artery remodeling, and hypertension. It was postulated that excess of free heme and insufficient release of CO are the main causes for this outcome. Indeed, application of hemin (free heme) dramatically increased fetal death (9). Further, the administration of low doses of CO (50 ppm) to HO-1 partial and total deficient dams at the time of placenta development (days 3-8 of pregnancy) was able to promote fetal viability, increase both placenta and pups' weights, and ameliorate the systolic blood pressure and wall-to-lumen ratios of the spiral arteries (9, 10). Accordingly, a possible role of the HO/CO axis in ovulation and fertilization was also investigated. It was previously shown that total-deficient (KO/*Hmox1*$^{-/-}$) female mice had a less efficient ovulation, and that the oocytes harvested from these dams were significantly less fertilized compared to those obtained from wild-type (WT) ones (20 vs. 60%) (6). Considering that sperm from WT and *Hmox1* knockout (KO) mice were proven to be equally fertile (11), these evidences proved that the total lack of HO-1 in the oocytes influences their ability to successfully complete fertilization, which includes several post-sperm penetration events. However, HO-1 human deficiency is a rare condition and up to date only 9 cases were described of patients who exhibited devastating and complex clinical courses (12). Nonetheless, it was described that *HMOX1* expression in the population may vary due to short tandem GT_n repeat (STR) region present in the promoter (13, 14). In particular, longer repeats were associated to decreased *HMOX1* expression in response to oxidative stress and to reduced HO-1 levels in serum and placenta (15-17).

In line with the described considerations and based on previous findings, the aim of the present study was to investigated whether a reduced maternal *Hmox1* expression in mice has an impact on ovulation and on the IVF success using heterozygous *Hmox1* knockout models (HET/*Hmox1*$^{-/-}$). Furthermore, we aimed to explore whether the exposure of oocytes to CO is able to improve their fertilization rate.

The progeny obtained by mating *Hmox1*$^{+/-}$ females with *Hmox1*$^{+/-}$ males in a BALB/c background was genotyped and accordingly assigned to the *Hmox1*-wild type (WT/*Hmox1*$^{+/+}$), *Hmox1* heterozygous KO (HET/*Hmox1*$^{+/-}$), or *Hmox1* total-KO (KO/*Hmox1*$^{-/-}$) mice groups that were used for the experiments (9). Mice were maintained in our barrier facility with a 12-h light/dark cycle, 22 ± 2°C, and 40-60% air humidity. They received water and food *ad libitum*. Experimental procedures were performed according to the institutional guidelines and approved by German authorities (Landesverwaltungsamt Sachsen Anhalt: 42502-2-1327 Uni MD).

The IVF protocol used in our experimental study included the following steps. Firstly, superovulation of WT (n=21) or *Hmox1*$^{+/-}$ (n=22) dams was induced by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG, Integonan®, Intervet, Germany), followed by 5 IU of Ovogest® (human chorionic gonadotropin, Intervet International B.V., Kenilworth, NJ, USA) 48 hours later. 12 hours later, sperm was collected from the cauda epididymis and the vasa deferentia of the males and transferred to a 35 mm petri dish containing 1 ml of ORIGIO® Handling™ IVF Medium (CooperSurgical®, Målov, Denmark), where it was incubated for 10 minutes at 37°C. Afterwards, 10 µl of the sperm were incubated (37°C, 5% CO_2) for 1 hour within 500 µl of pre-warmed IVF medium in 80 mm petri dishes ("IVF dishes") coated with mineral oil (Sigma Alderich, Steinheim, Germany), which were prepared the day before and incubated overnight (37°C, 5% CO_2). At this point-approximately 14 hours after Ovogest® injection-the dams were sacrificed by cervical dislocation. The oocyte complexes were collected from the ampullae and transferred to 35 mm petri dishes containing 1 ml of pre-warmed of IVF medium each. Oocytes that were obtained from each female were counted.

For the IVF experiments, we selected females from which we could harvest a minimum number of 5 oocytes. These females were randomly divided into 2 groups (control or CO-treated). This resulted in the following subgroups: WT-control (n=7), WT-CO-treated (n=7), *Hmox1*$^{+/-}$ -control (n=6), *Hmox1*$^{+/-}$ -CO-treated (n=7). Right after, the oocyte complexes were added to the IVF dishes in the same drop which contained the capacitated sperm. The sperm of one male was used to fertilize 4 females, each to cover all groups (WT-control, WT-CO-treated, *Hmox1*$^{+/-}$ -control, *Hmox1*$^{+/-}$ -CO-treated). The oocytes were then incubated for 4-6 hours at 37°C in a classic CO_2 incubator or in one implemented with CO at a concentration of 500 ppm, which was previously successfully used in one *in vitro* study (9). After, the oocytes were washed to remove the excess of sperm using a capillary to aspirate and move them in four 150 µl drops of IVF medium present in the IVF dishes. The washed oocytes were further incubated overnight at 37°C in the CO_2 incubator with or without CO (500 ppm). The morning after the number of fertilized ones was assessed via optical microscope observation. The fertilization rate was then calculated as the ratio of fertilized oocytes (two-cells stage embryos) divided by the total number of oocytes, multiplied by 100 (Fig.1).

GraphPad Prism software version 8.0 (GraphPad, Statcon, Witzenhausen, Germany) was used for the statistical analysis. For all the data collected mean ± SEM was calculated. In particular, normal distribution of the data using Shapiro-Wilk test was assessed. An unpaired t test was performed to establish the statistical difference in the number of oocytes harvested from the two groups of dams, while the data obtained from the fertilization experiments were analysed with Brown-Forsythe and Welch ANOVA test. All the groups' means were compared to each other using Dunnett's T3 test for multiple comparison as post-hoc test.

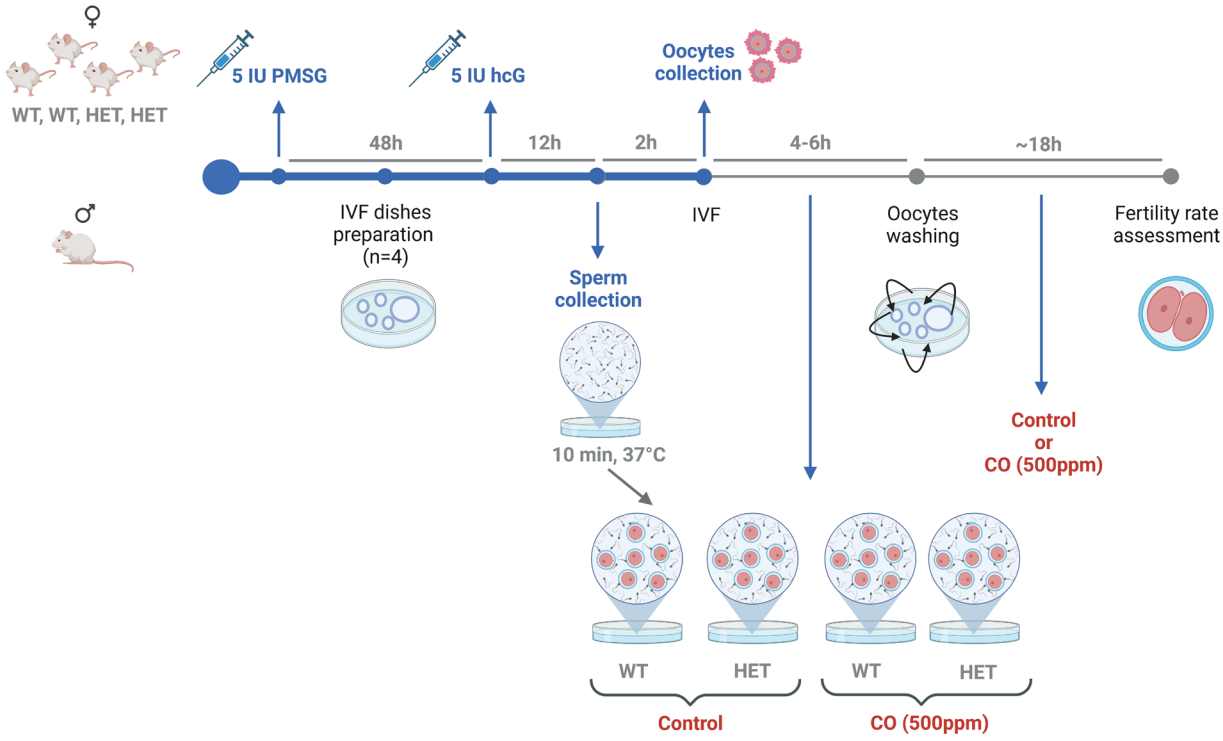

Fig.1: Graphical representation of the IVF procedure and experimental design. One experimental block comprised three rounds of IVF and was repeated three times in total. In each round the sperm from one male was used to fecondate the oocytes retrieved from 4 females (two WT and two HET dams) after the induction of superovulation by the intraperitoneal injection of 5 IU of PMSG, followed by 5 IU of Ovogest® . From the starting number of dams available, only those from which a minimum number of 5 oocytes could be harvested were used and randomly assigned to the control or CO-treated group. This resulted in the following dams number per subgroup: WT-control (n=7), WT-CO-treated (n=7), *Hmox*$^{+}$/$^{-}$-control (n=6), *Hmox*$^{+}$/$^{-}$-CO-treated (n=7). Their oocytes were correspondingly incubated with the capacitated sperm for 4-6 hours at 37°C either in a classic CO_2 incubator or in one implemented with CO at a concentration of 500 ppm. The oocytes were then washed to remove the excess of sperm and further incubated overnight at 37°C in the CO_2 incubator with or without CO (500 ppm). The morning after, the fertilization rate was then calculated as the ratio of fertilized oocytes (two-cells stage embryos) divided by the total number of oocytes, multiplied by 100. Created with BioRender.com. IVF; *In vitro* fertilization, WT; Wild type, HET; Heterozygous knockout for *Hmox1* (*Hmox*$^{+}$/$^{-}$), PMSG; Pregnant mare serum gonadotropin, CO; Carbone monoxide, h; Hours, and hCG; Human chorionic gonadotropin.

To begin with, we evaluated if the decreased dams *Hmox1* expression could have an impact on their ovulation. The mean of the oocytes obtained after the superovulation was comparable between the WT and *Hmox1*$^{+}$/$^{-}$ groups (Fig.2). This indicates that the ovulation process in *Hmox1*$^{+}$/$^{-}$ females is not impaired as it is in full KO mice (6). The oocytes retrieved (n=6-26) from WT or *Hmox1*$^{+}$/$^{-}$ mice were incubated with the capacitated sperm either in a classic incubator (5% CO_2, 37°C) or in an incubator implemented at a CO concentration of 500 ppm (9). To exclude falsification of the results due to differences in sperm fertility, the sperm of one male was used to fertilize 4 females, each to cover all groups (WT-control, WT-CO, *Hmox1*$^{+}$/$^{-}$-control, *Hmox1*$^{+}$/$^{-}$-CO). A total number of 3 males per genotype was used as seed donors. Besides WT and *Hmox1*$^{+}$/$^{-}$, also *Hmox1*$^{-}$/$^{-}$ males were available and used for the experiments. Indeed, it was showed before that sperm from *Hmox1* WT and KO mice is equally fertile (11), and this was confirmed in our experiments as well (data not showed). Moreover, it was found that HO-1 is only moderately expressed in male germ cells, while the prevalent HO isoform is the constitutively expressed HO-2, which is abundantly present in the testis (18).

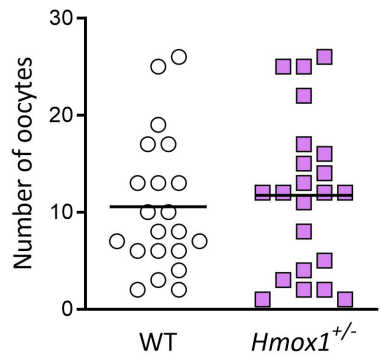

Fig.2: Number of oocytes harvested after superovulation from each wild-type (n=21) or *Hmox1*$^{+}$/$^{-}$ (n=22) dams. Data are shown as individual values with mean and were analysed for statistical differences by using the unpaired t test. WT; *Hmox1* wild type and *Hmox1*$^{+}$/$^{-}$; Heterozygous knockout for *Hmox1*.

As a result, the IVF experiments showed that the HO-1 partial deficiency of female mice did not lead to any statistically significant differences in the ability of their oocytes to be fertilized (Fig.3). This suggests that one maternal functional copy of the gene is enough to guarantee ovulation and fertilization at rates comparable with mice equipped with both copies.

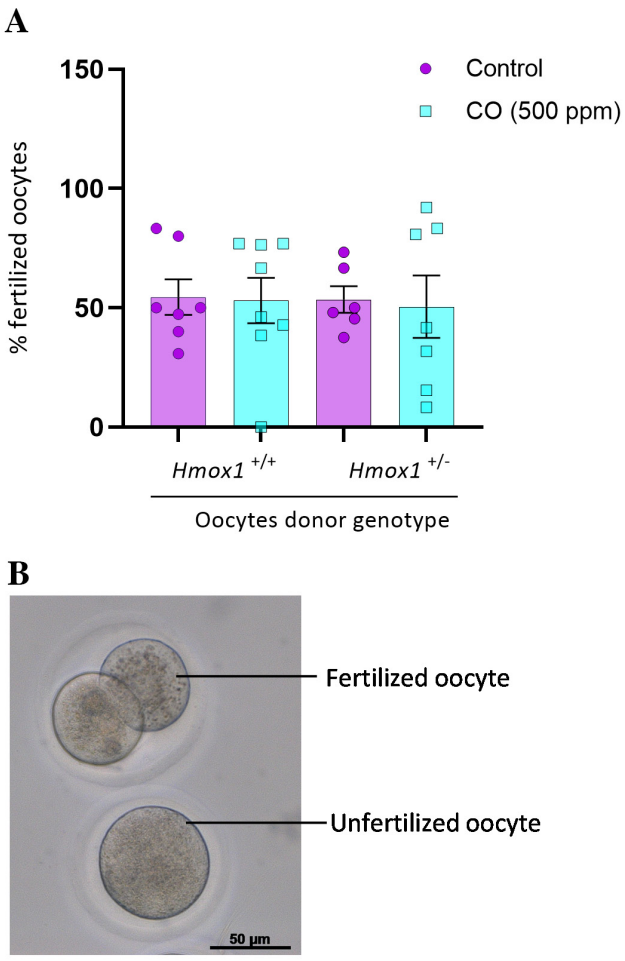

Fig.3: Percentage of fertilized WT or *Hmox*$^{+/-}$ dams- derived oocytes after CO treatment. **A.** Percentage of wild type (control n=7, CO-treated n=7) and *Hmox*$^{+/-}$ (control n=6, CO-treated n=7) dams-derived oocytes incubated in a CO_2 incubator without (control) or with CO (500 ppm). **B.** Microscopic image showing a fertilized (2-cells stage) and an unfertilized oocyte (scale bar: 50 µm). Data are presented as box plots showing the mean ± SEM and dots representing the individual values. The analysis for statistical differences was conducted using the Brown-Forsythe and Welch ANOVA test. Dunnett's T3 was used as post-hoc test. WT; *Hmox1* wild type and *Hmox*$^{+/-}$; Heterozygous knockout for *Hmox1*.

CO was demonstrated to mediate the protective functions of HO-1 enzyme in several pregnancy stages (5). Specifically, exogenous CO application was able to promote a normal placentation and fetal growth in mice which were totally defective for *Hmox1* expression and characterized by several pregnancy complications (9, 10). Additionally, the application of gaseous CO between gestation day 3 and 8 of pregnancy increases the percentage of viable fetuses in *Hmox1*$^{+/-}$ females mated with *Hmox1*$^{+/-}$ males (9). Dickson et al. (19) also demonstrated that pregnant dams exposure to CO (250 ppm) had a pro-angiogenic effect, leading to the enhancement of midgestational utero-placental vascular growth, with no negative impact on pregnancy outcomes. However, the effect of CO on oocytes or fertilization has been scarcely investigated. Interestingly, one study on *in vitro* oocytes aging showed that the delivery of CO to porcine oocytes was able to improve their viability and decrease the apoptotic rate mainly through the downregulation of caspase-3 activity. In the mentioned work different concentrations of CO donors (5-100 µM) with fast or slow release rates were used to implement the oocytes culture medium in presence of a heme oxygenase inhibitor (20).

In the presente study we wanted to explore whether CO exposure could improve the fertilization outcome in vitro. CO exposure during fertilization failed to improve the success rate of both WT and *Hmox1*$^{+/-}$ derived oocytes, since no statistically significant differences was observed between the groups' means (Fig.2). For future studies, different CO concentrations or alternative forms of exposure might be used to see if any remarkable changes are detected. A different question might be whether the formerly discovered low fertilization rate of oocytes derived from KO females would rather be improved by a treatment with CO. Unfortunately, the availability of female KO mice is very low. We previously found that 10 *Hmox1*$^{+/-}$×*Hmox1*$^{+/-}$ breeding pairs resulted in only 7 KO females within 1 year. In contrast to the expected 25%, only 8.46% of the pups exhibits a KO genotype, of which 5.68% males and 3.13% females (21). This low yield made logistically and technically impossible to answer the very interesting question formulated before.

For the IVF experiments, we used females from which we could harvest a minimum number of 5 oocytes.

For this reason, our initial mice cohort was reduced since we had to exclude few dams which produced a number of oocytes ranging from 1 to 4. Following this initial selection, in each single fertilization experiment the starting number of oocytes used ranged from 6 to 26. Still, we are aware that the sample size and the variation in the number of oocytes may have an influence on the calculated fertility success rate, and they might be considered limitations of our study. Also, it would be interesting to perform the same analysis taking into consideration the genotype of the embryos, to investigate whether the embryonic *Hmox1* expression at the very early beginning of pregnancy is crucial to terminate the whole fertilization process successfully and further steps of the IVF protocols. These aspects were not considered in the present study and should be taken into account in the future.

In conclusion, we demonstrated that the maternal *Hmox1*-heterozygous KO condition in mice did not affect the ability of their oocytes to be fertilized *in vitro*. Also, contradicting the initial hypothesis of therapeutic use, we showed that CO treatment did not affect the IVF rate, at least of WT or *Hmox1*$^{+/-}$ female derived oocytes. Nevertheless, it is conceivable that CO can be used for therapeutic purposes to compensate for various pathological consequences of a disturbed pregnancy, possibly at a later time in the course of a natural or IVF pregnancy.

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Authors' Contributions

F.R.; Performed writing (original draft, review and editing), Validation, Formal analysis, and Data visualization. N.M.; Contributed with conceptualization, Project administration, Investigation, Review and editing. M.L.Z.; Participated in the review and editing process. A.C.Z.; Contributed with methodology, Supervision, Funding acquisition, Review and editing. All authors read and approved the final manuscript.

References

1. Seidler EA, Murphy LA, Vaughan DA, Sakkas D. Fertilization and complete fertilization failure in human IVF. In: Kovacs G, Rutherford A, Gardner DK, editors. How to prep egg embryo to maximize IVF success. Cambridge: Cambridge University Press; 2019; 224-231.
2. Kahyaoglu I, Demir B, Turkkanı A, Cınar O, Dilbaz S, Dilbaz B, et al. Total fertilization failure: is it the end of the story? J Assist Reprod Genet. 2014; 31(9): 1155-1160.
3. Payne D, Flaherty SP, Jeffrey R, Warnes GM, Matthews CD. Successful treatment of severe male factor infertility in 100 consecutive cycles using intracytoplasmic sperm injection. Hum Reprod. 1994; 9(11): 2051-2057.
4. Swain JE, Pool TB. ART failure: oocyte contributions to unsuccessful fertilization. Hum Reprod Update. 2008; 14(5): 431-446.
5. Zenclussen ML, Linzke N, Schumacher A, Fest S, Meyer N, Casalis PA,et al. Heme oxygenase-1 is critically involved in placentation, spiral artery remodeling, and blood pressure regulation during murine pregnancy. Front Pharmacol. 2015; 5: 291.
6. Zenclussen ML, Jensen F, Rebelo S, El-Mousleh T, Casalis PA, Zenclussen AC. Heme oxygenase-1 expression in the ovary dictates a proper oocyte ovulation, fertilization, and corpora lutea maintenance. Am J Reprod Immunol. 2012; 67(5): 376-382.
7. Watanabe S, Akagi R, Mori M, Tsuchiya T, Sassa S. Marked developmental changes in heme oxygenase-1 (HO-1) expression in the mouse placenta: correlation between HO-1 expression and placental development. Placenta. 2004; 25(5): 387-395.
8. Schumacher A, Zenclussen AC. Effects of heme oxygenase-1 on innate and adaptive immune responses promoting pregnancy success and allograft tolerance. Front Pharmacol. 2015; 5: 288.
9. Zenclussen ML, Casalis PA, El-Mousleh T, Rebelo S, Langwisch S, Linzke N, et al. Haem oxygenase-1 dictates intrauterine fetal survival in mice via carbon monoxide. J Pathol. 2011; 225(2): 293-304.
10. Linzke N, Schumacher A, Woidacki K, Croy BA, Zenclussen AC. Carbon monoxide promotes proliferation of uterine natural killer cells and remodeling of spiral arteries in pregnant hypertensive heme oxygenase-1 mutant mice. Hypertension. 2014; 63(3): 580-588.
11. Poss KD, Tonegawa S. Heme oxygenase 1 is required for mammalian iron reutilization. Proc Natl Acad Sci USA. 1997; 94(20): 10919-10924.
12. Yachie A. Heme oxygenase-1 deficiency and oxidative stress: a review of 9 independent human cases and animal models. Int J Mol Sci. 2021; 22(4): 1514.
13. Hamilton F, Mitchell R, Ghazal P, Timpson N. Phenotypic associations with the HMOX1 GT(n) repeat in European populations. Am J Epidemiol. 2023: kwad154.
14. Hamilton FW, Somers J, Mitchell RE, Ghazal P, Timpson NJ. HMOX1 genetic polymorphisms and outcomes in infectious disease: a systematic review. PLoS One. 2022; 17(5): e0267399.
15. Saukkonen K, Lakkisto P, Kaunisto MA, Varpula M, Voipio-Pulkki LM, Varpula T, et al. Heme oxygenase 1 polymorphisms and plasma concentrations in critically ill patients. Shock. 2010; 34(6): 558-564.
16. Kaartokallio T, Utge S, Klemetti MM, Paananen J, Pulkki K, Romppanen J, et al. Fetal microsatellite in the heme oxygenase 1 promoter is associated with severe and early-onset preeclampsia. Hypertension. 2018; 71(1): 95-102.
17. Hirai H, Kubo H, Yamaya M, Nakayama K, Numasaki M, Kobayashi S, et al. Microsatellite polymorphism in heme oxygenase-1 gene promoter is associated with susceptibility to oxidant-induced apoptosis in lymphoblastoid cell lines. Blood. 2003; 102(5): 1619-1621.
18. Abdel Aziz MT, Mostafa T, Roshdy N, Hosni H, Rashed L, Sabry D, et al. Heme oxygenase enzyme activity in human seminal plasma of fertile and infertile males. Andrologia. 2008; 40(5): 292-297.
19. Dickson MA, Peterson N, McRae KE, Pudwell J, Tayade C, Smith GN. Carbon monoxide increases utero-placental angiogenesis without impacting pregnancy specific adaptations in mice. Reprod Biol Endocrinol. 2020; 18(1): 49.
20. Němeček D, Dvořáková M, Heroutová I, Chmelíková E, Sedmíková M. Anti-apoptotic properties of carbon monoxide in porcine oocyte during in vitro aging. PeerJ. 2017; 5: e3876.
21. Meyer N, Langwisch S, Scharm M, Zenclussen AC. Using ultrasound to define the time point of intrauterine growth retardation in a mouse model of heme oxygenase-1 deficiency†. Biol Reprod. 2020; 103(1): 126-134.

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Study on The Pedigrees of Three Cases of Whole-Arm Translocation in Hainan China and Literature Review: A Retrospective Study

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Abstract

In this study, in order to promote chromosome abnormality carriers eugenics, three patients with adverse pregnancy histories were examined by cytogenetics and their pedigrees further analyzed. In this retrospective study, approximately anticoagulant peripheral venous blood from the patients was collected for peripheral blood cell culture and chromosome analysis. Karyotypes were analyzed in the BEIONMED karyotype analysis system. The karyotypes of the three probands were all whole-arm translocations (WATs): case 1 (DatabaseNo.3591): 46, XY, t (7; 13) (p10; p10) dn, two years of marriage in which the spouse did not have pregnancy, with azoospermia; case 2 (Database No.3809): 46, XY, t(12; 17) (p10; q10), three spontaneous abortions within three years of marriage; case 3 (Database No.4914) 46, XX, t(2;6) (p10; q10) mat, 21ps+pat, a year of marriage without pregnancy. When the parents are carriers of WAT, the family should be considered to have a high reproductive risk, increasing the risk of producing offspring with chromosomal abnormalities. Three kinds of human chromosomal aberration karyotypes were reported for the first time providing an important basis for studying the occurrence and clinical consultation of chromosomal diseases.

Keywords: Case Report, Chromosomes, Genetic Counseling, Whole-Arm Translocation

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Introduction

Variations in the number and structure of chromosomes are linked to a variety of congenital abnormalities, intellectual disabilities and cancers. Chromosomal translocation can come from two sources: one is transmitted from the father or the mother-while the other is chromosomal aberrations occuring during gamete formation or zygote division. Because the breakage points of the two chromosomes are close to the centromere, the whole-arms will exchange when the chromosomes are translocated with each other, this process called whole-arm translocations (WATs). However, during the meiosis of germ cells, the translocated chromosomes can be paired to a quadriradial chromosome which is formed, and the related chromosomes can undergo para-location separation, ortho-1 separation, ortho-2 separation and 3:1 separation, resulting in 18 kinds of gametes. Among them, only one of them is normal; one is the carrier of balanced translocation; and the others are partial trisomy or monomer gametes. These gametes can cause abortion, stillbirth and birth defects, when combined with normal gametes (1).

Due to the same amount of genetic material, the phenotype and intelligence of patients with whole-arm chromosomal translocation can be normal. However, the genetic effect on the offspring of the translocation carriers will be obvious when they marry a normal person. Some studies (2, 3) have shown that when the parents are such carriers, the special chromosome rearrangement can affect the fertility of male offspring. Even though the composition of derivatives is unknown in two thirds of the cases, the circulation rate appears to be higher than that of other mutual translocations, possibly indicating that there are other factors, such as specific genomic polymorphisms. It has been reported (4) that hereditary WATs often appears in various tumors as unbalanced secondary changes, possibly caused by DNA repair errors. In this study, three patients with adverse pregnancy histories were examined by cytogenetics and their pedigrees further analyzed.

Materials and Methods

In this retrospective study, three cases with an adverse

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pregnancy history were examined by cytogenetics and also their pedigrees were further analyzed (Table 1). The karyotypes of the three cases were identified by the expert group of the Chinese Human Chromosome Abnormality Karyotype Database and no related reports were found by checking the Cytogenetics Database and the Chinese Human Chromosome Abnormality Karyotype Database. Therefore, the karyotype was included in the Chinese Human Chromosome Abnormal Nuclei Database. The patients provided informed consent for this study.

Table 1: Comparison among the three cases in family history of this study

Item	Case 1	Case 2	Case 3
Area	Wenchang City, Hainan Province	Qionghai City, Hainan Province	Haikou City, Hainan Province
Age (Y)	34	31	29
Sex	M	M	F
Nation	Han	Han	Han
Occupation	Company employee	Company employee	Company employee
Height (cm)	172	170	160
Visit time	2013	2014	2016
Reason	Married for two years; spouse had no pregnancy	Three spontaneous abortions within three years of marriage	PGD failed after a year of marriage without pregnancy
Pregnancy history of self or spouse	G0P0	G3P0	G0P0
Proband's karyotype	46,XY,t(7;13)(p10;p10)dn		
Database No.3591	46,XY,t(12;17)(p10;q10) Database No.3809	46,XX,t(2;6)(p10;q10)mat, 21ps+ patDatabaseNo.4914	
Spouse's karyotype	46, XX	46, XX	46, XY
Spouse's age (Y)	31	24	30

M; Male and F; Female.

Human peripheral blood lymphocyte culture medium (Guangzhou Baiyunshan Baidi Biomedical Co., Ltd.), low-osmosis solution (0.075 mol/LKCl), 10 μg/mL colchicine, fixative solution (methanol and glacial acetic acid), phosphate buffer solution, trypsin and Jimsa dye solution.

Karyotype analysis of peripheral blood lymphocyte culture

Approximately 3 mL of the peripheral patients blood was extracted to heparin sodium tubes for anticoagulation and evenly mixed. Approximately 1.5 mL of the blood was inoculated in the peripheral blood lymphocyte medium and evenly mixed. The cells were incubated in an incubator at 37°C for 72 hours, and colchicine was added to stop cell division. Then, the cells were harvested and microscope slides prepared for banding and analysis. Under an Olympus CX21 microscope, 30 cells in metakinesis were counted. Besides, 3-5 karyotypes were analyzed in the BEIONMED karyotype analysis system. For the patients with abnormal karyotypes, the modal number of cells was counted, and karyotype analysis conducted. The karyotype description referred to the International System for Human Cytogenetic Nomenclature (2016). In this study, karyotype analysis was also performed on the spouses and some family members of the probands. The pedigree chart was produced in PowerPoint (PPT).

Ethics approval and consent to participate

The patients provided informed consent to participate in this study, and their pedigrees were further investigated. This study protocol was approved by the Ethics Committee of Haikou Hospital of Traditional Chinese Medicine (HKSZYYYLL-2022(S)-08).

Results

Case 1: The proband was a 34-year-old man of Han nationality living in Wenchang City, (Hainan Province, China). The patient was a company employee, his height was 172-cm. He had a normal phenotype. Two Years of marriage without pregnancy. In September 2013, he was admitted to the Second Affiliated Medical Reproductive Center of Hainan Medical University. Karyotype(DatabaseNo.3591):46,XY,t(7;13)(p10;p10)dn (Fig.1A). Testicular biopsy indicated azoospermia. For the five items of coagulation, fibrinogen (FIB) was 1.88g/L (reference range: 2-4 g/L), while the rest was normal. The six sex hormones, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), progesterone (PRG), prolactin (PRL) and total testosterone (TES), were normal. Negative for Chlamydia trachomatis (CT-DNA), ureaplasmaurealyticum (UU-DNA) and Neisseria gonorrhoeae (NG-DNA). The blood-routine exam was normal. Positive for HBsAb. Negative for hepatitis C, human immunodeficiency virus (HIV) and Treponema pallidum antibody. The karyotypes of the proband's parents, younger brother and wife were normal. A telephone follow-up in 2020 revealed no pregnancy. The pedigree is shown in Figure 1B.

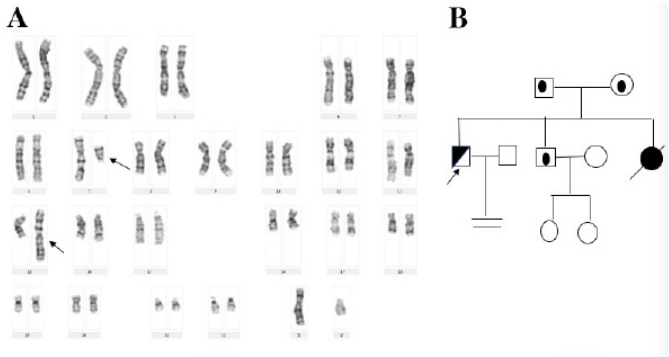

Fig.1: G-banding chromosome analysis and pedigree chart of case 1. **A.** Karyogram (Database No.3591) 46, XY, t(7;13) (p10;p10) dn and **B.** Pedigree chart.

Case 2: The proband was a 31-year-old man of Han nationality living in Qionghai City (Hainan Province, China). The patient was a company employee, his height was 170-cm. Normal phenotype and intelligence, three spontaneous

abortions within three years of marriage. In January 2014, he was admitted to the Second Affiliated Medical Reproductive Center of Hainan Medical University. Karyotype(Database No.3809):46,XY,t(12;17)(p10;q10) (Fig.2A). The blood-routine exam was normal. AB blood. Positive for RhD blood and RhC blood, negative for RhE blood. HBsAb was positive in hepatitis B screening, while the rest were negative for hepatitis C, HIV and Treponema pallidum antibody; negative for CT-DNA, UU-DNA and NG-DNA. Anti-sperm antibody was negative, and thalassemia genotyping was normal. The proband's wife had normal karyotype and became pregnant naturally as a telephone follow-up in 2014 showed. His older sister received a karyotype test, 46,XY,t(12;17)(p10;q10) at another hospital and had three spontaneous abortions. The pedigree is shown in Figure 2B.

Case 3: The proband was a 29-year-old female, of Han nationality living in Haikou City (Hainan Province, China). The patient was a company employee, her height was 160-cm. Normal in phenotype and intelligence, a year of marriage without pregnancy. The fallopian tubes were blocked and the uterus was 1.57 cm. In 2015, preimplantation genetic diagnosis (PGD) failed in the First Affiliated Medical Reproductive Center of Hainan Medical University. In January 2016, she was admitted to the Second Affiliated Medical Reproduction Center of Hainan Medical University. Karyotype (DatabaseNo.4914): 46,XX,t(2;6)(p10;q10)mat,21ps+ pat (Fig.3A). Cytology of the cervix was negative . All the biochemical indicators were normal. CT-DNA and UU-DNA were negative. The six sex hormones, including FSH, LH, E2, PRG, PRL and TES, were normal. The three items of hyperthyroidism were normal, namely, free triiodothyronine (FT3), free thyroid hormone (FT4) and the third-generation TSH. Insulin (INS) (reference range:2.8-24.7uU/mL):6.40uU/mL fasting, 105.50uU/mL one hour postprandial and 67.80uU/mL two hours postprandial. Blood glucose was normal (fasting, one hour postprandial and two hours postprandial). HBsAb was positive in hepatitis B screening, while the rest was negative. Negative for hepatitis C, HIV and Treponema pallidum antibody. Negative for the five items of eugenics. The anti-cardiolipin antibody was negative, and -A3.7 gene heterozygosity was detected in thalassemia genotyping. No pregnancy was found during a telephone follow-up in 2019. The husband's karyotype was normal, while the karyotypes of her mother and father were 46,XX,t(2;6)(p10;q10) and 46,XY,21ps+. The pedigree is shown in Figure 3B.

Comparison among WAT cases in 10 areas in China in pregnancy and childbirth history

Among the 29 probands from 10 areas in China, there were G85P9, wherein spontaneous abortions in the first trimester accounted for 48% (41/85); embryo arrest 31% (26/85); teratopia 6% (5/85); deaths 5% (4/85); amenorrhea 1% (1/85). There were two abortions, one case of unintended pregnancy and one case of biochemical one (Table 2).

Comparison of pregnancy history of whole arm translocation cases abroad

There were G25P5 probands from 7 families abroad, among which spontaneous abortion accounted for 72% (18/25) in early pregnancy, teratotypes accounted for 8% (2/25) and asthenospermia in 2 cases (Table 3).

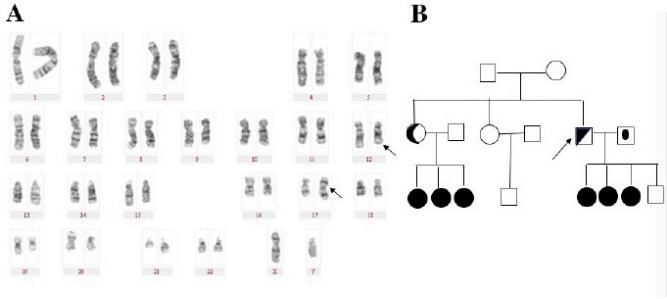

Fig.2: G-banding chromosome analysis and pedigree chart of case 2. **A.** Karyogram(DatabaseNo.3809) 46,XY,t(12;17)(p10;q10) and **B.** Pedigree chart.

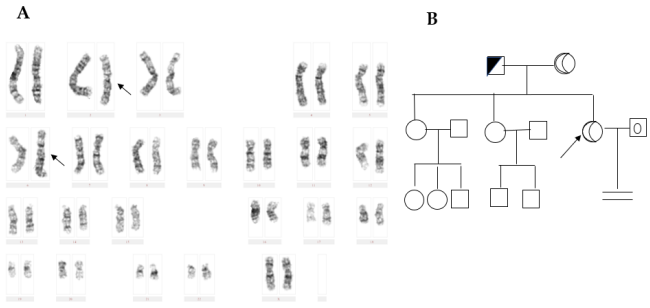

Fig.3: G-banding chromosome analysis and pedigree chart of case 3. **A.** Karyogram (DatabaseNo.4914) 46,XX,t(2;6)(p10;q10) mat,21ps+pat and **B.** Pedigree chart.

Table 2: Comparison among WAT cases in 10 areas in China in pregnancy and childbirth history

Literature	Chromosome karyotype	Area	Pregnancy history
Wang (1)	46,XX,t(1;6)(1pter→1p1-6q10→6qter;6pter→6p10-1q10→1qter)	Weihai City, Shandong Province	G3P1:Two spontaneous abortions and one deformed infant
Xiao (5)	46,XX,t(8;21)(p10;q10)(8pter→8p10::21q10→21qter;21pter→21p10::8q10→ 8qter)	Shenzhen City, Guangdong Province	G10P2:One postnatal death and eight spontaneous abortions
Lian (6)	46,XX,t(7;11)(7Petr→cen→11Pter;7qter→cen→11qter)	Shandong and Anhui Provinces	G2P0:Two spontaneous abortions
Liu (7)	46,XY,t(11;15)(11pter→cen→15pter;11qter→cen→15qter)	Shenzhen City, Guangdong Province	G2P0:Embryo arrest twice
Liu (7)	46,XY,t(1;16)(1pter→cen→16qter;1qter→cen→16pter)	Shenzhen City, Guangdong Province	G1P0:Unintended pregnancy
Tang (8)	46,XX,t(7,9)(7p9p.7q9q)(7pter→cen→9pter;7qter→cen→9qter)	Fuling Area, Sichuan Province	G3P0: Three spontaneous abortions
Zhang (9)	46,XY,t(11;15)(11pter→11p10::15p10→15pter;11qter→11q10::15q10→15qter)	Shenzhen City, Guangdong Province	G2P0:Two spontaneous abortions
Li (10)	46,XY,t(18;22)(p10;q10) mat	Linyi City, Shandong Province	G4P0: Four spontaneous abortions
Li (11)	46,XX,t(7:9)(p10;q10)	Beijing	Amenorrhea
Zhang (12)	46,XY,t(1;19)(1pter→1p11::19q11→19qter;19pter→19p11::1q11→1qter)	Guangzhou City, Guangdong Province	G2P1: One abortion
Mou (13)	46,XX.1(3;16)(3pter→3p10::16p10→16pter;3qter→3q10::16q10→16qter)	Nanchang City, Jiangxi Province	G0P0
Hu (14)	46,XY,t(7;12)(7pter→7p10::12p10→12pter::7qter→7q10::12q10→12qter)	Xuzhou City, Jiangsu Province	G1P0: Embryo arrest once
Liao (15)	46,XY,t(13;20)(13pter→13p10::20p10→20pter13qter→13q10::20ql0→20qter	Bengbu City, Anhui Province	G3P0: Three spontaneous abortions
Li (16)	46,XX,t(7:21)(7pter→7p10::21q10→21qter;21pter→21p10::7q10→7qter) mat,	Linyi City, Shandong Province	G2P0: Pregnant 1: hydrocephalus; Pregnant 2: fetal death
Li (16)	46,XX,t(7;14) (7pter→7p10::14p10→14pter::14qter→14q10::7q10→7qter) mat	Linyi City, Shandong Province	G4P0: Pregnancy 1: Abortion; Pregnancy 2: Embryo arrest; Pregnancy 3: Fetal death; Pregnancy 4: Spontaneous abortion
Li (16)	46,XY,t(9;18)(9pter→9pl0::18p10-18pter;18qter-18q10::9q10-9qter) mat	Linyi City, Shandong Province	G2P0: Pregnancy 1: Spontaneous abortion; Pregnancy 2: Omphalocele
Zhang (17)	46.XY,t(7;16)(7pter→7pl0::16q10→16qter;16pter→16p10::7q10→7qter) dn	Linyi City, Shandong Province	G3P0: Embryo arrest three times
Zhang (17)	46,XX,t(8;8)(8pter→8p10;:8p10→8pter;8qter-8q10::8q10-8qtcr)	Linyi City, Shandong Province	G5P0: Embryo arrest five times
Li (18)	46,XX,t(3;6)(3pter→3p10::6q10→6qter;6pter->6p10::3q10→3qter)pat	Linyi City, Shandong Province	G2P0: Two spontaneous abortions
Li (18)	46,XY,t(6;22)(6pter→6pl0::22q10→22qter;22pter→22pl0; :6q10→6qter)pat	Linyi City, Shandong Province	G2P0: Embryo arrest twice
Li (19)	46,XY,t(7;19)(7pter→7p10::19pl0→19pter;7qter→7q10::19q10→19qter)inv(9)(pter→pl2::q13→p12::q13→qter) mat	Linyi City, Shandong Province	G2P0: Two spontaneous abortions
Li (19)	46,XX,t(11;12(11pter→11p10::12pl0→12pter;11qter→11q10::12q10→12qter	Linyi City, Shandong Province	G2P0: Embryo arrest twice
Li (20)	46,XY,t(16;19)(16pter→16p10::19q10→19qter;19pter→19p10;:16q10→16qter)pat	Linyi City, Shandong Province	G2P0: Embryo arrest twice
Li (20)	46,XX,t(2;2)(2pter→2p10::2p10→2pter;2qter→2q10::2q10→2qter) dn	Linyi City, Shandong Province	G3P0: Embryo arrest three times
Li (21)	46,XY,t(18;22)(18pter→18p10::22q10→22qter;22pter→22p10::18q10→18qter) mat	Linyi City, Shandong Province	G4P0: Four spontaneous abortions
Li (21)	46,XY,t(18;22)(18pter→18p10::22q10→22qter;22pter→22p10::18q10→18qter) mat	Linyi City, Shandong Province	G2P2: The first baby girl had cleft lip and palate (CLP) and died a month later, due to eating difficulties. The second pregnancy was full-term normal delivery of a baby girl. She is now two years old, with normal intelligence and polydactylism.
Li (21)	46,XY,t(7;19)(7pter→7p10::19q10→19qter:19pter→19p10::7q10→7qter)pat	Linyi City, Shandong Province	G4P1: One spontaneous abortion and embryo arrest twice
Li (21)	46,XX,t(11;13)(11pter→11p10::13q10→13qter;13pter→13p10::11q10→11qter)pat	Linyi City, Shandong Province	G3P1: Embryo arrest twice
Liu (22)	46,XX,t(4;10)(4pter→4p10::10p10-10pter,4qter→4q10::10q10→10qter)	Linyi City, Shandong Province	G10P1: Six spontaneous abortions, induced labor once with single umbilical artery (SUA) and heart malformation, embryo arrest once and biochemical pregnancy once

Table 3: Comparison of pregnancy history of whole arm translocation cases abroad

Literature	Chromosome karyotype	Areas abroad	Pregnancy history
Safavi et al. (23)	46,XY,(13;18)(q10;q10)	Tehran, Iran	G1P1, one deformed infant
Fryns et al. (24)	46,XX,t(6plOq;6qlOp)	Leuven, Belgium	G4P1, Threespontaneous abortions
Fryns et al. (24)	46,XX,t(6plOq;6qlOp)mat	Leuven, Belgium	G4P1, Three spontaneous abortions
Vialard et al. (25)	46,XY,t(1;21)(q11;p13)	American	No refined disease
Vialard et al. (25)	46,XY,t(1;22)(q11;p11)mat	American	No refined disease
Tümer et al. (26)	46,XY,der(18),t(18;2)(p10;q10)pat	American	G2P1,one deformed infant
Smith et al. (27)	46, XX,t(1p5q;1q5p)	American	G9P2,7 spontaneous abortions
Smith et al. (27)	46,XX,-14,+derl4,t(10;14) (l0pl4q;14pl0q)mat	American	G5P01,5 spontaneous abortions

Discussion

The whole arm translocation is usually only a sporadic case report. However, there is no relevant literature on comparative statistics of a pregnancy and childbirth history with WAT. Chen et al. (28) reported and analyzed that the total detection rate of chromosomal abnormalities in patients with adverse pregnancies and childbirth histories in 20 provinces and cities in China and 16 countries was 5.62% (3,842/68,267), among which the incidence of autosomal balanced translocation was only 1.97% (1,325/68,267). Chromosome translocation is a significant cause of habitual abortion. Zhu (29) studied 42 balanced translocation carriers with a total of 90 pregnancies, among which 75 spontaneous abortions occurred during the first trimester, representing 83.4%. Chen (30) reported that the incidence of WATs was only 0.09% (3/3,353), and results of yunchun's study suggested that balanced chromosomal translocation carriers are associated with reproductive risks and a very high probability of abnormal pregnancy.

In this study, WATs was reported in 10 areas in China that were analyzed and their pregnancy and birth histories compared (1, 5-22). The 29 family probands had a total of 85 pregnancies and nine births. Specifically, spontaneous abortions accounted for 48% (41/85), while embryo arrest 31% (26/85); deformed infants 6% (5/85); deaths 5% (4/85). There were G25P5 (23-27), foreign family consents, among which spontaneous abortion in early pregnancy accounted for 72% (18/25), teratoma accounted for 8% (2/25), and azoospermia in 2 cases. WATs are relatively rare cytogenetic aberrations, mostly, these translocations are unbalanced accompanied by genomic imbalances (31). It has been reported (23) a rare cytogenetic variant of Monosomy 18p Syndrome as a consequence of WATs between chromosomes 13 and 18. Additionally, it has been reported (32) that, among 101 WAT cases, organized WATs seemed to occur more frequently than other translocations, which may explain that DNA promotes mismatches and unreasonably non homologous recombination. The three cases reported in this study also had an adverse pregnancy history for years, and case 1 even had azoospermia, indicating that WAT led to a severer pregnancy history.

As of December 2005, the Mitelman database (33, 34) included 933 cases of WAT chromosomes. WAT t(17;18) was detected in two cases (35) of acute monocytic leukemia and the conversion from acute to chronic granulocytic leukemia (CGL). Since these patients had either myeloid leukemia or myelodysplastic syndrome (MDS), it is suggested that t(17;18)(p10;q10) translocation is a novel nonrandom abnormality associated with myeloid cell proliferation. MDS(5;19)(p10;q10) cases (36). Moreover, der(5;19)(p10;q10) is a rare chromosomal abnormality of MDS that is genetically similar to 5q [del(5q)] deletion. These results indicate that der(5;19)(p10;q10) may play a significant role in the pathogenesis of high-risk MDS, because it is a rare but recurring translocation (36-38). One case (16) of MDS (refractory anemia) with karyotype 46,XY,+1,der(1;10)(q10;P10) resulted in abnormalities of trisomy 1q and monomer 10q, which may be necessary for tumor transformation. Clarifying the clinical features of myeloid neoplasms in patients with WATs would facilitate the elucidation of their tumorigenic mechanisms. But the pathogenesis of this cytogenetic anomaly still remains unresolved, an accumulation of cases with centromeric translocation may be still necessary.

Conclusion

When the parents are carriers of WAT, the family should be considered to have a high reproductive risk. Particularly, when the translocation area is a small chromosomal segment, the possibility of nonrandom involvement of some chromosomes in WAT and the risk of producing offspring with chromosomal abnormalities will increase. And this study is beneficial for better natal and prenatal care and better upbringing, conducting targeted inspections and guidance. The three cases of abnormal karyotype in this study have not yet been found in literature at home and abroad. This first report on the detection of the karyotype of human chromosomal aberration enriches clinical data on genetics for genetic counseling and prenatal diagnosis that serves as an important basis for research of the occurrence.

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Authors' Contributions

C.Y., L.Y., L.C.; Conceptualization, Methodology, Software, Writing, Reviewing and Editing, Writing the original draft, and Supervision. Z.Z., H.X., K.C.; Visualization and Investigation. All authors read and approved the final manuscript.

References

1. Wang P. t(1;6) a case of whole arm translocation associated with abortion. Chin J Med Genet. 2001; 18(3): 54.
2. Vázquez-Cárdenas A, Vásquez-Velásquez AI, Barros-Núñez P, Mantilla-Capacho J, Rocchi M, Rivera H. Familial whole-arm translocations (1;19), (9;13), and (12;21): a review of 101 constitutional exchanges. J Appl Genet. 2007; 48(3): 261-268.
3. Moreau N, Teyssier M. Whole arm translocation t(1;13) in an infertile man. J Med Genet. 1984; 21(3): 234-235.
4. Vialard F, Nouchy M, Malan V, Taillemite JL, Selva J, Portnoï MF. Whole-arm translocations between chromosome 1 and acrocentric G chromosomes are associated with a poor prognosis for spermatogenesis: two new cases and review of the literature. Fertil Steril. 2006; 86(4): 1001. e1-5.
5. Xiao XS. One case of chromosomal whole arm translocation with habitual abortion. Chin J Med Genet. 2004; 21(1): 13.
6. Lian ZZH. A case of spontaneous abortion induced by translocation of the whole arm of chromosome 7 and chromosome 11. J Anhui Med Coll. 1984; 1: 53-54.
7. Liu XY. Two cases of chromosome whole arm translocation. Chin J Eugen and Genet. 2004; 05: 69.
8. Tang XL. 9 A case of habitual abortion. Chin J Med Genet. 1995; 12(4): 247.
9. Zhang J. 46,XY,t(11;15) A case of whole arm translocation. Chin J Med Genet. 2004; 21(2): 13.
10. Li L. Three cases of balance translocation of whole arm of chromosome in two generations. Chin J Med Genet. 2004; 02: 13.
11. Li X. Chromosome balanced translocation and abnormal pregnancy. Chin J Med Genet. 2011; 19(11): 47-48.
12. Zhang HF. t(1;19) complete arm translocation occurred in 4 families. Chin J Med Genet. 1993; 10(4): 249
13. Mou HY. 46,XX,t(3;16) a case of whole arm translocation of chromosome. Chin J Med Genet. 2011; 28(2): 179.
14. Hu FF. 46,XY,t(7;12) a case of whole arm translocation of chromosome. Chin J Med Genet. 2009; 26(4): 373.
15. Liao YP. 46,XY,t(13;20) a case of arm translocation associated with habitual abortion. Chin J Med Genet. 2012; 29(2): 228.
16. Li L. Six cases of balanced translocation of whole arm in three families. Chin J Med Genet. 2011; 28(3): 350-351.
17. Zhang ChJ. Two cases of balanced Translocation of whole arm of chromosome. Chin J Med Genet. 2012; 29(2): 243.
18. Li L. Four cases of balanced translocation of whole arm in two families. Chin J Med Genet. 2012; 29(6): 742.
19. Li L. Two cases of balanced translocation of whole arm of chromosome. Chin J Med Genet. 2013; 30(2): 142.
20. Li L. Three cases of balanced translocation of whole arm in two families. Chin J Med Genetics. 2010; 27(5): 594.
21. Li L. Four families of balanced translocation of whole arm of chromosome. Chin J Med Genetics. 2007; 24(5): 610-611.
22. Liu ShL. Two cases of balanced translocation of whole arm of chromosome in one family. Chin J Med Genetics. 2014; 31(6): 756.
23. Safavi M, Haghi Ashtiani MT, Badv RS, Azari-Yam A, Vasei M. A rare cytogenetic variant of monosomy 18p syndrome as a consequence of whole-arm translocation between chromosomes 13 and 18. Arch Iran Med. 2019; 22(10): 627-628.
24. Fryns JP, Kleczkowska A, Van den Berghe H. Familial transmission of autosomal whole arm translocation. J Med Genet. 1988; 25(11): 783-784.
25. Vialard F, Nouchy M, Malan V, Taillemite JL, Selva J, Portnoï MF. Whole-arm translocations between chromosome 1 and acrocentric G chromosomes are associated with a poor prognosis for spermatogenesis: two new cases and review of the literature. Fertil Steril. 2006; 86(4): 1001. e1-5.
26. Tümer Z, Berg A, Mikkelsen M. Analysis of a whole arm translocation between chromosomes 18 and 20 using fluorescence in situ hybridization: detection of a break in the centromeric alpha-satellite sequences. Hum Genet. 1995; 95(3): 299-302.
27. Smith A, Murray R, den Dulk G. Two whole arm reciprocal translocations. J Med Genet. 1988; 25(1): 61.
28. Chen YC, Huang XN, Kong CY, Hu JD. Six families with balanced chromosome translocation associated with reproductive risks in Hainan Province: case reports and review of the literature. World J Clin Cases. 2020; 8(1): 222-233.
29. Zhu YY. Effects of balanced chromosomal translocation on fertility. China Mater Child Health Care. 2008; 23(2): 204-206.
30. Chen YC. Cytogenetic analysis of 3353 genetic consultants in Hainan region. Chin J Eugen Genet. 2019; 27(07): 817, 827-829.
31. Ha JS, Jeon DS. A case of myelodysplastic syndrome with a der(1;18)(q10;q10) translocation. Blood Res. 2014; 49(2): 132-134.
32. Vázquez-Cárdenas A, Vásquez-Velásquez AI, Barros-Núñez P, Mantilla-Capacho J, Rocchi M, Rivera H. Familial whole-arm translocations (1;19), (9;13), and (12;21): a review of 101 constitutional exchanges. J Appl Genet. 2007; 48(3): 261-268.
33. Mitelman F., Johansson B., Mertens F. (eds.). Mitelman database of chromosome aberrations in cancer, 2006. Available at: http://cgap.nci.nih.gov/Chromosomes/Mitelman. Accessed March 9, 2006.
34. Griffin CA, Burger P, Morsberger L, Yonescu R, Swierczynski S, Weingart JD, et al. Identification of der(1;19)(q10;p10) in five oligodendrogliomas suggests mechanism of concurrent 1p and 19q loss. J Neuropathol Exp Neurol. 2006; 65(10): 988-994.
35. Jonveaux P, Derré J, Berger R. Whole arm translocation t(17;18): a non-random abnormality of myeloid cell proliferation. Leukemia. 1993; 7(12): 1987-1989.
36. Manabe M, Takakuwa T, Nakano H, Harada N, Okamoto S, Aoyama Y, et al. Derivative (5;19)(p10;q10): a rare but recurrent whole-arm translocation in acute myeloid leukemia. Asia Pac J Clin Oncol. 2014; 10(2): e122-e126.
37. Ureshino H, Kizuka H, Kusaba K, Sano H, Nishioka A, Shindo T, et al. 5q- syndrome-like features as the first manifestation of myelodysplastic syndrome in a patient with an unbalanced whole-arm translocation der(5;19)(p10;q10). Int J Hematol. 2017; 105(5): 692-696.
38. Yamamoto K, Okamura A, Katayama Y, Shimoyama M, Matsui T. Unbalanced whole-arm translocation der(5;19)(p10;q10) is a novel and recurrent cytogenetic aberration in myelodysplastic syndrome. Leuk Res. 2009; 33(3): 377-383.

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A Case Report of Neuroendocrine Tumor in Presacral Region: How Can It Be Managed? Laparoscopy versus Laparotomy

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Abstract

Presacral or retrorectal tumors are rare, usually asymptomatic, and diagnosed accidentally during physical examination or imaging. Symptomatic tumors may present with perianal pain, bowel dysfunction, and urinary symptoms due to the mass compression or invasion of the surrounding tissues and organs. Surgical resection is the first choice for treating presacral tumors. Clinicians should choose surgical procedures based on the location and size of the tumors. We presented a 43-year-old woman who suffered from pelvic pain and primary infertility from two years ago. A large mass between the posterior vaginal wall and the rectum was found on recto-vaginal examination. Magnetic resonance imaging (MRI) revealed a large 120×115 mm benign multiloculated cystic mass. Eventually, the mass was removed through laparoscopic surgery. The pathology report indicated a carcinoid tumor (grade I) with no lymphovascular invasion. Thus, presacral tumors are resectable through laparoscopy with lower complications than open surgery.

Keywords: Carcinoid Tumor, Case Reports, Laparoscopy, Neuroendocrine Tumors

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Introduction

Presacral or retrorectal tumors are rare, with 1.4 to 6.3 reported cases per year, mostly discovered accidentally. Patients with presacral tumors are usually asymptomatic and are diagnosed during physical examination (digital rectal examination) or imaging. Moreover, symptomatic tumors may present with perianal pain, bowel dysfunction, and urinary symptoms due to the mass compression or invasion of the adjacent tissues. As a result, it is challenging for clinicians to diagnose and treat them in a timely manner (1).

The first choice for treating presacral tumors is surgical resection. Clinicians should choose surgical procedures based on the location and size of the tumors according to the preoperative examination and imaging (2).

Herein, we present a case diagnosed with a bulky presacral carcinoid tumor resected through laparoscopy.

Case report

A 43-year-old woman was referred to us from the infertility center with a history of pelvic pain and primary infertility from two years ago. The last menstrual period (LMP) of the patient was 15 days before the visit, and she had an irregular menstrual cycle with increased bleeding volume. Initially, the patient denied any complaint of abdominal pain and urinary symptoms. She did not complain of sweating, weight loss, or anorexia. The patient's familial history, past medical history, and drug history were negative. She was hemodynamically stable with a blood pressure of 110/80 mmHg, a pulse rate of 85/minutes, an axillary temperature of 37ºC, and a respiratory rate of 19 /minutes. The patient's body mass index (BMI) was 27.7 kg/m^2. Physical examination of the abdomen revealed a surgical scar resulting from a previous ovarian cystectomy about 11 months ago, without a palpable mass, and there was an exophytic wart on the labia major. On recto-vaginal examination, a large mass between the posterior vaginal wall and the rectum was found. On speculum examination, the cervix was invisible.

The patient's laboratory tests were normal (WBC: 6300 Mill/cumm, Hemoglobin: 12 g/dL, PLT: 299000×1000/cumm, Creatinine: 0.9 mg/dL, AFP: 3.4 IU/mL, CA-125: 19 IU/mL, CA19-9: 5.8 IU/mL). Ultrasound revealed a large multiloculated cystic 119×82 mm mass near the left ovary. magnetic resonance imaging (MRI) illustrated a large 120×115 mm benign multiloculated cystic mass, but some were restricted with thin peripheral enhancement (Fig.1). Eventually, the mass was removed through laparoscopic surgery. The pathology report indicated a grade I carcinoid tumor with mitosis <2/10 high-power fields (HPFs), with no lymphovascular

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invasion. The immunohistochemistry (IHC) result showed synaptophysin-positive/chromogranin: positive (Fig.2). The patient was discharged after the surgery with no complications, and after six months the patient had no complaints.

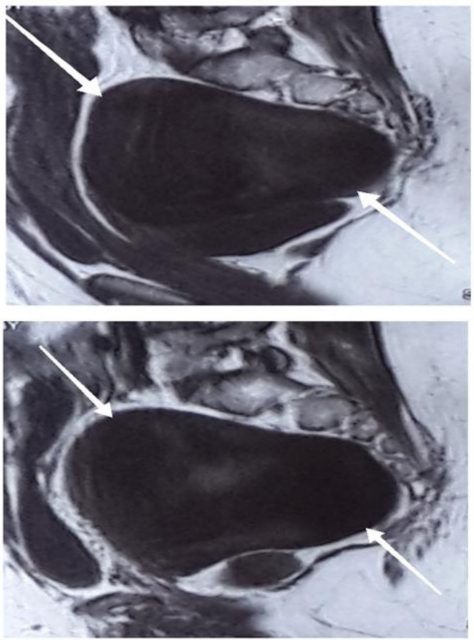

Fig.1: Magnetic resonance imaging (MRI) presents a large 120×115 mm benign multiloculated cystic mass that is attached to the thecal sac, some portion of the lesion is restricted with thin peripheral enhancement.

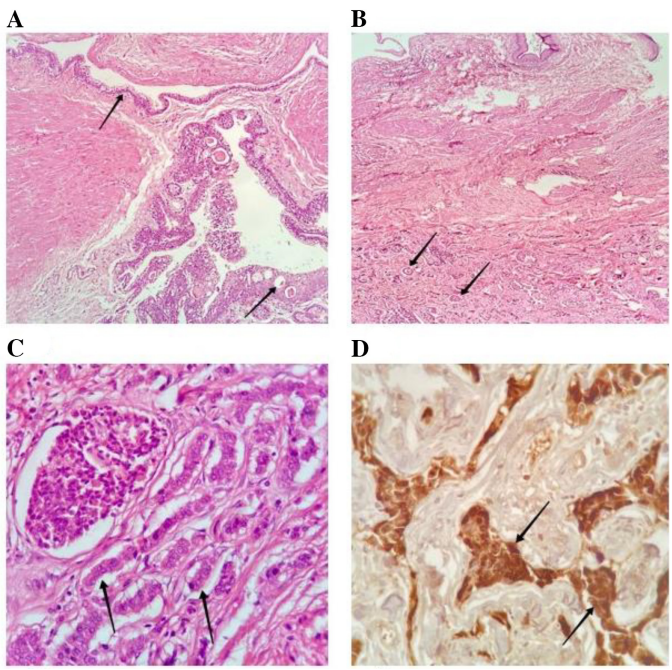

Fig.2: Pathological images of the mass. **A.** shows the cyst lining consists of cuboidal to columnar epithelium with intracytoplasmic mucin and foci of squamous metaplasia (100x, H&E staining). **B.** Focal neoplastic proliferation in the cyst wall is seen (40x, H&E staining). **C.** The neoplastic cells are composed of trabecular structures and nests of uniform polygonal cells with round to oval nuclei, salt and pepper chromatin, and moderate cytoplasm (100x, H&E staining). **D.** The neoplastic cells show immunopositivity for chromogranin (IHC study, 100x) and so the diagnosis of well-differentiated neuroendocrine tumor, grade 1 (carcinoid tumor) is confirmed.

This case report was approved by the Institutional Research Ethics Committee and Vice Chancellor in Research Affairs of Shahid Beheshti University of Medical Sciences (IR.SBMU.RETECH.REC.1401.638).

Discussion

Presacral space is an extraperitoneal fossa between the upper two-thirds of the rectum and the sacrum (3). There are several main vascular and neural structures in this fossa; thus, their injury may have considerable physiologic rectoanal, neurological, and musculoskeletal consequences (4). Due to the improvements in diagnostic methods, the number of reported cases of neuroendocrine tumors (NETs) has increased during the past decades (5).

NETs originate from neuroendocrine cells and usually occur in the gastrointestinal and bronchopulmonary tract (approximately 70 and 25%, respectively) (6). NETs account for about 0.5% of all newly diagnosed malignancies and are more common in women than in men. Also, NETs in the retroperitoneal space are extremely rare. They can arise from primary presacral neoplasms or metastasis of rectal carcinoids (7). The association between presacral carcinoid tumors and tailgut cysts suggests that both originate from the hindgut (8). Presacral NETs are usually asymptomatic; however, they may manifest with pelvic pain, rectal fullness, and constipation due to the mass effect (9). They usually present no carcinoid syndrome, such as flushing, sweating, or hypertension. Our patient suffered from chronic pelvic pain without other mass-related symptoms (e.g., constipation and rectal fullness) or carcinoid syndrome. Since NETs are usually asymptomatic, they are typically diagnosed accidentally through physical examination or imaging. Our patient's recto-vaginal examination showed a large mass between the posterior vaginal wall and the rectum (10).

Differential diagnoses of presacral NETs include presacral hypervascular masses, such as paraganglioma, extraintestinal gastrointestinal stromal tumor (GIST), and solitary fibrous tumor, distinguished by imaging. On MRI, paraganglioma is presented by a well-circumscribed, intensely enhanced mass. Extraintestinal GIST typically contains necrotic centers, hemorrhage, or cystic degeneration, tending to be aggressive. The solitary fibrous tumor presents as a well-circumscribed solid mass with intense heterogeneous enhancement (10).

On MRI, our case had a 120×115 mm benign multiloculated cystic mass with thin peripheral enhancement in the presacral region. MRI is the preferred modality for diagnosis before surgery because it designates local invasion and neural involvement with advanced contrast resolution compared to computed tomography (CT) (11).

In NETs, well-differentiated tumors, the cells produce neurosecretory granules, reflected in the strong and diffuse immunoexpression of neuroendocrine markers (e.g., chromogranin A and synaptophysin) (12). Our patient's mitotic index was<2/10 HPFs, so she had a G1

carcinoid tumor.

Presacral NETs are usually treated by resecting the primary tumor and closely following the patient after the procedure (5). Due to the location of presacral tumors, the treatment of choice for them is usually open surgery (13). There are three approaches for resecting presacral tumors: the anterior (transabdominal) approach, the posterior (transsacral or transanal) approach, and a combination of both (2). Nedelcu et al. (14) suggested laparoscopic surgery for presacral tumors that are supposed to be benign with no invasion to other organs or bones with a size of less than 6 cm. Laparoscopic surgery in presacral tumors has the following advantages: smaller incisions, milder postoperative pain, better field of vision, and the ability to precisely differentiate between the tumor and adjacent structures (15). Although our case had a 12 cm mass in the presacral region, the tumor did not invade other organs based on MRI results. Thus, we decided to resect it through laparoscopy surgery. Informed consent was obtained from the candidate patient for the laparoscopic anterior approach. Pre-surgery procedures, including bowel preparation and thromboprophylaxis were performed. The patient was placed in the lithotomy position. Prep and drape done, a 10 mm trocar was inserted from the umbilicus by a direct entry method; a 5 mm trocar was inserted into the lateral rectus and supra pubic muscles. Using the camera, the right side of the pelvis was observed. According to the mass location on the MRI, the surgeon noticed a brief bulging in the posterior cul de sac, which had caused the deviation of the rectum to the left side. Then, a peritoneal dissection was performed on the right side of the pelvis above the prominence of the mass. After identifying the right ureter, the right pararectal space and the recto-vaginal septum were dissected. When the mass became observable, an attempt was made to separate it from the space in front of the sacrum and behind the rectum.

At the bottom of the mass, a solid area of about 6-7 cm was visible, which was separated from the sacrum with difficulty. Despite careful hemostasis, venous bleeding occurred. Thus, the hemostatic powder was used, and tranexamic acid was injected. After the fixation of the hemovac drain, the peritoneum was repaired. The amount of bleeding during surgery was estimated to be 1000 cc. Due to a severe drop in hemoglobin (from 12 to 8.4 mg/dl), two units of packed red blood cells and two units of fresh frozen plasma (FFP) were injected. After 48 hours, she was discharged with good general condition. At the outpatient visit for follow-up, she complained of pelvic pain, which was formed in the re-MRI of an organized hematoma at the site of the mass, which was absorbed in the subsequent follow-ups.

Due to the anatomical proximities, bleeding from the tumor-feeding vasculature and the presacral venous plexus should be considered in presacral tumor surgeries. Previously, several patients with malignancies underwent laparoscopic surgeries. These cases are followed for possible uncontrolled bleeding and metastasis (2). The most common complications include bleeding, wound infection, and injury of the urethra or rectum (13). Another study showed among 11 reported cases of primary presacral NETs with a mean follow-up time of 41.8 months, the patients with well-differentiated NETs and local disease had good prognoses (5). Similarly, we followed up of our case for six months, and her conditions turned out to be good, with no complaint or recurrence.

Conclusion

We reported a rare presacral carcinoid tumor of about 12 cm in size. Finally, the patient underwent laparoscopic resection without any complications. Our case revealed that laparoscopic surgery is a feasible option for resecting presacral tumors because of its advantages (less invasive and small skin incision); however, this finding requires further investigations.

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Authors' Contributions

B.N.; Conception, design, and supervision. H.N.; Drafting of the manuscript and critical revision of the manuscript. Sh.H.Ch; Analysis and interpretation of data. All authors read and approved the final manuscript.

References

1. Li Z, Lu M. Presacral tumor: insights from a decade's experience of this rare and diverse disease. Front Oncol. 2021; 11: 639028.
2. Kodera K, Eto S, Fukasawa N, Kai W, Matsumoto T, Hirabayashi T, et al. Laparoscopic resection of a neuroendocrine tumor that almost fully replaced tailgut cysts: a case report. Surg Case Rep. 2020; 6(1): 269.
3. Patel N, Maturen KE, Kaza RK, Gandikota G, Al-Hawary MM, Wasnik AP. Imaging of presacral masses--a multidisciplinary approach. Br J Radiol. 2016; 89(1061): 20150698.
4. Neale JA. Retrorectal tumors. Clin Colon Rectal Surg. 2011; 24(3): 149-160.
5. Zhang R, Zhu Y, Huang XB, Deng C, Li M, Shen GS, et al. Primary neuroendocrine tumor in the presacral region: A case report. World J Clin Cases. 2019; 7(14): 1884-1891.
6. Kim MR, Shim HK. Long-term follow-up of a patient with primary presacral neuroendocrine tumor: a case report with literature review. Am J Case Rep. 2019; 20: 1969-1975.
7. Oronsky B, Ma PC, Morgensztern D, Carter CA. Nothing but NET: a review of neuroendocrine tumors and carcinomas. Neoplasia. 2017; 19(12): 991-1002.
8. Luong TV, Salvagni S, Bordi C. Presacral carcinoid tumour. Review of the literature and report of a clinically malignant case. Dig Liver Dis. 2005; 37(4): 278-281.
9. Matrood S, Apostolidis L, Schrader J, Krug S, Lahner H, Ramaswamy A, et al. Multicenter analysis of presacral neuroendocrine neoplasms-clinicopathological characterization and treatment outcomes of a rare disease. Front Endocrinol (Lausanne). 2021; 12: 709256.
10. Kim JM, Lee NK, Kim S, Ahn SJ, Suh DS, Han GJ, et al. Primary presacral neuroendocrine tumor: a case report and review of MRI

Findings. J Korean Soc Radiol. 2017; 77(3): 187-191.
11. Hosseini-Nik H, Hosseinzadeh K, Bhayana R, Jhaveri KS. MR imaging of the retrorectal-presacral tumors: an algorithmic approach. Abdom Imaging. 2015; 40(7): 2630-2644.
12. Klimstra DS, Modlin IR, Coppola D, Lloyd RV, Suster S. The pathologic classification of neuroendocrine tumors: a review of nomenclature, grading, and staging systems. Pancreas. 2010; 39(6): 707-712.
13. Dziki Ł, Włodarczyk M, Sobolewska-Włodarczyk A, Saliński A, Salińska M, Tchórzewski M, et al. Presacral tumors: diagnosis and treatment - a challenge for a surgeon. Arch Med Sci. 2019; 15(3): 722-729.
14. Nedelcu M, Andreica A, Skalli M, Pirlet I, Guillon F, Nocca D, et al. Laparoscopic approach for retrorectal tumors. Surg Endosc. 2013; 27(11): 4177-4183.
15. Wang L, Hirano Y, Ishii T, Kondo H, Hara K, Ishikawa S, et al. Laparoscopic surgical management of a mature presacral teratoma: a case report. Surg Case Rep. 2019; 5(1): 144.

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Pregnancy with Intrauterine Device Perforation: A Case Report

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Abstract

Although intrauterine devices (IUDs) are known for their low failure rate in pregnancy prevention, potential risks associated with their use include uterine perforation and migration through the abdomen. In this particular case, the patient experienced simultaneous IUD failure and perforation, with the device becoming embedded in an omentum. A 28-year-old woman who was 39 weeks plus one day gestation presented for caesarean section. During the caesarean section, it was discovered that the IUD had entered the omentum through a hole in the posterior part of the uterus. The result of the birth was a live boy. IUD perforation is most commonly observed during the insertion procedure; however, it may rarely occur at a later stage. IUD perforations are frequently asymptomatic and remain undetected until follow-up assessments are conducted or clinical manifestations become apparent. The presence of gastrointestinal symptoms is commonly observed in cases with intraperitoneal migration of the IUD. Although the occurrence of abdominal pain, diarrhoea, and fever with a missing IUD are indicative, they may not always be present. Nonspecific signs and symptoms lead to misdiagnosis and the consequent delay in initiating appropriate treatment. In the present case, the co-occurrence of IUD embedded in the omentum and pregnancy posed a challenge due to the device's rapid and imprecise shift, which complicated its accurate localisation. In such scenarios, ultrasonographic guidance can serve as a valuable tool to enhance accuracy and decrease adverse outcomes.

Keywords: Intrauterine Device, Omentum, Uterine Rupture

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Introduction

Intrauterine devices (IUDs) are one of the most frequently used contraceptive methods. Severe and rare complications include uterine perforation and displacement into the abdomen (1). There have been reports of intra-abdominal IUDs that caused abdominal wall embedment, bowel injury, fistula formation, and bladder perforation. The rate of uterine perforation is 0.4 to 2.2 out of 1000 copper IUD insertions (2). IUD failures can result in preterm labour and miscarriage. Additionally, uterine perforation can result from the inserter, or the uterus (3). Pregnancy and reaching term despite rupture of the uterus are rare (4). It is the aim of this study to describe a rare case of pregnancy with IUD perforation and embedment within the omentum found during a caesarean section.

Case report

A 28-year-old woman, [gravida (G), para (P), live child (Lch), dead child (Dc), abortion (Ab)] G4 P2 Lch1 Dch1Ab 1 presented to the Ba'ath Hospital in Sanandaj, Iran, on May 24, 2022 at 39 weeks plus one day gestation for a caesarean section because of her history of two previous caesarean sections. She reported a copper IUD (TCu380A) placement nine months prior to presentation, which had not been removed or expelled. Her primary care physician performed a physical examination and history during the first visit, and they were unremarkable. As far as the IUD was concerned, she was completely symptom-free. She was sexually active and had not conceived. She did not suffer from abdominal or pelvic pain.

In the nine months following insertion, a follow-up examination revealed that the patient was pregnant. She was scanned with ultrasonography and a live embryo was spotted with cardiac activity. The average gestational age according to FL/AC/HC, BPD was 16 weeks+2 days (± 2 weeks). The foetus weighed 143 grams. The amount of amniotic fluid was within normal limits, and the placenta was located in the fundus of the uterus with a normal relationship to the internal cervical os. An IUD was seen in the lower posterior uterine segment that penetrated the myometrium, but not the serosa, and there were no signs of uterine rupture. During the caesarean section, a hole was seen in the posterior region of the uterus through which the IUD had entered the omentum (Fig.1). This hole might

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have been caused by an IUD because, except for caesarean section, her only history was manipulation of the uterus. The birth resulted in an apparently healthy, live baby boy who weighed 3500 g [A1] and had an Apgar score of 9.10. An operation was performed to repair the posterior hole in the uterus. The patient and the baby were discharged in good general condition three days after surgery.

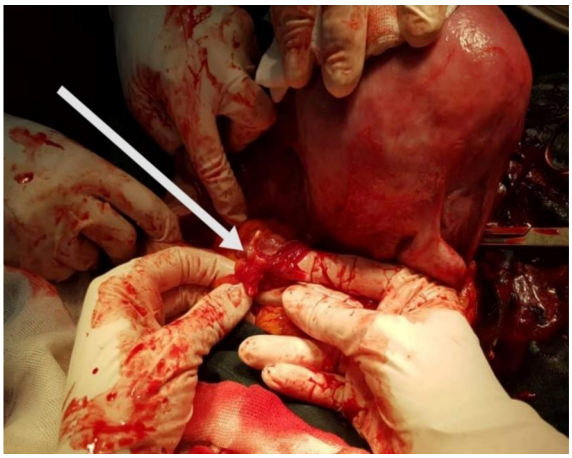

Fig.1: Intrauterine device (IUD) in a part of the omentum (white arrow).

According to our institutional policies, this Case Report required patient consent to participate which has been filed in the patient chart for our records. At Kurdistan University of Medical Sciences, Sanandaj, Iran approved this case study (IR.MUK.REC.1401.247).

Discussion

IUDs are one of the most commonly used long-acting contraceptives worldwide (5). Despite the fact that IUDs are highly effective, they may not work all the time. There are very few instances of IUDs that perforate the uterus (3). However, significant harm associated with perforation may be the loss of the contraceptive effect, which results in unplanned and often unwanted pregnancies (6). The migration of a perforated IUD can occur to various sites within the pelvis, such as adhesions, the omentum, the pouch of Douglas, or it may adhere to the colon sigmoid (7).

The majority of perforations occur during the insertion of an IUD, although they can occur later. Often these perforations remain unnoticed until follow-up examinations are performed or they become symptomatic. Missing IUDs can sometimes cause gastrointestinal symptoms, especially when the device migrates intraperitoneally. A combination of abdominal pain, diarrhoea, and fever may be indicative, but not always present (8). Misdiagnosis and delay in treatment is attributed to nonspecific signs and symptoms (9). The patient in this case complained of a four months history of amenorrhoea. A vaginal examination was performed and the IUD strings were not observed. A beta human chorionic gonadotropin (hCG) test was positive and she underwent a vaginal ultrasonography. The ultrasonography showed a single alive intrauterine foetus with normal movements, an average gestational age according to FL/AC/HC, BPD of 16 weeks+2 days (± 2 weeks). The IUD was seen in the posterior lower uterine segment with penetration into the myometrium but not through the serosa. IUD migration is most commonly associated with “missing strings” (8).

Failure to visualize or feel strings on examination should not be interpreted as an indication that the IUD has been expelled through the cervix or vagina. It is vital to proceed with ultrasound in this scenario (7). If ultrasonography does not detect the IUD within the uterus, an x-ray of the pelvis and abdomen should be obtained to determine if the IUD is in the peritoneal cavity. If both an ultrasound and a pelvic and abdominal x-ray do not detect the IUD, the IUD was probably expelled from the patient (10). Due to the patient's pregnancy, the only modality that could be used was ultrasonography. During surgery, the device was barely visible and cloaked in the omentum. Furthermore, there was a hole in the posterior part of the uterus. There is a low incidence of rupture of the posterior wall of the uterus. This rupture may be covered by the intestinal loop or omentum, which makes some of the minor symptoms difficult to detect (9). Both the patient and the newborn were fortunate to have a favourable outcome. The occurrence of pregnancy and the continuation of pregnancy until term with uterine rupture is rare, but it can have severe consequences (4).

Conclusion

This case highlights the potential risks associated with IUD use, particularly perforation and migration. While these complications may be rare, they can lead to significant morbidity and require prompt management, particularly in cases where the patient is asymptomatic. It is imperative that healthcare providers remain vigilant and promptly investigate any signs or symptoms that suggest an IUD-related complication to ensure optimal patient care. The use of ultrasound in IUD-related complications should be further encouraged to improve patient outcomes.

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Authors’ Contributions

Sh.Z.; Investigation, Conceptualization, Methodology, and Data curation. H.S.; Data curation, Writing of the original draft preparation, and Supervision. R.S.; Writing - reviewing and editing, and Validation. All authors read and approved the final manuscript.

References

1. FaridMojtahedi M, Khezri A. Cecum perforation following intrauterine device insertion: a case report. IJOGI. 2022; 24(12): 132-136.
2. Gao T, Lacue A, Brandi K. Intra-abdominal IUD requiring bowel resection in pregnancy. J Minim Invasive Gynecol. 2021; 28(11): 1812-1813.

3. Tarafdari A, Malek M, Pahlevan Falahy E, Hadizadeh A. IUD perforation and embedment within omentum: A rare and perplexing incidence. Clin Case Rep. 2022; 10(4): e05732.
4. Sohrabi H, Yousefi Sharmi SR. Pregnancy with uterine perforation: a case report. Sci J Kurdistan Univ Med Sci. 2021; 25(6): 127-132.
5. Gündüz R, Ağaçayak E, Akkılıç Dönmez D, Findik FM, Evsen MS, Gül T. Evaluation of patients with uterine perforation after intrauterine device placement and determination of risk factors: a retrospective case-control study. Eastern J Med. 2022; 27(2): 264-271.
6. Rowlands S, Oloto E, Horwell DH. Intrauterine devices and risk of uterine perforation: current perspectives. Open Access J Contracept. 2016; 7: 19-32.
7. Carroll A, Paradise C, Schuemann K, Schellhammer SS, Carlan SJ. Far migration of an intrauterine contraceptive device from the uterus to the small bowel. Clin Case Rep. 2022; 10(3): e05589.
8. Rahnemai-Azar AA, Apfel T, Naghshizadian R, Cosgrove JM, Farkas DT. Laparoscopic removal of migrated intrauterine device embedded in intestine. JSLS. 2014; 18(3): e2014.00122.
9. Chen SH, Du XP. Silent spontaneous posterior uterine rupture of a prior caesarean delivery at 36 weeks of gestation. BMC Pregnancy Childbirth. 2019; 19(1): 23.
10. Robert L, Barbieri M. How common is IUD perforation, expulsion, and malposition? OBG Management 2020; 34(4): 8-22.

International Journal of Fertility and Sterility (Int J Fertil Steril)
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It emphasizes the new and important aspects of the study. All conclusions are justified by the results of the study.

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