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A Review of The Society for Assisted Reproductive Technology Embryo Grading System and Proposed Modification

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Abstract

The Society for Assisted Reproductive Technology (SART) method of embryo grading is unique, simple, and widely practiced, and its use has been mandatory for SART membership programs since 2010. Developed by SART in 2006, the current embryo grading system categories, “good, fair, and poor,” are limited because they do not describe the best 1-2 embryos in the interest of keeping pace with the shift in clinical practice to be more selective and to transfer fewer embryos. This inspired us to conduct a review on the SART embryo grading system.

In this retrospective study, the literature on evaluation of human embryo quality in general, and the SART method of evaluation in particular, were reviewed for the period of 2000 to 2014. A multifaceted search pertaining to methods of embryo grading and transfer using a combination of relevant terms [embryo, mammalian, embryo transfer, grade, grading, morphology, biomarkers, SART, and in vitro fertilization (IVF)] was performed. The inclusion and exclusion in this review were dictated by the aim and scope of the study. Two investigators independently assessed the studies and extracted information. A total of 61 articles were reviewed.

Very few studies have evaluated the efficacy of the SART embryo grading method. The present study suggests the necessity for revision of the current SART grading system. The system, as it is now, lacks criteria for describing the cohort specific best embryo and thus is of limited use in single embryo transfer. The study foresees heightened descriptive efficiency of the SART system by implementing the proposed changes.

Strengths and weaknesses of the SART embryo grading were identified. Ideas for selecting the best cohort-specific embryo have been discussed, which may trigger methodological improvement in SART and other embryo grading systems.

Keywords: Embryo, SART, Grading, Transfer

Introduction

Embryo selection for embryo transfer (ET) is a crucial step of in vitro fertilization (IVF). Selecting the best embryo for achieving pregnancy from an embryo cohort has been a challenge for embryologists (1). In the early use of IVF for infertility treatment, morphological assessment of embryo quality was the method for choosing embryos and remains the mainstay of embryo selection today (1-3), but different morphological methods for grading IVF-generated embryos...
have been developed over time (4-12). Recently, biochemical and time-lapse analyses of embryo quality have been under investigation, but they are not yet fully ready for clinical application (13-16). Information about the efficiency and usefulness of these grading methods is important for improving ET success.

Embryologists also recognize the necessity of developing a unifying standard method of grading embryos (17-19). The European Society of Human Reproduction and Embryology (ESHRE) is working to develop one such unifying embryo grading method (17, 18, 20, 21). Some European countries such as the United Kingdom and Spain have already started to utilize a national standardized grading method (19, 22, 23). Likewise, embryologists in the United States under the banner of the Society for Assisted Reproductive Technology (SART) took the initiative to establish a uniform embryo grading method (1, 24, 25). The SART task force devised a grading system, applying a 3-point grading scale of “good, fair, and poor” in 2006 (24, 25). The present study is a review of the current SART 3-point embryo grading method. The objective of this review was to find whether the SART method is fulfilling embryologists’ needs in selecting embryos for transfer. The review makes some suggestions which we believe will improve the SART embryo grading method’s usefulness for selecting the best embryo(s) for transfer.

Materials and Methods

In this retrospective study, a review of the literature relevant to SART embryo grading system was conducted to assess its strengths and limitations. Information on evaluation of human embryo quality in general, and the SART method of evaluation in particular, was used. Several strategies were adopted to identify the pertinent articles. First, a multifaceted search performed for the period of 2000 to 2014 generated a total of 113 citations (Fig.1). The search utilized combinations of the following terms and subject headings: embryo, mammalian, ET, grade, grading, morphology, morphological parameters, biomarkers, SART, and IVF. Special emphasis was given to articles dealing with the efficiency of the SART grading system. Reference lists of relevant articles were searched manually to find additional reports which led us to select several articles prior to 2000. Proceedings of selected scientific meetings, book chapters, and monographs on embryo assessment were also reviewed. Articles found not relevant to the aim and scope of the present study were excluded from the review. Articles on the other embryo grading methods were included only if found pertinent to the scope of the study. Some of the search-generated items were excluded stepwise from the study if they were i. Duplicates (n=7) or ii. Irrelevant after reading the title and abstract (n=34) or the entire article (n=11).

Fig.1: Flow chart showing selection and exclusion of articles in the systematic review. SART; Society for Assisted Reproductive Technology.
Institutional review board approval was not requested, as this was a review of published literature and not human research.

Results

Characteristics of the studies retrieved and reviewed

The literature search had 2 components. The first component, which specifically focused on the SART grading method, produced 22 articles, of which 9 were not relevant to the objective of the study. The review findings of the remaining 13 articles are shown below under the section “Synopsis of the SART grading system”. Two authors (A.H. and M.M.) independently reviewed the articles and reached similar conclusions. Another 48 articles covering other grading methods and advances in IVF technologies, specifically those that had an association with embryo evaluation, comprised the second component of the search. This second set of 48 articles was reviewed, and the extracted information was collated with the first set (13 articles) to prepare the other sections of the manuscript (Fig.1).

Synopsis of the SART grading system

The review found that the SART members realized the necessity of developing a unifying standard method of grading embryos, and SART established a task force to explore such a possibility (24). In 2005, the task force developed a 3-point grading system using “good, fair, and poor” as grades. Three preconditions—must be simple, must have a basis in scientific inquiry, and must be easily adoptable in laboratories—guided the SART scheme. The grading utilized morphologic features applicable to 3 growth phases: cleavage, morula, and blastocyst (24, 25). Compared to other grading methods, the SART method was found to have 2 unique attributes. First, the SART system uses words, such as “good, fair, and poor,” as grades, while other methods apply alphabet letters (A/a, B/b, C/c) and numerals (1/I, 2/II, 3/III) or their combinations as grades (1, 26). Second, implementation of the SART grading system is endorsed by the nationally recognized organization that created it, while the majority of grading methods lack the advantage of being supported by a professional organization (18, 24, 26).

Proposed SART Embryo Grading System Modifications

The voluntary collection of embryo data employing the SART method began in 2006 and became mandatory in 2010 (24, 25). The task force claimed an association between implantation and SART grades based on the initial set of SART embryo data. This relationship of SART grades and implantation was first reported at the 2009 American Society of Reproductive Medicine (ASRM) meeting and then in a number of journal articles (27-30). In the consensus workshop on embryo assessment sponsored by ALPHA scientists (an organization of scientists in reproduction) and ESHRE, a member of the SART task force made a presentation that highlighted the SART’s stand on standardized embryo grading (18).

The Centers for Disease Control (CDC) has been responsible for publishing the SART embryo data since 2009 (31-33). The American Association of Bioanalysts (AAB) implemented a proficiency test, based on the SART method of grading, to standardize the grading skills of embryologists (34). Both the CDC and AAB remain committed to sharing the SART embryo grading outcomes with the public (31, 32, 34). Apart from those conducted by SART, CDC, and AAB, there were no evaluation studies, clinical trials, comparative analyses, or review studies on the SART grading system. The only studies outside of SART that made comments on the SART system were those of our group (35, 36). Our study found SART grading applicable to all developmental stages from oocyte to blastocyst.

Limitations of the SART grading system and potential resolution

The SART system sorts the embryos of a cohort into 3 groups: good, fair, or poor (24, 25). Since many IVF procedures produce a large number of embryos, obtaining several good embryos in each cohort is likely, and the same is true for the fair and poor categories (31, 36, 37). The dilemma, however, is determining which good embryo(s) to select for ET when several of the same grade are in the pool. The SART system does not have any provision for further discriminating the single best embryo from the available good embryos (24, 25). Second, the SART method selects embryos based on static observation (1, 24). This type of single snapshot examination may miss or overlook in-depth details, making the grading insufficient (38-41).
Recent publications demonstrate that new knowledge and technological advancements that have occurred in the field, particularly in the assessment of embryo viability and implantation, are powerful enough for refining SART’s embryo selection strategy to overcome the challenge of embryo selection for ET (6, 42-49). Specifically, knowledge on sequential assessment, time-lapse monitoring, and profiling by “-omics” technology has grown significantly and shows great promise to add a new dimension to the embryo evaluation (7, 50-57). In addition to this literature-based projection, we have come up with specific ideas of our own to make the SART system a better fit to tackle the challenge of embryo selection for transfer (Table 1). In our proposal, we advocate for 2 different upgrades to the SART grading method (Table 1).

### Discussion

The SART grading system was based on 3 preconditions: must be simple, must have a basis in scientific inquiry, and must be easily adoptable in laboratories. Such preconditions were imposed for better standardization and easy execution of the system globally. The goal apparently has been achieved as SART grading became one of the most widely practiced grading methods.

In the era of highly efficient ovulation induction, yields of multiple embryos in all 3 SART grades—good, fair, and poor—became common (32, 33, 36). The SART method classifies the embryos into 3 broad groups instead of selecting the best embryo for ET. By identifying embryos as good, fair, and poor, the SART system prepares a list of transfer-suitable embryos, not a rank-ordered list of embryo(s) for transfer. Ideally, the number of embryos for ET should be narrowed down to 1 embryo (32, 58, 59)—the best in the cohort—which is not achieved using the SART system.

Our vision of the SART upgrade has been briefly outlined in Table 1. It presents 2 alternate suggestions to overcome the above indicated limitations of the SART system in embryo selection for ET. This proposal provides a guiding principle to rank a sequential list of embryos in the cohort. In option 1 of the proposal (Table 1), we suggest grading the embryos as “good, fair, and poor,” as it is currently done by the SART method; however, we recommend adding a second tier of ranking for the graded embryos. For example, in the event of ET, the embryos in the “good” group should be ranked further for selection for ET. If the “good” group has no embryo or has an insufficient number of embryos, the embryos of the lower group should be ranked for ET. The target is to find the best embryo in the cohort. In the alternate plan (option 2), the SART system could be expanded to 5 grades instead of the current 3. Increasing the number of grades from 3 to 5 and simultaneously restricting the number of embryos to 1 in the top 2 grades (best and better) would compel the embryologist to serially tag the embryos, particularly the top 2. Emphasis is placed on 2 embryos because 1-2 embryos are commonly used in ET (21, 32, 33, 37). In either plan (option 1 or option 2), in lieu of the one-time evaluation, the cumulative grade obtained by sequential monitoring, manual or electronic, should be favored for individualizing the cohort-specific embryos. The SART method utilizes a set of parameters (cell number,
fragmentation, and symmetry) for grading the cleaving embryos and another set of parameters (expansion, inner cell mass [ICM], and trophectoderm [TE]) for blastocyst grading (24, 25). Many other studies, including our own, whose primary focus were embryo grading, found the following growth phase-specific morphological parameters ideal for embryo evaluation: zona pellucida (ZP), perivitelline space (PS), ooplasm, and polar body (PB) for oocyte; ZP, PS, pronucleus, and cytoplasm for zygote; number, quality, symmetry, fragmentation, and compaction of blastomeres in the cleaving embryos; and size (expansion), ICM, and TE for blastocysts (3, 4, 6, 9, 10, 12, 18, 23, 36, 39). In our proposed upgrade, we emphasize continuous monitoring of the cohort members in the pool by employing the above mentioned growth phase-specific morphological parameters so that the cohort members can be ranked reflecting the differences in their quality, specifically their vigor and implantation potential. Although time-lapse and “-omics” technologies may have advantages in monitoring and ranking the embryos, many laboratories lack these advance technologies. These laboratories have to sharpen their embryo ranking skills based on the methodological resources available to them. No matter what method a laboratory applies in evaluation of embryos, conventional or advanced, the primary goal-ranking the embryos in the cohort-can eventually be achieved by the embryologist’s embryo monitoring skills. Based on this optimism, we suggest embryo ranking in both of our proposed upgrade options. Embryo selection for ET will hopefully be better served by the proposed changes in SART grading simply because they require the embryologist to rank the embryos in the respective cohort. Future studies will cultivate this important concept of ranking embryos to develop a comprehensive upgrade plan for the SART system.

Selecting the best embryo for transfer could perhaps be achieved if the SART system would rank the embryos the way students in a class are ranked based on cumulative assessments. A successful embryo ranking would improve the ability to assess the relative vivacity and implantation potential of individual embryos within a cohort, perhaps lessening the need to transfer more than 1 embryo. With accurate embryo ranking, it is not unreasonable to assume that if the best ranked embryo cannot result in implantation, the lower-ranked embryos will be less likely to implant in an equitable uterine environment. Thus, if validated embryo ranking can be achieved, the practice of transferring lower quality embryos with the thought of improving pregnancy rates may become less common.

The primary aim of embryo ranking should be to discriminate the viability and implantation potential among embryos of a cohort (6, 42, 44, 45, 48, 53, 55). Two concepts are becoming increasingly evident from recent studies: i. Improved understanding of embryo implantation is necessary to enhance success in selecting the best embryo, (1, 5, 12, 40, 52-54, 57, 60) and ii. Sequential assessment has an advantage over single assessment in finding the best embryo (8, 11, 38, 42, 51). In addition, recent studies also suggest that advanced high-technology IVF techniques, compared to conventional IVF, are more effective for investigating the viability and implantation potential of embryos (3, 12, 26, 46, 49). In the near future, 2 of these advanced IVF techniques, 1 using time-lapse monitoring technology (3, 16, 46, 49, 57, 61) and the other using “-omics” technology, (14, 43, 48, 52-54) may become capable of efficiently discriminating the embryo viability and implantation.

Conclusion

The present study shows the strengths and weaknesses of the SART grading system. SART grading was established for the noble mission of developing a unifying standard method of grading human embryo. It has helped immensely in standardizing grading systems among clinics. The joint effort of SART and AAB in developing an embryo grading-related proficiency test homogenizes the embryo grading skills of the embryologists. However, with the shift in clinical practice to transfer fewer embryos, the current SART system falls short in fulfilling its ultimate goal-selecting the right embryo for ET. Apart from SART itself, we found no evaluation studies or clinical trials on the efficiency of the SART grading method. The authors of this manuscript humbly suggest that the time for upgrading the current SART grading system to include a more descriptive ranking of embryos is due. Our proposed additions to the current SART grading system are simple and can be implemented by any IVF laboratory without the need for additional equipment. Moreover, it would better permit a descriptive process to delineate the best embryos...
for transfer rather than a cohort of embryos.

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Resurgence of Minimal Stimulation \textit{In Vitro} Fertilization with A Protocol Consisting of Gonadotropin Releasing Hormone-Agonist Trigger and Vitrified-Thawed Embryo Transfer

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Abstract

Minimal stimulation \textit{in vitro} fertilization (mini-IVF) consists of a gentle controlled ovarian stimulation that aims to produce a maximum of five to six oocytes. There is a misbelief that mini-IVF severely compromises pregnancy and live birth rates. An appraisal of the literature pertaining to studies on mini-IVF protocols was performed. The advantages of minimal stimulation protocols are reported here with a focus on the use of clomiphene citrate (CC), gonadotropin releasing hormone (GnRH) agonist trigger for oocyte maturation, and freeze-all embryo strategy. Literature review and the author’s own center data suggest that minimal ovarian stimulation protocols with GnRH agonist trigger and freeze-all embryo strategy along with single embryo transfer produce a reasonable clinical pregnancy and live birth rates in both good and poor responders. Additionally, mini-IVF offers numerous advantages such as: i. Reduction in cost and stress with fewer office visits, needle sticks, and ultrasounds, and ii. Reduction in the incidence of ovarian hyperstimulation syndrome (OHSS). Mini-IVF is re-emerging as a solution for some of the problems associated with conventional IVF, such as OHSS, cost, and patient discomfort.

Keywords: \textit{In Vitro} Fertilization, Clomid, GnRH Agonist

Introduction

The widespread increase in the daily dosage of gonadotropins was introduced in \textit{in vitro} fertilization (IVF) protocols in the late 1980s and early 1990s. Some of the reasons for this increase include: i. High doses of gonadotropins increased the number of oocytes retrieved in good and poor responders, and ii. It allowed the formation of more embryos providing excess embryos for cryopreservation (1, 2). The introduction of gonadotropin-releasing hormone (GnRH) agonists and antagonists as a suppression for the premature luteinizing hormone (LH) surge/ovulation further allowed clinicians to use higher doses of gonadotropins (3, 4). Although there is no doubt that the high oocyte yield in conventional IVF contributed to better success rates, it has resulted in several drawbacks such as: i. High treatment cost (5), ii. Increased incidence of multiple pregnancies when more than one embryo is transferred (6), and iii. Increased risk of the potentially life-threatening ovarian hyperstimulation syndrome (OHSS) when human chorionic gonadotropins (hCG) is used for final oocyte maturation (7). The conventional long-stimulation protocol uses GnRH agonists for suppression of the anterior pituitary thus preventing the LH surge (4). The long GnRH agonist stimulation protocol became accepted as the standard protocol in many countries. GnRH agonist is usually started in the mid-luteal phase of the preceding menstrual cycle followed by stimulation with high doses of gonadotropins leading to multifollicular recruitment (8). However, the GnRH agonist protocol has many side effects including longer duration of treatment, formation of ovarian cysts and symptoms of estrogen deprivation (mood changes, hot flushes, and headaches) (9). Additionally, some of the known side effects of conventional IVF include the intake.
of several daily injections by the patient what causes pain, frustration, and skin/muscle irritation. These side effects, along with the exponential improvement in the embryology field with its associated better implantation rates, have led many to question the continued need for aggressive stimulation and to encourage revisiting minimal stimulation protocols (10-12). Minimal stimulation consists of a gentle controlled ovarian stimulation that produces a maximum of five to six oocytes (13). Minimal stimulation IVF (mini-IVF) is re-emerging as a solution for some of the problems associated with conventional IVF. The purpose of this report is to revisit the advantages of minimal stimulation protocols over conventional IVF with a focus on a protocol that uses GnRH agonist trigger rather than hCG for oocyte maturation and that utilizes a freeze-all embryo strategy rather than fresh embryo transfer.

Materials and Methods

A literature review of clinical prospective and retrospective available studies in PubMed for relevant publications in English through January 2015 was performed. In addition to IVF, the following search terms were used: conventional, mini-IVF, mild ovarian stimulation, clomiphene citrate (CC), freeze-all embryo, and GnRH agonist trigger. In addition, references from all relevant articles were reviewed. Titles and abstracts of all citations identified in the search were reviewed. The full-text article was retrieved if the citation was potentially relevant to mini-IVF.

Results

Minimal stimulation in vitro fertilization protocol

After oral contraceptive pill pre-treatment for 14 days, adequate suppression is usually confirmed with a low estradiol (E₂) level of <75 pg/mL. Minimal ovarian stimulation is started with an extended regimen (from day 3 of the cycle until the day before triggering) of CC (50-100 mg/day orally) in conjunction with gonadotropin injections starting on days 4-7 of the cycle with 75-150 IU of human menopausal gonadotropins (hMG) daily. Patients usually receive both CC and low dose gonadotropins, and the dose given depends on the ovarian reserve status and the body mass index (BMI) of the patient. The final maturation of oocytes is usually induced by either intramuscular hCG or GnRH agonist (intramuscularly or nasally) (Fig.1). When patients desire fresh embryo transfer, hCG is the preferable method for oocyte maturation. Zarek and Muasher (13) have reported a protocol in 31 patients where they used 100 mg of oral CC on days 3 to 7 of the cycle followed by 150 IU of gonadotropins daily started on day 8 of the cycle. They have used GnRH antagonist (0.25 mg of ganirelix acetate) daily for LH surge suppression and 10,000 IU of hCG for final oocyte maturation trigger. In this study, the mean number of mature oocytes retrieved was 4.2, the mean number of embryos transferred was 2.4, and the clinical pregnancy rate per cycle was 42%. Williams et al. (14) compared mini-IVF protocol, using sequential protocol of CC (100 mg of orally on days 3 to 7) and gonadotropins (150 IU of gonadotropin daily starting on day 9) ± GnRH antagonist to suppress the LH surge (n=50 participants), to conventional IVF protocol, using the standard long GnRH agonist protocol (n=52 participants). Despite the fact that mini-IVF yielded significantly lower number of oocytes compared to the conventional stimulation IVF protocol (3.7 vs. 13.1, respectively, P<0.05), both protocols produced similar pregnancy rates.

At our institution (New Hope Fertility Center, USA) retrieved oocytes are fertilized and subsequently cultured until the cleavage stage or preferably the blastocyst stage. Figure 1 represents a summary diagram that summarizes a protocol that has been used at our institution over the last five years and used by Kato et al. (15) and Teramoto and Kato (16) in Japan. This protocol uses mini-IVF with GnRH agonist trigger and freeze-all embryo strategy, as suggested by previously published literature (14, 15, 17-19). Good quality blastocysts are vitrified and a single thawed blastocyst is typically transferred in a subsequent artificially prepared frozen embryo cycle. At our center, 564 infertile women (age<39) undergoing their first IVF cycle between February 2009 and August 2013 were randomly allocated to either mini-IVF with single embryo transfer as seen in Figure 1 (n=285) or conventional IVF (n=279) with double embryo transfer (20). The primary outcome was cumulative live birth rate per woman over a 6-month period. We found that the cumulative live birth rate was 49% (140/285) for mini-IVF and 63% (176/279) for conventional IVF [relative risk (RR): 0.76; 95% confidence interval (CI): 0.64-0.89]. There were no cases of OHSS after mini-IVF compared to 16 (5.7%) moderate/severe OHSS cases after conventional IVF. Gonadotropin consumption was significantly lower with mini-IVF compared to conventional IVF (459 ± 131 vs. 2079 ± 389 IU, P<0.0001) (20).
Advantages of using clomiphene citrate in mini-in vitro fertilization

CC has traditionally been used as the most fundamental drug for ovulation induction in the treatment of infertility worldwide (21). Chemically, CC is a nonsteroidal triphenylethylene derivative that exhibits both estrogenic agonist and antagonist properties (14, 15, 17-19). Approximately, 85% of CC dose is eliminated from the blood after 6 days, although traces may remain in the circulation for months. CC is a mixture of 2 geometric isomers, enclomiphene and zuclomiphene, in a 3:2 ratio. Enclomiphene is the more potent isomer that is primarily responsible for ovulation induction. Zuclomiphene is the less-active isomer and cleared far more slowly from the blood (21, 22). At the cellular level, CC binds nuclear estradiol receptor (ER) for a long period of time thus depleting ER concentration by slowing down and ultimately depleting ER replenishment (22). In ovulation induction, CC depletes ER at the level of the hypothalamus thus suppressing the usual negative feedback by circulating E2 (22, 23). This triggers the hypothalamus to secrete high levels of GnRH secretion that will stimulate endogenous release of follicle-stimulating hormone (FSH) and LH by the pituitary (23). The increase in serum gonadotropins will then stimulate follicular activity at the level of the ovary. In usual ovulation induction, CC is used for 5 days; a period of time that increases both endogenous LH and FSH (22). On the contrary, when CC is administered for more than 5 days, LH release decreases. Thus, clinicians around the world started using CC, instead of GnRH agonists.
or antagonists, as a suppressive agent for premature LH surge in many IVF protocols, especially mini-IVF (15, 16). Messinis and Templeton (24) reported that prolonged administration of CC inhibited positive feedback and thus prevented the LH surge. In that study, they demonstrated that CC administration for 15 days (days 2 to 16 of the cycle) produced a continuous and progressive increase of basal LH levels with no LH surge and no ovulation.

**Trigger with nasal gonadotropin-releasing hormone agonist**

Conventional IVF typically uses an intramuscular or a subcutaneous injection of hCG at 5000-10,000 IU. It is well known that hCG can trigger OHSS, especially in high-risk groups such as women with polycystic ovary syndrome (PCOS), low BMI, young, and good responders (25). Rather than using hCG, protocols described in the mini-IVF and conventional IVF literature have used nasal administration of GnRH agonist as the maturation trigger (15, 16, 26, 27). The reasoning behind using GnRH agonist is first to avoid OHSS, and second to have a natural maturing process of oocytes based on the endogenous LH/FSH surge thus maintaining a natural luteal function (28). GnRH agonist trigger have been shown to be beneficial in situations like repeated IVF failure, empty follicle syndrome and repeated retrieval of immature oocytes, with the hypothesis that some patients may require the FSH surge, in addition to the LH surge, to promote final oocyte maturation resembling the natural midcycle surge of gonadotropins (29, 30). Several studies have reported that cycles where GnRH agonist was used as the maturation trigger produced comparable number of mature oocytes to cycles where hCG was used (31, 32). Griesinger et al. (33) demonstrated that the use of GnRH agonist to trigger final oocyte maturation yields a comparable number of mature oocytes and comparable embryonic development to that achieved with hCG trigger. Interestingly, Humaidan et al. (34) demonstrated that GnRH agonist trigger produced higher number of metaphase II (MII) oocytes than hCG trigger. However, in that same study, GnRH agonist trigger as a final oocyte maturation was associated with a lower pregnancy rate, a lower live birth rate, and a higher rate of early miscarriage (28). It seems more likely that GnRH agonist induces a luteal phase defect. This luteal phase defect may result from the short half-life of the induced LH surge, leading to premature luteolysis of corpus luteum and significantly lower steroidal and non-steroidal hormones, thus affecting endometrial receptivity (35). However, these evaluations become less relevant in protocols where a freeze-all embryo strategy, rather than transferring embryos in the same fresh cycle, is employed. Finally, GnRH agonist can be administered nasally making it a patient-friendly drug by avoiding the injectable hCG (15, 16).

**Freeze-all embryo strategy**

The general success rates for frozen-thawed embryo transfers have increased in the past few years. The Centers for Disease Control and Prevention (CDC) collected data on assisted reproductive technology (ART) success rates of all American fertility clinics from 1997 to 2011. According to those data, success rates of both fresh and frozen-thawed embryo transfer cycles (donor eggs not included) have increased over the past 14 years for women of all ages. Interestingly, the increase in success rates seems to be greater for frozen-thawed embryos compared with fresh embryos (36). Furthermore, data from children born from frozen-thawed embryo transfer cycles show fewer perinatal complications of preterm birth, small for gestational age, low birth weight, and perinatal mortality compared with children born from fresh embryo transfers (37-39). Additionally, outcomes of singletons born after frozen-thawed embryo transfer seem more similar to naturally conceived singletons (40). So far, the etiology of these differences is unknown, although suboptimal endometrial development has been suggested to be a risk factor for the adverse outcomes of ART (41). Proper placentation with fresh embryo transfer may be jeopardized by the supraphysiological concentrations of estrogen and progesterone ($P_4$), leading to worse perinatal outcomes compared with frozen-thawed embryo transfers in a more neutral physiological environment. There seems to be a less receptive endometrium in cycles with ovarian stimulation, and children born from the transfer of frozen-thawed embryos had better outcome compared with children born from cycles with fresh embryo transfer. Several recent studies have shown that the success rates of frozen-thawed embryo transfer are similar, if not better, to the success rates of fresh
Embryo transfer (42, 43). Whether frozen-thawed embryo transfers should be performed in natural or in artificial cycles is not clear. Several studies compared frozen-thawed embryo transfers in natural or artificial cycles and found no differences in pregnancy rates or live birth rates (44, 45).

Conclusion

There is still resistance to use of minimal stimulation protocols because of the fear of having few oocytes. There is also a misbelief that minimal stimulation severely compromises pregnancy and live birth rates. It is clear that IVF treatment is stressful and costly to lots of patients. Multiple office visits including injections, blood draws, and ultrasounds can add to the stress. Additionally, stress could emanate from the high cost of IVF medications and procedures, as well as IVF outcomes that include complications of multiple pregnancies (such as preterm birth, preeclampsia, cesarean section, etc.). Although severe OHSS is rare, it constitutes a real threat for high-risk patients. Interestingly, there is a lack of literature pertaining to the outcome of mini-IVF in obese older women, especially those with diminished ovarian reserve. We have recently shown that female adiposity might impair oocyte number and maturity in conventional IVF but not in minimal stimulation IVF, suggesting that gentle ovarian stimulation might yield healthier oocytes in obese women. Thus, future studies are needed to address the optimal IVF protocol in this patient population. There is no doubt that minimal stimulation protocols is disadvantageous to oocyte donation and elective oocyte cryopreservation, and that it limits the number of available embryos. However, molecular differences at the level of the oocyte seem to be real between conventional IVF versus mini-IVF. Additionally, molecular differences between frozen-thawed embryos and freshly cultured human embryos have been reported. Interestingly, success rates after frozen-thawed embryo transfer are now nearing the success rates of fresh embryo transfer supporting the hypothesis of so called freeze-all embryo strategies in IVF to optimize success rates. Finally, there is a critical need for high quality randomized controlled trials to determine which cryopreservation protocol is best and whether a freeze-all embryo strategy is justified in IVF treatments.

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Bone in The Endometrium: A Review

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Abstract

To provide a comprehensive review of the published literature of patients with endometrial bone or osseous fragments with a view to critically examine the antecedent clinical presentation, investigations and prognosis after treatment.

This systematic review of the literature includes full text articles of published case reports and case series from the following computerized databases: PubMed, Ovid, and Medline between 1928 and 2013. We reviewed a total of 293 patients in 155 case reports and case series.

The mean ± SD age at presentation was 32.7 ± 8.9. Approximately 88% of patients had at least one prior surgical uterine evacuation relating to pregnancy termination or loss at a median gestational age of 14 weeks (range of 4-41 weeks). The most common presenting symptom was infertility (56.2%). One hundred twenty-four (66.0%) of the 188 patients attempting pregnancy after treatment achieved pregnancy prior to article publication and the majority (82.3%) were spontaneous. Spontaneous miscarriage rate remains high (43%); however, most pregnancies ended in live-birth (55%).

Bone fragments in the endometrium are most commonly found after pregnancy termination, present with infertility and/or irregular menses, and upon removal, patients rapidly conceive spontaneously.

Keywords: Infertility, Female, Surgery, Metaplasia, Ossification


Introduction

Following the first description of bone in the endometrium by Meyer (1), several other authors have reported cases and case series relating to calcified material in the endometrium representing bone. Reports have originated from across the globe and spanned several countries and ethnicities. Although the prevalence of this entity remains unknown, the advent of newer imaging techniques such as ultrasonography meant that the presence of bone in the endometrium is being increasingly diagnosed. Nevertheless the majority of information on this subject is still from case reports and case series (2-4).

The origin (5, 6) of and the effects of bone in the endometrium on endometrial function remain a mystery. The argument centers around whether these calcified tissues are retained fetal bone from prior termination of pregnancy (7) or osseous endometrial metaplasia (6, 8). The later may result from endometrial multipotent stem cell activation (9), chronic endometritis, trauma (8, 10, 11), heterotopia, strong endometrial estrogenic stimulation, dystrophic and metastatic calcification.
among others (12). Authors of previous works on the subject have suggested that the bony fragments may function as a type of intrauterine contraceptive device (13, 14) thereby leading to subfertility. Indeed, the available literature have suggested that patient complaints include not only infertility but also irregular vaginal bleeding, chronic pelvic pain and even persistent vaginal discharge (10, 14, 15). There is a need for a clear description of the demographic information of patients who present with this condition, specifically their antecedent pregnancy information, the complaints with which they present, and the duration of time the condition has persisted before diagnosis. Accordingly, our objectives are: first, to provide a comprehensive review of the published literature with a view to gleaning the epidemiological characteristic of patient found to have bone on radiological imaging. Second, we describe the possible pathogenesis of the condition. Third, we evaluate the different published treatment modalities for this condition and their outcome.

Materials and Methods

In this systematic review of the literature, we performed a comprehensive literature search on PubMed, Ovid, and Medline using medical subject headings (MESH) words including endometrium, fetal, bone, osseous fragments, pelvic imaging (X-ray and ultrasound) and osseous metaplasia and all combination of the aforementioned words. All articles referenced in searched articles were also reviewed for cases missed from the original search. Only full text English, Spanish, Portuguese, Italian, French, and Turkish articles were included. One article in German (16), one in Danish (17), and one Hebrew (18) were excluded, as we were unable to find translators for these articles. Data from all articles published in French describing this phenomenon in 1928 (19) and the latest in 2013 (20) were included. One article in German (16), one in Danish (17), and one Hebrew (18) were excluded, as we were unable to find translators for these articles. Data from all articles published in French describing this phenomenon in 1928 (19) and the latest in 2013 (20) were included. One article in German (16), one in Danish (17), and one Hebrew (18) were excluded, as we were unable to find translators for these articles. Continuous data were summarized using mean (SD) for age, and gestational age at delivery, while median (range) was used for gravidity and parity. Percentages were calculated for categorical data. As this was an analysis of published cases, institutional review board (IRB) approval was not necessary for this study.

Results

A total of 293 patients with endometrial bone or osseous materials were analyzed from a total of 155 articles. Of the factors analyzed, only patient’s age was reported in all of the 293 cases. The mean (SD) age for the cohort was 32.7 ± 8.9 years (range of 15-73 years). Similarly, 209 (71.3%) cases contained information regarding gravity and parity with a median gravity and parity of 2 (range of 0-12) and 1 (range of 0-10), respectively. Of 218 (74.4%) patients in which ethnicity was re-
ported, the majority (65, 29.8%) were Hispanic/Latino, while other ethnicities were less common: 52 (23.8%) were Black or of African descent; 39 (17.9%) were Caucasian from Europe and Australia, while 13 (6%) were Caucasian from North America; 18 (8.3%) were Asian/Pacific Islander, 16 (7.3%) were from the Indian Subcontinent, while 14 (6.4%) were of Middle Eastern (including Turkey) ethnicity; and the least common reported ethnicity in 1 case [0.5, 95% confidence interval (CI): 0.02-2.9%] was Native American. The United States of America (USA) and France reported 21 articles each (13.6%), followed in descending order by; the United Kingdom (UK) (12.3%), Turkey (6.5%), Italy and India (5.8% each), Brazil and Canada (3.9% each), Netherlands and Chile (3.2% each), China and Mexico (2.6% each), Romanian and Venezuela (1.9% each), Pakistan, Greece, Spain, Hong Kong, Sweden, Columbia and Tunisia (1.3% each), while Korea, Ireland, Australia, Ivory Coast, Jamaica, Japan, Jordan, Germany, Ghana, Qatar, Morocco, Israel, Algeria, Democratic Republic of Congo and Denmark all had one publication relating to bone in the endometrial in our study (0.6% each). It is noteworthy that majority of the cases were reported by large cases series from Brazil (6), Korea (22) and the UK (10). The majority of the patients were being investigated for infertility which was reported in 150 cases out of a total of 267 (56.2%) patients in whom the absence or presence of presenting symptoms were reported. Of the 170 patients in whom the menstrual history was reported or can be deduced from the report, only 53 (31.2%) reported regular menses, 105 (61.8%) reported irregular menses, while 12 (7.1%) were postmenopausal. Other presenting symptoms are reported in Table 1.

<table>
<thead>
<tr>
<th>Table 1: Presenting symptoms</th>
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<tbody>
<tr>
<td><strong>Symptom</strong></td>
</tr>
<tr>
<td>Infertility</td>
</tr>
<tr>
<td>Irregular bleeding</td>
</tr>
<tr>
<td>Vaginal discharge</td>
</tr>
<tr>
<td>Dysmenorrhea</td>
</tr>
<tr>
<td>Dyspareunia</td>
</tr>
<tr>
<td>Pelvic pain</td>
</tr>
<tr>
<td>Recurrent pregnancy loss</td>
</tr>
<tr>
<td>Asymptomatic</td>
</tr>
</tbody>
</table>

The median number of prior terminations or losses was 1 (range of 0-6). In other words, the vast majority of the 239 patients in whom number of pregnancy terminations or losses was reported, 175 (73.2%) had only 1 preceding termination or loss, 29 (12.1%) had 2 terminations or losses and 25 (10.5%) had 3 or greater. Of note, 10 cases (4.2%) reported no prior pregnancy termination or loss. Two hundred forty-four cases reported on whether termination or loss was spontaneous or surgically accomplished. Of these, twenty-nine cases (11.9%) reported spontaneous losses with no surgical intervention, while the remaining 215 cases (88.1%) reported surgical terminations or curettage. The median gestational age of preceding pregnancy was 14 weeks (range of 4-41 weeks). The median duration between last pregnancy and presentation to care or incidental diagnosis was 5 years (range of 1-40 years). Diagnostic modalities used to diagnose the presence of endometrial bone or osseous materials are shown in Table 2.

<table>
<thead>
<tr>
<th>Table 2: Different diagnostic tests used for the diagnosis of endometrial bone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diagnostic test</strong></td>
</tr>
<tr>
<td>Ultrasound</td>
</tr>
<tr>
<td>Dilation and curettage</td>
</tr>
<tr>
<td>Radiography</td>
</tr>
<tr>
<td>HSG</td>
</tr>
<tr>
<td>Hysteroscopy</td>
</tr>
<tr>
<td>Visualization on exam</td>
</tr>
<tr>
<td>Endometrial biopsy</td>
</tr>
</tbody>
</table>

HSG: Hysterosalpingogram.

Interestingly, the initial diagnostic method was reported as normal in 11 of 123 (8.9%) cases that had ultrasound as the initial imaging modality. Figure 1 demonstrates an ultrasound image of bone in the endometrial cavity. Another interesting finding was the presence of uterine anomalies in the cohort. Of all cases reviewed, 4 (1.4, 95% CI: 0.4-3.7%) patients were noted to have Müllerian duct anomalies (MDA) including: 1 uterine didelphys and 3 septate uteri. Of the patients evaluated for infertility, assessment of tubal patency was reported in 82 cases. Of these, 61 (74.4%) had bilateral tubal patency, 5 (6.1%) had unilateral tubal patency, while 16 (19.5%) had bilateral tubal occlusion. History of previous pelvic inflammatory disease was rarely reported.
The presence or absence of such a history was reported in only 15 case reports, of which only 6 (40%) had a positive previous history. Endometrial location of the bone fragment was recorded in 33 cases (11.3%) (Table 3).

Table 3: Location of bone fragments within endometrium and/or endometrial cavity

<table>
<thead>
<tr>
<th>Location of fragments</th>
<th>Frequency (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Posterior</td>
<td>27/67 (40.3%)</td>
<td>28.6-52.0%</td>
</tr>
<tr>
<td>Intracavitary</td>
<td>19/67 (28.3%)</td>
<td>17.6-39.2%</td>
</tr>
<tr>
<td>Anterior</td>
<td>8/67 (11.9%)</td>
<td>4.2-19.7%</td>
</tr>
<tr>
<td>Multiple surfaces</td>
<td>13/67 (19.4%)</td>
<td>9.9-28.9%</td>
</tr>
</tbody>
</table>

CI: Confidence interval.

Two hundred seventy cases (92.2%) included information about treatment, of which the most common treatment modality for removal of bone fragments was hysteroscopic based excision in 182 cases (67.4%), whereas non-hysteroscopic treatments (biopsy, forceps, dilation and curettage, and hysterectomy) occurred in 87 (32.2%) patients. Figure 2 shows calcified tissue located within the endometrium, which was pathologically determined to be bone. It is noteworthy that in 61.2%, the bony fragment involved the posterior uterine wall. Most patients had 4 or more bony fragment (58.6%) with 68.9% of these measuring 1 cm or greater. Two hundred eighty-nine (98.6%) cases reported pathologic evaluation and all (100%) detected the presence of bone. In two cases, fragments were not removed; therefore, pathologic confirmation was never obtained. Of the 289 cases reporting pathology, only 17 (5.9%) were noted to have marrow formation and 11 (3.8%) were noted to have cartilage. Of the 289 cases, the presence or absence of inflammation in the retrieved endometrial specimen was reported in 224 (76.5%), of which 151 (67.4%) showed evidence of inflammation or infection.

Symptom resolution was reported in 52 of 64 (81.3%) patients who did not present with infertility. Symptom non-resolution was reported in 3 (4.7%), while 9 patients (14.1%) were lost to follow up. One hundred and eighty-eight (64.2%) cases reported on whether patients did or did not attempt to get pregnant after treatment and of these, 124 (66.0%) attempted to get pregnant. Of these, 90 (72.6%) achieved pregnancy prior to article publication, 16 (12.9%) reported persistent infertility, while 3 (2.4%) stopped attempting pregnancy for reasons including age, decreased ovarian reserve and tubal non-patency. In the remaining 15 patients (12.1%), the outcome of fertility was not reported. Of the 90 (72.6%) patients who achieved pregnancy, 74 (82.2%) achieved pregnancy spontaneously, while 10 (11.1%) needed infertility treatment or assisted reproductive technology (ART), whereas in 6 cases (6.7%), how pregnancy was achieved was not specified. Of the
90 patients who achieved pregnancy, information on duration of attempt prior to success was provided in 25 cases. The majority of patients who were attempting to achieve pregnancy after treatment conceived within 3 months (36%), followed by 2 months (16%) with durations from 1 to 9 months making up the remainder. A paucity of information was reported regarding pregnancy outcomes. Thirty cases reported the outcomes of pregnancies after treatment of endometrial bone. Of those, 17 resulted in live births with only one preterm case, which was a twin pregnancy. Of the remaining 13, 11 were spontaneous abortions and 2 were ectopic pregnancies (Table 4).

Table 4: Pregnancy outcomes

<table>
<thead>
<tr>
<th>Pregnancy outcome</th>
<th>Frequency (%)</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous pregnancy</td>
<td>74/90 (82.2%)</td>
<td>72.9 to 88.9%</td>
</tr>
<tr>
<td>Pregnancy with infertility treatment</td>
<td>10/90 (11.1%)</td>
<td>5.9-19.4%</td>
</tr>
<tr>
<td>Pregnancy (unspecified if spontaneous of with infertility treatment)</td>
<td>6/90 (6.7%)</td>
<td>2.8-14.1%</td>
</tr>
<tr>
<td>Full term birth</td>
<td>16/30 (53.3%)</td>
<td>36.1-69.7%</td>
</tr>
<tr>
<td>Preterm birth</td>
<td>1/30 (3.3%)</td>
<td>&lt;0.01-18.1%</td>
</tr>
<tr>
<td>Ectopic</td>
<td>2/30 (6.6%)</td>
<td>0.8-22.4%</td>
</tr>
<tr>
<td>Spontaneous abortion</td>
<td>11/30 (36.6%)</td>
<td>21.8-59.8%</td>
</tr>
</tbody>
</table>

CI: Confidence interval.

Discussion

A clearer characterization of patients with bone in the endometrium has emerged from this comprehensive review of published cases reports and case series. Most reports were of patients of South American, North American/European, or African descent; however, the majority of published reports originated from the UK and the USA. Most patients who were in their earlier thirties reported having undergone at least 1 pregnancy termination or loss often in the early second trimester. Graham et al. (10) from the UK reported on 11 West African women who underwent termination of pregnancies (TOPs) in their countries of origin and presented with infertility related to retained intrauterine bone. Termination of pregnancies in developed countries is often illegal; therefore, such operations are more likely to be performed by inexperienced practitioners and those with no medical qualifications. Due to associated social stigma in these communities, TOPs tend to be performed much later than usual, carrying a greater risk of being incomplete. It, therefore, seems that the bone or osseous materials are more likely of fetal origin related to such TOPs. The USA and the UK have a large population of immigrants; therefore, it is tempting to speculate that in the majority of cases, sub-optimal management in underdeveloped countries prior to immigration might be responsible for this disorder. The prevalence of endometrial fetal bone following TOPs is unknown. However, ultrasounds are increasingly being used in the western world to evaluate patients with infertility, uterine fibroids, other pelvic masses and cancer, endometriosis and pelvic pain; therefore, the presence of endometrial bone is more likely to be identified. The high number of reported cases in the western world may be because gynecologists in the USA and the UK are more willing to subject patients to pelvic ultrasound, and are more likely to publish such cases when encountered.

A major point of debate regarding this topic is the pathophysiology of bone in the endometrium. The prevailing hypothesis posits that these bone fragments are retained fetal bone fragments left embedded in the endometrium (13-15) following uterine evacuation after pregnancy termination or a miscarriage.

The reasons for the findings of endometrial osseous material in the 9 reported nulligravidaes and those with early first trimester terminations or loss when fetal bone formation is not known to occur are unknown. Endometrial bony fragments in these cases may be due to metaplasia of the stromal cells of the endometrium into osteoblastic cells that mature to produce bone (25-27). In support of this theory, 16 articles in the current series reported the presence of marrow formation on pathologic evaluation of the retrieved osseous fragments. It noteworthy that the reported gestational age at time of loss or termination in these cases was less than 20 weeks and as early as 10 weeks, which is prior to when fetal medullary hematopoiesis is known to occur. In addition, multiple groups have confirmed the presence of multipotent cells in the endometrium. It is known that endometrial stem cells can differentiate into bony material (9, 28). Consistent with this, metaplasia of the endometrial stromal cells (usually fibroblasts) into osteoblasts...
has been proposed as a bone-derived mechanism (29). Moreover, Parente et al. (6) and Cayuela et al. (30) performed genetic analysis on the bony fragments retrieved from endometrial curettage (8 of 14 cases and 1 case, respectively) and found them to be genetically identical to cells from the mother in all cases analyzed. These findings provide support to the possibility that some endometrial osseous material might come either from maternal endometrial stem cells or from metaplasia of maternal stromal cells. Collectively, these suggest that bone fragments in some of these patients may not be of fetal but of maternal origin.

Several studies have tried to relate the pathophysiology of the condition with the presenting symptoms. Over 70% of patients presented with irregular menstrual periods and/or infertility; however, the range of presenting complaints also included pelvic pain and vaginal discharge, while a small minority was asymptomatic. Abnormal menstruation in patients with retained bony fragment(s) may be due to higher prostacyclin concentrations noted in their menstrual fluid, which is known to cause vasodilation and increased uterine bleeding. Also it was proposed that the physical presence of ossified intrauterine material may cause uterine irritation and that the ensuing pelvic pain may result from associated increased prostaglandins levels (15). However, in the present study, we found that <2% of patients with retained endometrial bone presented with dyspareunia and pelvic pain. The presence of osseous material within the endometrial lining or in the uterine cavity (10, 29) may act in a similar manner to a non-hormonal intrauterine contraceptive device (13) by increasing menstrual fluid prostaglandin and prostacyclin (15) or by causing chronic endometritis (CE)-like reaction (31). Indeed, over 52% of the patients who had their endometrial curettages subjected to histopathological examination had evidence of inflammation. Interestingly, despite the fact that all patients had bone in the endometrium, the presence or absence of pelvic inflammatory disease was only reported in 15 patients and of these, only 6 (40%) reported a history of prior infections causing pelvic inflammatory disease. Reasons for these findings may include underreporting in the case reports as well as a failure to elicit this history from patients. One theory is that bone in the endometrium acts as a foreign body (10), which can then be a nidus for infection. However, based on the available evidence, it appears that patients with bone in endometrium do not have an increased propensity for pelvic inflammatory disease.

The most common method of diagnosis was ultrasound; the use of other modalities such as hysterosalpingography (HSG) (23) may represent earlier time period when sonography was not as widespread. Interestingly, there were several reports of prior ultrasound evaluation (8.9% in this series) and HSG that were reported as normal prior to the definitive diagnosis. These would suggest that either the prior studies were inadequate or that the process through which endometrial bone ensues is chronic in nature as with osseous metaplasia. Unfortunately, our numbers are too small to make a clear assessment of this finding.

One very interesting finding in our study was the presence of MDA in the cohort. Of all cases reviewed, there were 4 cases of MDA with one uterine didelphys and three septate uteri. This finding has been previously noted as well as the importance of characterizing the anomaly prior to any instrumentation (32). The question of whether patients with uterine anomalies are more predisposed to retention or development of bone in the endometrial cavity was suggested by Chervenak et al. (33). To address this question, we analyzed the frequency in our cohort and compared this with frequency data from other populations. In a large case series published in 2008, Saravelos et al. (34) determined the frequency of uterine anomalies in the general population to be 6.7%, in the infertile population to be 7.3% and in the recurrent miscarriage population to be 16.7%. In our cohort, the frequency was much less than any of the previously described populations at 4/293(1.4%), suggesting that there is not an increased risk of the finding of bone in the endometrium in patients with uterine anomalies.

Most treatments were hysteroscopy based (68%), as it is the most appropriate method to remove intrauterine pathology because it is less invasive, more efficacious and less costly than other options (35). Of the other 32% treated cases, the most common treatment was dilation and curettage, which may be a function of the time period prior to the widespread use of hysteroscopy. Although the first description of hysteroscopy was
published in the 1920s (36), widespread use of this technique did not appear until the mid-1970s (37). Of note, 29 cases in our series were published prior to 1980. Interestingly, 12 cases reported hysterectomy for the treatment of bone in the endometrium. Most of these cases described patients who were perimenopausal and who had completed childbearing. However, at least three patients from different articles were in their early-mid 20s and presented with complaints of infertility but underwent hysterectomy; the exact indication(s) for such an operation in these young women was not stated in these reports.

Perhaps the most important finding of the present study was that the majority of patients with this condition were able to become pregnant spontaneously after treatment. Furthermore, most patients who did conceive were able to do so quickly, indicating that the majority conceived in less than 6 months after treatment. Additionally, in over 50% of these pregnancies, the outcomes were live births. The rate of spontaneous miscarriage of 36% is higher than even the most robust incidence estimates, raising concern for remnants of osseous material still retained in the endometrium (38, 39). Additionally, for those patients who did not desire pregnancy, the majority did experience symptom relief after treatment.

The purpose of this study is to characterize these patients, increase awareness that the condition exists, and provide guidance on how to counsel patients regarding prognosis after treatment. The information therein is intended for use by practitioners when they encounter this problem, because most will never encounter a case judging by the rarity of the condition. Given that most cases resulted from uterine evacuation in the second trimester, surgical uterine evacuation under ultrasound guidance at this stage of pregnancy may help and perhaps should be routinely used in these cases. However, there is no scientific evidence for such a recommendation as retained products conception could still be encountered despite concomitant use of ultrasound (40).

There are several strengths to this study, the most important one being the number of cases included for analysis, which to our knowledge is the largest in the literature. Furthermore, we included cases reported in all languages except 5 case reports that were written in languages in which we were unable to obtain help in translation; therefore, our list cannot be regarded as complete.

We wish to emphasize several limitations associated with this study. This is a synopsis of case reports and case series often with different emphasis and heterogeneity on facts presented; however, the study design is necessary for studying very rare conditions such as bone in the endometrium. Retrospective epidemiologic studies are susceptible to being biased, and data entries into patient’s health record are uncontrolled or unsupervised often with several important missing data as shown in this study. Furthermore, the bias to describe favorable as opposed to unfavorable outcomes is well documented and may contribute to the success rate of conception after treatment. Therefore, caution is needed to interpret the data presented here. By its nature, it was impossible to estimate the prevalence of this condition in the general public as this is not a cohort or prospective study. Also, the relatively small number of cases reported may make the conclusions resulting from sub-analysis flawed. In addition, as cases went as far back as 1928 (19), it is unclear how accurate cases reported in early years were, because it was only during the 1960s that the first medical applications of ultrasound were being tested and it was not until the 1970s that the technology became widely available (41).

Conclusion

The present study is the largest evaluation of patients with findings of bone in the endometrium. We describe the most common demographic information as well as the presentation and sequel after treatment. Based on available research, it appears that the pathogenesis of this condition involves at least some component of osseous metaplasia; however, further studies are warranted to better understand the pathophysiology of this condition.

Acknowledgements

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References

 Bone in Endometrium


Comparison of Risk of Preterm Labor between Vaginal Progesterone and 17-Alpha-Hydroxy-Progesterone Caproate in Women with Threatened Abortion: A Randomized Clinical Trial

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Abstract

Background: Threatened miscarriage is a common complication in pregnancy that leads to adverse pregnancy outcomes such as preterm labor. This study aimed to compare the vaginal progesterone (Cyclogest) versus 17-alpha-hydroxyprogesterone caproate (Proluton) on preventing preterm labor in pregnant women with threatened abortion at less than 34 weeks’ gestational age.

Materials and Methods: This balanced randomized, double-blind, single-center controlled clinical trial included 190 women with threatened abortion. They were then randomly allocated into Cyclogest (n=95) and 17-alpha-hydroxyprogesterone caproate (Proluton, n=95) groups. Interested outcome was preterm labor less than 34 weeks. The Pearson chi-square and Student’s t test were used to compare two groups. The data were analyzed by Stata software version 13.

Results: The risks of preterm labor less than 34 weeks in Proluton and Cyclogest groups were 8.6 and 6.52%, respectively. There was no significant difference for risk of preterm labor less than 34 weeks [relative ratio (RR): 1.31, 95% confidence interval (CI): 0.47-3.66, P=0.59] between two groups.

Conclusion: Risk of preterm labor in the vaginal progesterone group and 17-alpha-hydroxyprogesterone caproate group in pregnant women with threatened abortion is the same (Registration Number: IRCT2014123120504N1).

Keywords: Progesterone, 17-Alpha-Hydroxyprogesterone Caproate, Premature Labor, Threatened Abortion


Introduction

Preterm labor is defined as babies born alive at less than 37 weeks’ gestational age (259 days), (1-3) which is divided into three groups, including extremely preterm (less than 28), very preterm (28 to less than 32 weeks) and moderate to late preterm (32 to less than 37 weeks) (1). Based on the study by Blencowe et al. (4), 14.9 million babies are born worldwide before 37 weeks’ gestational age, indicating about 11.1% of all live births, which is more than 1 in 10 babies. Preterm labor rates across 184 countries in the world range from 5 to 18 % (5).

Also, preterm labor rates in Iran were estimated from 5.1-8.4% (6, 7).

The preterm labor is the most important cause of neonatal mortality and morbidity, estimated 27% of neonatal deaths, and several studies have revealed mortality and morbidity rates are inversely associated with gestational age at delivery time (3, 8-12). Several risk factors are related to preterm labor such as shortened cervical length, infection, previous preterm labor, socioeconomic status, nutritional status, threatened abortion, etc. (13-15). Threatened abortion refers to vaginal bleeding be-
fore 20 weeks’ gestational age and occurs in 20% of pregnancy (a fifth of cases) (16-18).

Progesterone has been shown to be an important factor for maintaining uterine quiescence in the latter half of pregnancy, probably by restricting the production of prostaglandins and inhibiting the expression of contraction-associated protein genes within the myometrium, including oxytocin and prostaglandin receptors, ion channels and gap junctions (19, 20). The weeks before labor, levels of progesterone in the maternal circulation remain unchanged, although the onset of labor is directly associated with a functional withdrawal of progesterone activity (20-22). In both basal and pro-inflammatory conditions, progesterone stops apoptosis in fetal membrane explants (23). This may help to prevent preterm premature rupture of membranes, which is a frequent cause of preterm birth. However, application of progesterone has the US Food and Drug Administration (FDA) approval in preterm labor. In liver, progesterone is metabolized through reduction to pregnanetriol, pregnanediol, and pregnanolone, and it was then excreted through the urine. It is noted that function of progesterone receptors are important for development of pregnancy, while the serum level of progesterone less than 45 nmol/l is considered as useful tool to predict nonviable pregnancy (24).

Moreover, a systematic review and meta-analysis of randomized controlled trials showed that there is a significant reduction in risk of preterm delivery when using progesterational agents (25). Additionally, several studies revealed that administration of progesterone in women with threatened abortion increases the fetus survival rate (26-28).

Threatened miscarriage is known as a risk factor for preterm labor. In a number of studies such as Hassan et al. (29) and Meis et al. (30) in which they applied weekly injections of 250 mg of 17P starting from weeks 16 to 36 (delivery), and Foneseca et al. (31) in which they applied vaginal progesterone (200 mg/each night) from weeks 24 to 34, a long-term progesterone therapy is considered as a preventive therapy for preterm labor. In this study, spontaneous delivery before 34 weeks of gestation was less common in the progesterone group than in the placebo group [19.2 vs. 34.4%, relative risk (RR): 0.56], indicating that treatment with progesterone prevents spontaneous preterm birth in women with a short cervix.

Also, in a multicenter randomized controlled trial conducted by Grobman et al. (32), they revealed that there is no significant difference regarding the frequency of preterm birth between the 17-alpha-hydroxyprogesterone caproate (225.1%) and placebo groups (24.2%).

Progesterone is usually given as injection and vaginal suppository forms that are based on a patient’s choice. Therefore, this study aimed to compare the vaginal progesterone (Cyclogest) versus injection of 17-alpha-hydroxyprogesterone caproate (Proluton) in preventing preterm labor in pregnant women with threatened abortion at less than 34 weeks’ gestational age.

Materials and Methods

Trial design

This study was a balanced randomized, double-blind, single-center controlled clinical trial. Eligible patients were recruited from the women with threatened abortion referred to the Department of Gynecology and Obstetrics, Arash Hospital, Tehran, Iran, from January 2014 to March 2015. The study was approved by Institutional Review Board for Human Research of Tehran University of Medical Sciences (Ethical Committee code: 93/d/130/556). Patients were informed about the aim of the study as well as benefits and harms of treatments before being randomly allocated into two groups. An informed consent was obtained from all patients. The trial was registered on the Iranian website for registration.

Eligibility criteria

The inclusion criteria were as followings: i. Genital abnormalities, ii. Confirmed singleton pregnancy, iii. Between 6 and 20 weeks gestational age at enrollment time, and iv. Threatened abortion (vaginal bleeding or any bloody vaginal discharge during the first half of pregnancy. Exclusion criteria were as followings: i. Uncontrolled medical diseases (hypertension, diabetes, cardiovascular, renal or hepatic disease), ii. History of drugs or alcohol abuse, iii. Lack of pregnancy sac at week 5, iv. Lack of yolk sac at weeks 5.5-6, v. Lack of fetus at weeks 6-6.5, vi. Lack of fetal heart rate at 16-24 weeks of gestational age, vii. Cervical length of less than 25 mm that was measured by transvaginal ultrasound at 16-24 weeks, viii. Fetal abnor-
Sample size

Based on an expected occurrence of the primary endpoint (preterm labor) of 6% in the Proluton group and 20% in Cyclogest group, we estimated that a minimum sample size of 91 patients in each treatment group is necessary to give 80% power to detect a significant difference between two groups (with a two-sided type I error of 5%). Our hypothesis was that Proluton would decrease the preterm labor rate in patients compared to Cyclogest. Therefore, 190 patients were randomly assigned into two groups [Cyclogest (n=95) and Proluton; n=95], and five patients were then excluded from the analyses because they failed to meet inclusion criteria. Finally, 185 patients were included in the analysis [Cyclogest group (n=93) and Proluton group (n=92)].

Randomization

Patients (n=190) were randomly assigned into two groups, Cyclogest (n=95) and 17-alpha-hydroxyprogesterone caproate (Proluton, n=95) groups, using a computer-generated program that was provided by a statistician, who was independent of the clinical study team. To avoid imbalance of patients in two groups during study, randomization was performed in blocks of six (three received either Cyclogest or Proluton).

To ensure allocation concealment, an independent subject prepared the randomization list and the sequence was protected in a sealed envelope. Whenever a patient was found qualified, the numbered envelope was opened to determine the intervention technique. The outcome assessors, caregiver and data analysts were blinded to the assigned treatment throughout the study.

Interventions

The Proluton group (n=92) underwent a weekly administration of 17-alpha-hydroxyprogesterone caproate, 250 mg/intramuscular injection/weekly. The Cyclogest group (n=93) was treated daily with vaginal progesterone suppository at a dosage of 400 mg (in the analysis, vaginal progesterone was considered as baseline group). They were applied every night to the end of week 34 of gestation. Patients were trained to use the suppositories. The medications were supplied by the pharmacy of Arash Hospital and administrated by the ward nurse who was blinded to the study. The patients were registered and followed up in the hospital using an electronic record of the prenatal clinic. Data gathering was done by two educated residents.

Outcome measures

At study entry, a trained physician performed standardized interviews that included information about demographic and clinical data. In addition, maternal weight and height were assessed and a routine laboratory work-up was carried out. The primary outcome measure was the preterm labor. Preterm refers to a birth that occurs at less than 34 weeks of gestation. Gestational age was calculated on the basis of the last menstrual period and the results of ultrasound scan, indicating the crown-rump length and the gestational sac dimension. Postoperative side effects related to two medications were recorded.

Statistical analysis

Categorical and continuous variables were summarized as proportions and mean ± SD, respectively. The Pearson chi-square and Student’s t test were used to compare variables at baseline between two groups based on type of variables. Results are presented as RR, 95% confidence interval (CI). The association was considered significant when the P value was <0.05 or when the 95% CI for RR did not include 1.0 (equivalent to P<0.05). Data analysis was undertaken using the SPSS (SPSS Inc., USA) version 13.0.

Results

A total of 223 women were interviewed, of which thirty-three women did not meet the inclusion criteria and 190 were eligible to participate in this study, so they were randomly assigned into Proluton (n=95) and Cyclogest (n=95) groups. Five women were then excluded from the analyses because they failed to complete the study, so 185 participants completed the study. The statistical analysis was also performed according to an intention to treat approach.
The distribution of demographic data and pregnancy history variables are reported in Table 1, indicating that no significant differences were found between the two groups of patients on the basis of age, body mass index (BMI), gestational age at enrollment, cervical length in the third trimester, history of abortion, history of ectopic pregnancy and parity.

Table 1: Baseline demographics and clinical characteristics of participants randomly allocated

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cyclogest group</th>
<th>Proluton group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (Y)</strong></td>
<td>28.37 (5.21)</td>
<td>28.56 (5.31)</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>25.59 (4.83)</td>
<td>24.68 (4.05)</td>
<td>0.17</td>
</tr>
<tr>
<td>Marital duration</td>
<td>5.98 (4.98)</td>
<td>4.85 (0.73)</td>
<td>0.07</td>
</tr>
<tr>
<td>Gestational age at enrollment</td>
<td>11.15 (5.35)</td>
<td>10.96 (6.02)</td>
<td>0.82</td>
</tr>
<tr>
<td>Cervical length in the second trimester</td>
<td>28.03 (5.34)</td>
<td>28.3 (6.73)</td>
<td>0.79</td>
</tr>
<tr>
<td>Parity</td>
<td>0.15 (0.44)</td>
<td>0.2 (0.71)</td>
<td>0.5</td>
</tr>
<tr>
<td>History of ectopic pregnancy</td>
<td>4 (4.34)</td>
<td>2 (2.15)</td>
<td></td>
</tr>
<tr>
<td><strong>Yes</strong></td>
<td>88 (95.66)</td>
<td>91 (97.85)</td>
<td>0.39</td>
</tr>
<tr>
<td><strong>No</strong></td>
<td>24 (26.08)</td>
<td>20 (21.5)</td>
<td>0.46</td>
</tr>
<tr>
<td>History of abortion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Yes</strong></td>
<td>68 (73.92)</td>
<td>73 (78.5)</td>
<td></td>
</tr>
</tbody>
</table>

BMI: Body mass index, *; Data are presented as mean (SD) for continuous variables, and #; Number (percent) for categorical variables.

As shown in Table 2, the number of preterm labor less than 28 completed weeks in women treated with Proluton was 6 out of 93 (6.45%), whereas in the Cyclogest group, there were 4 out of 92 (4.35%). The number of preterm labor less than 34 completed weeks in Proluton group was 8 out of 93 (8.60%), whereas there were 6 out of 92 (6.52%) in the Cyclogest group, and also preterm labor less than 37 completed weeks was observed in 16.30% of Cyclogest group and 15.05% of Proluton group. There were no significant differences regarding risk of preterm labor less than 28 completed weeks (RR: 1.48, 95% CI: 0.43-5.08, P=0.52), preterm labor less than 34 completed weeks (RR: 1.31, 95% CI: 0.47-3.65, P=0.59), and preterm labor less than 37 completed weeks (RR: 0.92, 95% CI: 0.47-1.80, P=0.81) between two groups.

No significant differences were found for termination of pregnancy in Cyclogest (mean=36.82, SD=4.9) and Proluton groups (mean=36.56, SD=5.4) (mean difference: 0.26 month, 95% CI: -1.2-1.7, P=0.73). In terms of side effects of treatment and pregnancy complications, in group treated with Cyclogest, eclampsia was observed only in two cases, intrauterine growth restriction (IUGR) in two cases, and membrane rupture in one case, whereas in the group treated with Proluton, no adverse effects were reported.

Table 2: Relative risk (RR) and 95% confidence interval (CI) of preterm labor in two groups

<table>
<thead>
<tr>
<th>Preterm labor</th>
<th>Proluton group</th>
<th>Proluton group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 28 weeks</td>
<td>0.043 (4)</td>
<td>0.064 (6)</td>
<td>1.48 (0.43-5.08)</td>
</tr>
<tr>
<td>Less than 34 weeks</td>
<td>0.065 (6)</td>
<td>0.086 (8)</td>
<td>1.31 (0.47-3.65)</td>
</tr>
<tr>
<td>Less than 37 weeks</td>
<td>0.163 (15)</td>
<td>0.150 (14)</td>
<td>0.92 (0.47-1.80)</td>
</tr>
</tbody>
</table>

Table 2: Relative risk (RR) and 95% confidence interval (CI) of preterm labor in two groups

Discussion

The present study compared the risk of preterm labor between vaginal progesterone (Cyclogest) and 17-alphahydroxy-progesterone caproate (Proluton). The results of this study indicated that there is no statistically and clinically significant difference in risk of preterm birth (less than 34 weeks) between Cyclogest and Proluton groups. Also, there was no significant difference in risk of preterm labor less than 28 completed weeks and preterm labor less than 37 completed weeks between two groups.

Threatened abortion occurred in one of five pregnancies and 10% of women with threatened abortion will experience abortion (16). Progesterone decreases preterm labor and abortion rates via suppressing luteinize hormone in luteal phase and improving implantation and regulation of immune responses, while progesterone decreases the prostaglandins synthesis and inhibits inappropriate contractions of uterus.

In this study, the risks of preterm labor less than 34 weeks in women with and without histo-
ry of abortion were 0.068 and 0.078, respectively, and there is no significant difference between two groups. Tita and Rouse (33) in a review study showed that the 17-alpha-hydroxyprogesterone effectively reduces the incidence of recurrent preterm labor in women with a history of spontaneous preterm labor. Also in a study by Meis et al. (30), they declared that weekly injections of 17-alpha-hydroxyprogesterone led to considerable reduction in the recurrent preterm delivery rate among high risk women for preterm delivery.

In the previous study, women were enrolled at 19 clinical centers at 16 to 20 weeks of gestation and randomly assigned to receive weekly injections of 250 mg of 17P that injections were continued until delivery or to 36 weeks of gestation (a long time progesterone therapy) (32). Also in the study by Fonseca et al. (31), spontaneous delivery before 34 weeks of gestation was less frequent in the progesterone group receiving vaginal progesterone (200 mg each night) from 24 to 34 weeks of gestation. However, the results of previous studies are inconsistent with each other. Some studies stated that 17-alpha-hydroxyprogesterone had no additional advantage for prevention of preterm labor in women with prior spontaneous preterm labor and did not improve neonatal and maternal outcomes (34).

Negligible adverse effects are related to the route of administration, and include injection site reactions and vaginal discharge. The only important side effect that reported in some studies is a threefold increase in risk of developing gestational diabetes (35), but this finding was not confirmed in large data study (36). The main concern about synthetic progesterone is binding to androgen receptors, but 17-alpha-hydroxyprogesterone as a natural progesterone metabolite is produced by the corpus luteum and placenta with minimal to no androgenic activity. One concern is a probable increase in risk of hypospadias in male offspring exposed to exogenous progestins (37). The benefit of vaginal progesterone is its high uterine bioavailability since uterine exposure occurs earlier than pass through the liver.

In a systematic review and meta-analysis by Mackenzie et al. (25), they revealed that use of prostational agents, initiated in the second trimester of pregnancy, for women at risk of preterm labor possibly decreases the rate of preterm labor, but the effect of prostational agents on neonatal outcome is doubtful. In contrast, a study by Chawanpaiboon et al. (38) conducted on 150 pregnant women with threatened preterm labor between 28 and 35 weeks of gestation revealed progesterone is not the most effective agent as compared to other agents (Nifedipine) and also bed rest.

In this study, in group treated with Cyclogest, eclampsia was observed only in two cases, IUGR in two cases, and membrane rupture in one case, whereas in the group treated with Proluton, no adverse effects were reported, indicating that complications arising as side effects are not attributable to progesterone agents. The observed effects in this group can be caused by other factors.

As regards, there was no difference between two groups in term of preterm labor risk reduction, whereas vaginal progesterone (Cyclogest) is safe for mother and fetus, not painful, cost effective and more compliance, suggesting that Cyclogest is highly recommended as compared with Proluton.

Due to rarity of preterm labor, further studies with more sample size or multicenter design are, therefore, suggested in order to have an appropriate statistical power.

One of the limitations of this study was the lack of a control group which was not possible for ethical reasons. Also some other unmeasured confounder variables like stress, anxiety, family history of abortion and preterm labor may affect the results. In this study, the interested outcome was an objective outcome, while the lack of patients blinding to the treatment results in less complication for subjective outcomes.

**Conclusion**

Based on the findings, there was no significant difference in the risk of preterm labor between the vaginal progesterone (Cyclogest) and 17-alpha-hydroxy progesterone caproate (Proluton).

**Acknowledgements**

We cordially appreciate the nursing, as well as administrative and secretarial staff of the Department of Obstetrics and Gynecology and the Clinic at Arash Hospital and also Tehran University of

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References

Use of Follicular Output Rate to Predict Intracytoplasmic Sperm Injection Outcome

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2. Department of Obstetrics and Gynecology, United Medical and Dental College, Karachi, Pakistan
3. Department of Biochemistry, Faculty of Medicine, Rabigh, King Abdul Aziz University, Jeddah, Saudi Arabia
4. House Officer Civil Hospital, Karachi, Pakistan
5. House Officer, Services Hospital, Lahore, Pakistan

Abstract

**Background:** The measurement of follicular output rate (FORT) has been proposed as a good indicator for evaluating follicular response to the exogenous recombinant follicle-stimulating hormone (rFSH). This places FORT as a promising qualitative marker for ovarian function. The objective of the study was to determine FORT as a predictor of oocyte competence, embryo quality and clinical pregnancy after intracytoplasmic sperm injection (ICSI).

**Materials and Methods:** This prospective study was carried out on a group of infertile females (n=282) at Islamabad Clinic Serving Infertile Couples, Islamabad, Pakistan, from June 2010 till August 2013. Down-regulated females were stimulated in injection gonadotropins and on ovulation induction day, pre-ovulatory follicle count (PFC) was determined using transvaginal ultrasound scan (TVUS), and FORT was determined as a ratio of PFC to antral follicle count (AFC)×100. Group I consisted of females with a negative pregnancy test, while group II had a positive pregnancy test that was confirmed with the appearance of fetal cardiac activity. Linear regression analyses of categorical variables of clinical pregnancy along with other independent variables, including FORT, were performed using SPSS version 15.0.

**Results:** Pregnancy occurred in 101/282 women who were tested, recording a clinical pregnancy rate of about 35.8%. FORT values were higher in group II as compared to group I females (P=0.0001). In multiple regression analysis, 97.7, 87.1, 78.2, and 83.4% variations were explained based on the number of retrieved oocytes per patients, number of metaphase II oocytes retrieved, number of fertilized oocytes, and number of cleaved embryos, respectively, indicating FORT as an independent predictor.

**Conclusion:** FORT is a predictor of oocyte competence in terms of a number of retrieved, mature and fertilized oocytes. It also gives information about the number of cleaved embryos and clinical pregnancy rate.

**Keywords:** Intracytoplasmic Sperm Injection, Assisted Reproductive Techniques, Infertility, Ovarian Follicles, Follicular Output Rate

include in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) for the treatment of infertile couples (2, 3).

In ICSI, after the down-regulation of ovaries, controlled ovarian hyperstimulation (COH) is followed by ovulation induction (OI) and oocyte pickup, after which a single sperm is introduced in the ooplasm, placed under a microscope. The procedure has shown a greater success rate as compared to IVF. Ovarian stimulation is a key procedure necessary to achieve success in assisted reproductive techniques. Stimulation is achieved by the administration of exogenous gonadotropins in the form of recombinant follicle stimulating hormone (rFSH) in order to increase the follicular recruitment and oocyte yield. Confirmation of responsiveness of ovarian reservoir to FSH in terms of the development of the antral follicles is so far a challenge for reproductive endocrinologists at clinics (1, 4). The appropriate response of antral follicles to FSH and a high-quality oocyte may result in a positive impact on outcomes of IVF and ICSI. Poor response to COH results in retrieval of few oocytes with reduced number of embryos available for transfer, leading to a decrease in pregnancy rates (2).

The endocrinologists, thus, devised various methods to assess the ovarian reservoir and the expected responsiveness. Antral follicle count (AFC) is one of the non-invasive methods used for the assessment of the sensitivity of antral follicles to FSH (5). AFC represents the number of remaining primordial pool which corresponds to the number of oocytes retrieved; however, it does not influence the number of oocytes, embryo quality and the outcome of ICSI (6). The number of pre-ovulatory follicle count (PFC) obtained at the end of COH is estimated to be the best indicator of the number of retrieved oocytes (2, 3, 7).

However, it also includes the number of small antral follicles available before treatment (8, 9). It means that follicular output rate (FORT) determines follicular response to exogenous rFSH by the ratio of pre-ovulatory follicles to the existing pool of small antral follicles. The index has been investigated as an indicator of existing ovarian reservoir in response to stimulation and oocyte competence by a number of researchers (1, 4, 5). In addition to assessing ovarian function, we designed this study in order to identify the value of FORT for the prediction of oocyte competence, embryo quality and the likelihood of clinical pregnancy after ICSI.

**Materials and Methods**

This prospective study was carried out at Islamabad Clinic Serving Infertile Couples Islamabad, Pakistan, between June 2010 and August 2013 after the ethical approval was obtained from the Ethical Review Board of Islamabad Clinic Serving Infertile Couples, Islamabad, Pakistan. The study was conducted in accordance with the principles of the Declaration of Helsinki, and an informed written consent was taken from all study participants. The women (n=282) undergoing ICSI were included in this study with the following criteria: infertility duration of more than 2 years, age range of 21-40 years, regular menstrual cycle, normal morphological ovaries, body mass index (BMI) of 18-35 kg/m², and serum FSH levels lower than 8 IU/ml. The exclusion criteria were as follows: polycystic ovary syndrome (PCOS), uterine fibroids, down-regulation with short gonadotropin-releasing hormone (GnRH)-agonist or -antagonist protocol, sperm retrieval by testicular biopsy, and use of IVF cycles.

**Treatment protocol**

The treatment protocol of females was carried out as mentioned by Rehman et al. (3). Total number of AFC with a mean diameter of 3-8 mm was determined using a transvaginal ultrasound scan (TVUS) with a 7.5 MHz probe (Aloka Co., Japan). COH was carried out using subcutaneous administration of 50 IU/day rFSH (Puregon, NV Organon, The Netherlands). The initiation dose was calculated based on the of the age of the subject, basal serum FSH concentrations, AFC and BMI (6). The number of pre-ovulatory follicle count (PFC) obtained at the end of COH is estimated to be the best indicator of the number of retrieved oocytes (2, 3, 7).
probe, after which luteal support was provided with the progesterone vaginal pessaries (Cyclogest 400 mg, Actavis Co., UK) twice a day. Transfer of blastocysts was performed seven days after hCG injection using Edwards-Wallace embryo replacement catheter (SIMS Portex Ltd., UK) under ultrasound guidance. Two blood samples were taken on days of hCG injection and blastocyst transfer for estimation of peak and mid-luteal levels of estradiol (E₂) and progesterone using enzyme linked immuno sorbent assay (ELISA). Serum E₂ and progesterone as well as ELISA kits were provided by MP Biomedicals, USA and BioSource, Belgium, respectively.

**Outcome parameters**

The marker for detection of pregnancy outcome was beta-hCG (β-hCG) levels that were measured 14 days after egg collection. Clinical pregnancy was identified by the appearance of a gestational sac with cardiac activity using TVUS. Therefore, the participants were divided into the following groups: group I (n=181) including non-pregnant women with β-hCG<25 mIU/ml, and group II (n=101) including pregnant women with β-hCG>25 mIU/ml and the presence of cardiac activity confirmed by TVUS. Pregnancy rate was calculated by the presence of an intrauterine gestational sac observed by TVUS per number of patients in the cycle (5, 7).

**Statistical analysis**

Data was analyzed by Statistical Package for Social Science software (SPSS, SPSS Inc., USA) version 15.0. The stepwise backward method was used to estimate the best model using FORT as a predictor that was adjusted with other clinical risk factors affecting clinical pregnancy outcome, including female age, BMI, FSH, duration of infertility, the length of stimulation, AFC, and PFC. A variable is entered into the model if the significance level of its F value is less than 0.05 and removed if the significance level is greater than 0.10. Four different models were developed showing that FORT gave significant effect on the number of oocytes/patient, the number of metaphase II oocytes retrieved, the number of cleaved embryos and number of fertilized oocytes. In regression analysis, R-squared (R², coefficient of determination) described variation in dependent variable with respect to independent variable(s), while Spearman’s Rank Correlation was used to investigate the correlation of clinical pregnancy with different parameters. A P value less than 0.05 was considered to be statistically significant.

**Results**

Baseline characteristics of study participants are given in Table 1. Pregnancy occurred in 101/282 women who were tested, recording a clinical pregnancy rate of about 35.8%. Table 2 explains the significant difference regarding FORT values in group I (49.31 ± 12.83) and II (64.17 ± 8.96).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age (Y)</td>
<td>32.18</td>
<td>4.72</td>
</tr>
<tr>
<td>Duration of infertility (Y)</td>
<td>7.46</td>
<td>4.01</td>
</tr>
<tr>
<td>Length of stimulation (days)</td>
<td>14.16</td>
<td>0.96</td>
</tr>
<tr>
<td>AFC</td>
<td>13.07</td>
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<tr>
<td>PFC</td>
<td>8.31</td>
<td>1.71</td>
</tr>
<tr>
<td>FORT</td>
<td>64.09</td>
<td>8.97</td>
</tr>
<tr>
<td>No. of oocytes/patient</td>
<td>8.21</td>
<td>1.53</td>
</tr>
<tr>
<td>No. of metaphase II oocytes retrieved</td>
<td>8.03</td>
<td>1.45</td>
</tr>
<tr>
<td>No. of fertilized oocytes</td>
<td>6.65</td>
<td>1.07</td>
</tr>
<tr>
<td>No. of cleaved embryos</td>
<td>6.56</td>
<td>1.04</td>
</tr>
<tr>
<td>Peak progesterone (ng/ml)</td>
<td>0.85</td>
<td>0.45</td>
</tr>
<tr>
<td>Mid-luteal progesterone (ng/ml)</td>
<td>89.17</td>
<td>32.84</td>
</tr>
<tr>
<td>Peak E₂ (pg/ml)</td>
<td>2529.06</td>
<td>193.99</td>
</tr>
<tr>
<td>Mid-luteal E₂ (pg/ml)</td>
<td>1109.46</td>
<td>136.29</td>
</tr>
<tr>
<td>Endometrial thickness (mm)</td>
<td>1.74</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD. AFC; Antral follicle count, PFC; Pre-ovulatory follicle count, FORT; Follicular output rate, and E₂; Estradiol.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-pregnant participants n=181</th>
<th>Pregnant participants n=101</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age (Y)</td>
<td>32.11 ± 4.61</td>
<td>32.107 ± 4.74</td>
<td>0.952</td>
</tr>
<tr>
<td>Duration of infertility (Y)</td>
<td>6.94 ± 3.78</td>
<td>7.40 ± 4.02</td>
<td>0.409</td>
</tr>
<tr>
<td>Length of stimulation (days)</td>
<td>14.33 ± 0.96</td>
<td>14.15 ± 0.95</td>
<td>-0.454</td>
</tr>
<tr>
<td>Dose of rFSH/day</td>
<td>3.89 ± 1.43</td>
<td>4.29 ± 2.96</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 1: Baseline characteristics of the infertile subjects

Table 2: Comparison of the parameters between group I (non-pregnant participants) and group II (pregnant participants)
Table 2: Continued

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Non-pregnant participants n=181</th>
<th>Pregnant participants n=101</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFC</td>
<td>15.57 ± 2.51</td>
<td>13.04 ± 2.55</td>
<td>0.559</td>
</tr>
<tr>
<td>PFC</td>
<td>7.52 ± 1.90</td>
<td>8.30 ± 1.69</td>
<td>0.364</td>
</tr>
<tr>
<td>FORT</td>
<td>49.31 ± 12.83</td>
<td>64.17 ± 8.96</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>No. of cleaved embryos</td>
<td>5.45 ± 1.53</td>
<td>6.56 ± 1.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Endometrial thickness (mm)</td>
<td>1.26 ± 0.44</td>
<td>1.74 ± 0.43</td>
<td>0.819</td>
</tr>
<tr>
<td>No. of oocytes/patient</td>
<td>7.40 ± 1.68</td>
<td>8.20 ± 1.51</td>
<td>0.296</td>
</tr>
<tr>
<td>No. of mature oocytes/patient</td>
<td>6.61 ± 2.03</td>
<td>8.02 ± 1.43</td>
<td>0.004</td>
</tr>
<tr>
<td>No. of fertilized oocytes</td>
<td>5.54 ± 1.64</td>
<td>6.65 ± 1.06</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Peak E₂ (pg/ml)</td>
<td>2207.97 ± 283.73</td>
<td>2529.06 ± 193.35</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mid-luteal E₂ (pg/ml)</td>
<td>899.49 ± 122.05</td>
<td>1109.46 ± 135.83</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Peak progesterone (ng/ml)</td>
<td>1.96 ± 0.67</td>
<td>1.12 ± 0.59</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Mid-luteal progesterone ng/ml</td>
<td>192.44 ± 20.34</td>
<td>116.51 ± 48.60</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are presented as mean ± SD. rFSH; Recombinant follicle stimulating hormone, AFC; Antral follicle count, PFC; Pre ovulatory follicle count, FORT; Follicular output rate, and E₂; Estradiol.

It indicates that the length of stimulation, AFC (r=-0.412, P<0.01), as well as peak (r=-0.640, P<0.01) and mid-luteal progesterone levels (r=-0.802, P<0.01) showed a significant negative association with clinical pregnancy outcome. However, FORT (r=0.532, P<0.001), PFC (r=0.265, P<0.01), number of cleaved embryos (r=0.365, P<0.01), peak (r=0.640, P<0.01) and mid-luteal (r=0.679, P<0.01), E₂ (r=0.640, P<0.01) levels, as well as endometrial thickness (r=0.412, P<0.01) showed a significant positive correlation with clinical pregnancy outcome (Table 3). Linear regression analysis of FORT as an independent predictor described variations of 97.7, 87.1, 78.2 and 83.4% for the number of retrieved oocytes per patients, number of metaphase II oocytes retrieved, the number of fertilized oocytes, and the number of cleaved embryos, respectively (Table 4). Furthermore, Table 4 shows an increase in FORT value by one unit increased the mean number of oocytes/patients (β coefficient: 0.135), while the model was adjusted for length of stimulation and AFC with an adjusted R² value of 97.7%. The model describes that an increase in FORT value by one unit increased the mean number of metaphase II oocytes retrieved (β coefficient: 0.128), while the model was adjusted for FSH and AFC with an adjusted R² value of 87.1%. The model also describes that an increase in FORT value by one unit will give a positive impact on the mean number of oocytes fertilized (β coefficient: 0.089), while the model was adjusted for FSH and AFC with an adjusted R² value of 78.2 %. However, the model suggested that one unit increase in FORT value decreased the mean number of cleaved embryos (β coefficient: -0.025), while it was adjusted for AFC and number of metaphase II oocytes retrieved (Table 4).

Table 3: Comparison of the parameters for clinical pregnancy outcome using Spearman rank correlation

<table>
<thead>
<tr>
<th>Predictors</th>
<th>Clinical pregnancy r(P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age (Y)</td>
<td>0.001(0.981)</td>
</tr>
<tr>
<td>Duration of infertility (Y)</td>
<td>0.052(0.380)</td>
</tr>
<tr>
<td>Length of stimulation (days)</td>
<td>-0.140(&lt;0.01)</td>
</tr>
<tr>
<td>AFC</td>
<td>-0.412(&lt;0.01)</td>
</tr>
<tr>
<td>PFC</td>
<td>0.265(&lt;0.01)</td>
</tr>
<tr>
<td>FORT</td>
<td>0.532(&lt;0.01)</td>
</tr>
<tr>
<td>No. of cleaved embryos</td>
<td>0.365(&lt;0.01)</td>
</tr>
<tr>
<td>Peak progesterone (ng/ml)</td>
<td>-0.652(&lt;0.01)</td>
</tr>
<tr>
<td>Mid luteal progesterone (ng/ml)</td>
<td>-0.802(&lt;0.01)</td>
</tr>
<tr>
<td>Peak E₂ (pg/ml)</td>
<td>0.640(&lt;0.01)</td>
</tr>
<tr>
<td>Mid luteal E₂ (pg/ml)</td>
<td>0.679(&lt;0.01)</td>
</tr>
<tr>
<td>Endometrial thickness</td>
<td>0.628(&lt;0.01)</td>
</tr>
</tbody>
</table>

AFC; Antral follicle count, PFC; Pre-ovulatory follicle count, FORT; Follicular output rate, E₂; Estradiol. *; Correlation with P<0.05 considered as significant, and r; Spearman rank correlation.

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Discussion

The successful outcome in assisted reproductive clinics depends on embryos and endometrial qualities. The quality of embryos depends on number and quality of oocytes, which in turn is considered as a measure of antral follicles responsiveness to FSH; however, no pointer can predict both ovarian response and oocyte competence. FORT, in this regard, can be used as an innovative marker to predict ovarian response and oocyte competence, leading to increase the chances of clinical pregnancy (8).

AFC as a non-invasive method of assessment plays a key role in the prediction of ovarian response and provides a valuable insight into the sensitivity of antral follicles to FSH (5). In this study, AFC showed significant negative association with the pregnancy outcome. Similar to our findings, a study reported that AFC represented the actual ovarian reserve and were highly correlated with the number of oocytes retrieved (6). Furthermore, other studies concluded that AFC cannot predict the oocyte/embryo quality or IVF/ICSI outcome and showed better FORT results in subjects with decreased AFC (4, 11). A study declared that AFC obtained at the end of stimulation is the best indicator of the number of retrieved oocytes and good clinical outcome (7). However, a contrasting observation was noted in another study where pre-ovulatory follicles were not observed as an appropriate reflector of antral follicle sensitivity to FSH (12). The conflict can thus be resolved by the use of FORT index, which can reflect ovarian follicular competence in response to stimulation (4, 5).

High FORT showed a higher rate of clinical pregnancies that can be explained by increased retrieval, maturity and fertilization of oocytes, which potentially lead to an increase in clinical pregnancy rate (10, 13, 14). Enhanced mid-luteal E$_2$ concentration in females with high FORT increases the chance of pregnancy, which is also supported by other studies (3-8, 15).

The exact role of progesterone in determining the success of IVF/ICSI is a subject of debate. Our results showed that females with high FORT had low peak progesterone levels, which is similar to another study in which pregnancy rate was inversely related to serum progesterone levels (16). However, in contrast to these results, a study proved that progesterone enhances uterine receptivity by the maturation of mast cells that produce cytokines and growth factors (17, 18). Hofmann et al. (19) observed no significant difference in pregnancy rate in patients undergoing IVF/embryo transfer with high or low progesterone concentrations on the day of hCG administration and in patients receiving oocytes donated from women with high or low progesterone concentrations. The role of FORT in the prediction of clinical pregnancy can thus be emphasized on the basis of its effect on hormones of implantation.

It seems that FORT is a qualitative indicator of ovarian follicles competence and has a significant correlation with the clinical pregnancy outcome. Our results showed that it has positive effects on a number of oocytes/patient, the number of metaphase II oocytes retrieved, the number of cleaved embryos and number of fertilized oocytes. Similar to other two studies that reflected the rates of good quality embryos and embryo implantations, clinical pregnancies increased dramatically in accordance with FORT values (4, 20). Besides the importance of the impact of FORT on clinical pregnancy, it was a unicentric study in which different sonologists determined AFC and PFC which must be standard-

### Table 4: Regression models with FORT as an independent predictor

<table>
<thead>
<tr>
<th>Regression models</th>
<th>Independent predictor</th>
<th>Beta coefficient</th>
<th>SE</th>
<th>Adjusted $R^2$</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of oocytes/patients$^a$</td>
<td>FORT</td>
<td>0.135</td>
<td>± 0.003</td>
<td>97.7%</td>
<td>&lt;0.01$^*$</td>
</tr>
<tr>
<td>No. of metaphase II oocytes retrieved$^b$</td>
<td>FORT</td>
<td>0.128</td>
<td>± 0.006</td>
<td>87.1%</td>
<td>&lt;0.01$^*$</td>
</tr>
<tr>
<td>No. of fertilized oocytes$^c$</td>
<td>FORT</td>
<td>0.089</td>
<td>± 0.006</td>
<td>78.2%</td>
<td>&lt;0.01$^*$</td>
</tr>
<tr>
<td>No. of cleaved embryos$^d$</td>
<td>FORT</td>
<td>-0.025</td>
<td>± 0.01</td>
<td>83.4%</td>
<td>0.019</td>
</tr>
</tbody>
</table>

FORT: Follicular output rate, $R^2$: Coefficient of determination, AFC: Antral follicle count, FSH: Follicle stimulating hormone. $^a$: Adjusted with length of stimulation and AFC; $^b$: Adjusted with FSH and AFC; $^c$: Adjusted with AFC and number of metaphase II oocytes retrieved and $^d$: $P<0.05$ was considered as significant.
ized for uniformity. The variation in marking AFC and PFC by different sonographers is unlikely to be ruled out.

Conclusion

FORT is a predictor of oocyte competence in terms of a number of retrieved, mature and fertilized oocytes. The indicator can be used for prediction of clinical pregnancy rate after ICSI. It also gives information about the number of cleaved embryos and clinical pregnancy rate which needs to be further explored.

Acknowledgements

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References

The Role of Residential Early Parenting Services in Increasing Parenting Confidence in Mothers with A History of Infertility

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Abstract

Background: Mothers with a history of infertility may experience parenting difficulties and challenges. This study was conducted to investigate the role of residential early parenting services in increasing parenting confidence in mothers with a history of infertility.

Materials and Methods: This was a retrospective chart review study using the quantitative data from the clients attending the Karitane Residential Units and Parenting Services (known as Karitane RUs) during 2013. Parenting confidence (using Karitane Parenting Confidence Scale-KPCS), depression, demographics, reproductive and medical history, as well as child’s information were assessed from a sample of 27 mothers who had a history of infertility and who attended the Karitane RUs for support and assistance. The data were analyzed using SPSS version 19.

Results: More than half of the women (59.3%) reported a relatively low level of parenting confidence on the day of admission. The rate of low parenting confidence, however, dropped to 22.2% after receiving 4-5 days support and training in the Karitane RUs. The mean score of the KPCS increased from 36.9 ± 5.6 before the intervention to 41.1 ± 3.4 after the intervention, indicating an improvement in the parenting confidence of the mothers after attending the Karitane RUs (P<0.0001). No statistically significant association was found between maternal low parenting confidence with parental demographics (including age, country of birth, and employment status), a history of help-seeking, symptoms of depression, as well as child’s information [including gender, age, siblings, diagnosis of gastroesophageal reflux disease (GORD) and use of medication].

Conclusion: Having a child after a period of infertility can be a stressful experience for some mothers. This can result in low parenting confidence and affect parent-child attachment. Our findings emphasized on the role of the residential early parenting services in promoting the level of parenting confidence and highlighted the need for early recognition and referral of the mothers with a history of infertility to such centers.

Keywords: Infertility, Postnatal, Parenting Confidence, Residential Units, Parenting Services

Introduction

With the improvement of assisted reproduction technologies (ART) and public awareness, there is an increasing rate of children born to infertile parents (1). Having a baby after short- or long-term infertility can be joyful to all parents. This form of transition to parenthood may be most likely to be considered as a challenging experience for some parents, especially for mothers during early parenthood (2). Some women with a history of infertility experience less severe symptoms of postnatal mental health problems and easily adjust to the
changes, which is likely due to their socioeconomic advantages and supportive partners (3). Nevertheless, different studies have reported higher levels of postnatal depression in infertile women as they underwent infertility treatment, experienced obstetric complications associated with ART pregnancy and had idealistic expectations about their parenting role (3-5). As compared with women without a history of infertility, another study has shown that infertile women conceiving through ART experienced greater level of psychological disorders such as distress, over-protectiveness, fatigue, anxiety, depression, as well as inability to develop parenting skills (6). Furthermore, feelings of guilt or shame may affect the mother’s mental health, relationship with the partner, parenting skills, parent-child relationship and child’s development (3, 5).

Different studies have also shown the association of early parenting difficulties with negative birth experiences, parental physical and mental health problems, sleep deprivation, distress, mental and physical disabilities of the child, insufficient family-child interactions, inadequate support from partner, family and friends (7, 8), intimate partner violence, as well as divorce (9, 10). Another research has revealed that 20-30% of mothers referred to the parenting centers met the diagnostic criteria for major depression, anxiety disorder and severe fatigue. These negative experiences have been reported to affect mental, emotional, physical and social development of the child (11). It is becoming increasingly evident that the infant’s brain development is shaped by the positive and negative experiences of early parenting (12). New evidence suggests that the infant’s unsettled behaviour is associated with the maternal characteristics and psychosocial environment (13). These indicate the importance of early intervention in cases when the parenting experiences are less favourable (12).

**Karitane Residential Units and Parenting Services**

In order to facilitate a smooth transition to parenthood and minimize its undesirable effects on the parents’ well-being and child’s development, the Karitane Residential Units and Parenting Services (known as Karitane RUs) have been established in New South Wales (NSW), Australia, under the name of the Australian Mother Craft Society in 1923. These centers aim to provide sufficient parenting support to families struggling with the demands of parenting, help them promote their knowledge and skills, as well as enhance their confidence in raising their children. The families (parents and their children) reside in these centers for a limited period of time (3-5 days). Their parenting skills and children’s behaviours are observed, and sufficient advice and support are provided on a case by case basis. The Karitane RUs, as tertiary level public hospitals, provide services including psychology services, day stay, residential services and outreach services to families in order to promote children’s wellbeing and family-child emotional attachment. They are staffed by a team of child and family health nurses, parent-craft/mother-craft nurses, general practitioners, pediatricians, psychiatrists, psychologists and social workers. Parents are commonly referred to the Karitane RUs for the following reasons: establishment of a bedtime routine, feeding problems and struggling with managing unsettled infants. In order to provide the most effective and high quality services, the professionals work with parents through a partnership approach. Through this model of care, the clinicians work as facilitators assisting the parents to promote their problem-solving skills, self-esteem, self-efficacy and confidence.

Parenting confidence is a feeling of competence in their parenting role. The partnership-approach focuses on the development of relationship, encouragement of the parents to choose the best intervention that works for the family and improvement of their parenting confidence (13, 14).

Assessment of parenting confidence in the mothers with a history of infertility can provide better knowledge about how infertility can affect early parenting behaviours of these women. To the knowledge of the authors, there is a lack of research on the parenting confidence of the mothers with a history of infertility who receive support and education in the parenting centers. Thus, this study aimed to evaluate the effect of residential early parenting services on the parenting confidence of the mothers with a history of infertility during the first postpartum year. We hypothesized that attending these centers can improve parenting confidence of these mothers. The study also intended to investigate the factors associated with low parenting confidence.

**Materials and Methods**

This was a retrospective chart review study using the quantitative data from the clients’ records of the
Karitane RUs during 2013. The study was part of a larger project in which 144 records of the mothers with infants of 0-12 months were randomly selected. Among them, 27 mothers with a history of infertility attended Karitane RUs for support and assistance. The data of these entire 27 mothers were considered for further evaluations.

The project was approved by the South Western Sydney Local Health District (SWSLHD) Human Research Ethics Committee (HREC) prior to its commencement. Since this was a retrospective study using the previously recorded data of the clients’ files and the results of the study were de-identified and analyzed collectively, we were not required by the SWSLHD HREC to obtain an informed consent from clients to analyze the data and publish the results.

Karitane Parenting Confidence Scale

The Karitane Parenting Confidence Scale (KPCS) as a validated 15-item self-report measure was developed to assess parental confidence in caring for their child. The internal consistency (Cronbach’s alpha=0.81), test-retest reliability (r=0.88), and discriminant and convergent validity of the KPCS have already been documented in a literature (15). Every item on the KPCS is scored from 0 to 3. After summing the points, the total score can range from 0 to 45. The cut-off score for the KPCS is 39 or less, indicating clinically significant low levels of parenting confidence.

Edinburgh Postnatal Depression Scale

The Edinburgh Postnatal Depression Scale (EPDS) as a validated 10 item self-report questionnaire was designed to screen and identify mothers for symptoms of depression during pregnancy and after childbirth (16). Each item is scored from 0 to 3, while items 3 and 5-10 are scored reversely. The total score varies from 0 to 30. A score between 10 and 12 indicates probable minor depression, and a score greater than 12 indicates probable major depression.

Additional data collected from clients’ files

After a comprehensive review of the literature, the following variables were extracted from the clients’ records: history of infertility, KPCS and EPDS scores on both admission and discharge, parental demographics (age, education, career, marital status, residence, country of birth, and main language spoken at home), obstetrics and gynaecological history, medical history, child’s information [gender, age, siblings, diagnosis of gastroesophageal reflux disease (GORD), and use of medication], history of mental health problems, domestic violence, alcohol use, cigarette smoking, and history of parenting intervention.

Demographics of the families referred to the Karitane Residential Units

The following inclusion criteria were used to refer families to the Karitane RUs: unable to establish bedtime routines, feeding problems, and difficulties in parent-child relationship, while the exclusion criteria was mothers with serious mental illness. The clients referred to the Karitane RUs by a health care professional were contacted by a clinical nurse who explained the admission process and procedure. After collecting relevant information over the phone and setting an admission date, an admission package was sent to the clients. On the admission day, the clients with their children attended the Karitane RUs and completed relevant forms. They also completed the EPDS and the KPCS before and after (on day four of their stay) the intervention. Then education, support and guidance in an intensive program were provided for parents by a multidisciplinary team consisting of nurses and allied health staff on an inpatient basis. The details of the service in the Karitane RUs were documented in the earlier publications (10, 17).

Service summary in Karitane Residential Units

A summary of the day-by-day care provided to the families in the Karitane RUs is as follows:

Monday: After admission, all parents were required to sign a consent form and complete the measurement tools (EPDS and KPCS), while the mothers were screened for domestic violence. The children were also examined by a pediatrician. If they took certain medications, a management plan would be prepared according to the findings at the time of assessment and their medications would be reviewed. Reason for admission and goals were identified during the comprehensive assessment, and a family management plan (including strategies) was discussed in partnership with the parents on a case-by-case basis. These strategies were implemented on Monday afternoon.
**Tuesday:** The mothers made appointments with allied health professionals if needed. The discussion about how to prepare formula for the infants, also known as “Formula Preparation Talk”, was held in the food room. A pram-walking group supervised by a clinician and a playroom coordinator was done either on Tuesday or Wednesday dependent on the weather conditions. Otherwise, a DVD on effective parenting was played for parents. The mothers attended both the toddler and relaxation groups in the afternoon.

**Wednesday:** Mothers were offered massages. Adjustment to parenthood group was held in order to encourage the parents to practice independently, support given as required. On Wednesday evening, a group, called Ready, Set, Go, was held to prepare the mothers for the discharge where they could ask their questions and find out what to expect at home. Father group was held at night.

**Thursday:** Clinicians strongly encouraged parents to use the different strategies trained more independently, support given as required. Relevant forms and documents were also completed. Discharge summary, including plans for discharge, follow-ups and appointments, was completed and finalized in partnership with a clinician. Those parents who stayed in the RUs until Friday attended the relaxation group and watched movie at night along with other parents.

**Friday:** Mothers completed an exit survey and left the residential units with their children by 10.30 am.

**Data analysis**

The quantitative data were analyzed by Statistical Package for Social Science software (SPSS, SPSS Inc., USA) version 19.0. The data validation occurred prior to the data entry, during the data entry, and post data entry as follows; visual review, value range checks, field type checks, and logical checks. Descriptive statistics were calculated for the variables of interest. Chi-square test ($\chi^2$) (Fisher’s exact test if applicable) was used to investigate the frequency distribution of all variables between the clients with low and high levels of parenting confidence. Paired t test was carried out to investigate the difference in the scores of the KPCS before and after the intervention. To test our hypothesis, we used independent samples t test to measure the differences in the parenting confidence and symptoms of depression at 0-6 months and 7-12 months postpartum. A P value less than 0.05 was considered to be statistically significant.

**Results**

Table 1 shows that 14.8% of the mothers reported symptoms of depression on admission to the Karitane RUs, and 59.3% reported low level of parenting confidence.

<table>
<thead>
<tr>
<th>Symptoms of Depression</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-depressed</td>
<td>23</td>
<td>85.2</td>
</tr>
<tr>
<td>Depressed</td>
<td>4</td>
<td>14.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parenting confidence before the intervention</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low level of parenting confidence</td>
<td>16</td>
<td>59.3</td>
</tr>
<tr>
<td>High level of parenting confidence</td>
<td>11</td>
<td>40.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parenting confidence after the intervention</th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low level of parenting confidence</td>
<td>6</td>
<td>22.2</td>
</tr>
<tr>
<td>High level of parenting confidence</td>
<td>21</td>
<td>77.8</td>
</tr>
</tbody>
</table>

The rate of low parenting confidence, however, dropped to 22.2% after receiving 4-5 days support and training. Results of the Chi-square test revealed no statistically significant association between the low parenting confidence with maternal age, education, career, country of birth, level of support, depression, recent major stressor, parity, antenatal and postnatal health complications, mode of delivery, as well as child information (including gender, age, siblings, diagnosis of GORD and use of medication) (P>0.05, Table 2). Results of the paired t test showed that the mean score on the KPCS increased from $36.9 \pm 5.6$ before the intervention to $41.1 \pm 3.4$ after the intervention, indicating an improvement in the parenting confidence of the mothers after attending the Karitane RUs, which was statistically significant (P<0.0001). This was also confirmed by a large effect size [degree of freedom (df)=0.905], indicating clinically meaningful changes in the parenting confidence of the mothers with a history of infertility (Table 3).
Parenting Confidence in Mothers with A History of Infertility

Table 2: Parenting confidence level and demographics of the mothers with a history of infertility on admission to Karitane RUs using chi-square test

<table>
<thead>
<tr>
<th>Table 2: Parenting confidence level and demographics of the mothers with a history of infertility on admission to Karitane RUs using chi-square test</th>
<th>Parenting confidence on admission</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low level of confidence’ (n=16)</td>
<td>High level of confidence’ (n=11)</td>
</tr>
<tr>
<td>Age group (ranged from 20 to 45)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-29</td>
<td>7 (43.7%)</td>
<td>6 (54.5%)</td>
</tr>
<tr>
<td>30-45</td>
<td>9 (56.2%)</td>
<td>5 (45.5%)</td>
</tr>
<tr>
<td>Country of birth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Australia</td>
<td>10 (62.5%)</td>
<td>10 (90.9%)</td>
</tr>
<tr>
<td>Other</td>
<td>6 (37.5%)</td>
<td>1 (9.1%)</td>
</tr>
<tr>
<td>Education</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No formal education</td>
<td>0</td>
<td>1 (9.1%)</td>
</tr>
<tr>
<td>Diploma or lower</td>
<td>4 (25%)</td>
<td>1 (9.1%)</td>
</tr>
<tr>
<td>Tertiary education</td>
<td>5 (31.2%)</td>
<td>5 (45.5%)</td>
</tr>
<tr>
<td>Unknown</td>
<td>7 (43.7%)</td>
<td>4 (36.4%)</td>
</tr>
<tr>
<td>Career</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not employed</td>
<td>1 (6.3%)</td>
<td>2 (18.2%)</td>
</tr>
<tr>
<td>On maternity leave</td>
<td>1 (6.3%)</td>
<td>4 (36.4%)</td>
</tr>
<tr>
<td>Currently working</td>
<td>14 (87.5%)</td>
<td>5 (45.5%)</td>
</tr>
<tr>
<td>Support</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partner only</td>
<td>3 (18.8%)</td>
<td>3 (27.3%)</td>
</tr>
<tr>
<td>Partner/Family/Friend/Clinicians</td>
<td>13 (81.3%)</td>
<td>8 (72.7%)</td>
</tr>
<tr>
<td>Depression</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-depressed</td>
<td>13 (81.3%)</td>
<td>10 (90.9%)</td>
</tr>
<tr>
<td>Depressed</td>
<td>3 (18.8%)</td>
<td>1 (9.1%)</td>
</tr>
<tr>
<td>Recent major stressors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>7 (43.7%)</td>
<td>5 (45.5%)</td>
</tr>
<tr>
<td>Yes</td>
<td>9 (56.2%)</td>
<td>6 (54.5%)</td>
</tr>
<tr>
<td>Parity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primiparous</td>
<td>8 (50%)</td>
<td>6 (54.5%)</td>
</tr>
<tr>
<td>Multiparous</td>
<td>8 (50%)</td>
<td>5 (45.5%)</td>
</tr>
<tr>
<td>Antenatal health complications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>13 (81.3%)</td>
<td>8 (72.7%)</td>
</tr>
<tr>
<td>Yes</td>
<td>3 (18.7%)</td>
<td>3 (27.3%)</td>
</tr>
<tr>
<td>Postnatal health complications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>14 (87.5%)</td>
<td>10 (90.9%)</td>
</tr>
<tr>
<td>Yes</td>
<td>2 (12.5%)</td>
<td>1 (9.1%)</td>
</tr>
<tr>
<td>Mode of delivery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal delivery(spontaneous or assisted)</td>
<td>12 (75%)</td>
<td>8 (72.7%)</td>
</tr>
<tr>
<td>Caesarean section (C/S)</td>
<td>4 (25%)</td>
<td>3 (27.3%)</td>
</tr>
<tr>
<td>Child</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-6 months old</td>
<td>12 (75%)</td>
<td>4 (36.4%)</td>
</tr>
<tr>
<td>7-12 months old</td>
<td>4 (25%)</td>
<td>7 (63.6%)</td>
</tr>
<tr>
<td>GORD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>10 (62.5%)</td>
<td>10 (90.9%)</td>
</tr>
<tr>
<td>Yes</td>
<td>6 (37.5%)</td>
<td>1 (9.1%)</td>
</tr>
<tr>
<td>Use of medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>9 (56.3%)</td>
<td>9 (81.8%)</td>
</tr>
<tr>
<td>Yes</td>
<td>7 (43.8%)</td>
<td>2 (18.2%)</td>
</tr>
</tbody>
</table>

Karitane RUs; Karitane Residential Units, GORD; Gastroesophageal reflux disease, and ‘; Values are given as number (%).
The mean scores of the KPCS and EPDS at 0-6 and 7-12 months postpartum were calculated using independent samples t test. It was shown that the mothers were less likely to have symptoms of depression at 7-12 months postpartum compared with 0-6 months after childbirth (5.1 ± 4.4 vs. 7.4 ± 4.6, respectively). Nevertheless, no statistically significant difference was found between the two time points (P=0.219). Similar results were shown for the mean score of the KPCS, indicating lower level of parenting confidence at 0-6 months postpartum compared with 7-12 months after childbirth (39.4 ± 3.8 vs. 35.2 ± 6.08, respectively). However, the difference between the two time points was not statistically significant (P=0.054) (Table 4).

### Discussion

This study is the first to demonstrate the risk factors of low parenting confidence in the mothers with a history of infertility and the impact of residential early parenting service on their parenting confidence. A research has shown that the prevalence of cry-fuss problems decreases from 19.1% at 2 months to 5.6% at 4 months after birth. In addition, the prevalence rate of sleep problems decreases from 21.2% at 8 months to 16.2% at 12 months (18). It has also been reported that the unsettled infant behaviour is associated with poor parental maternal health (18, 19) and less sense of competence and self-efficacy (20). Similarly, our findings showed that the parenting confidence and depression of the mothers with a history of infertility were affected by the infant’s age as they are more likely to display unsettled behaviours during 0-6 months of age. Our study also demonstrated that 59.3% of the women reported low level of parenting confidence. This finding supports the results of the study by Pearlman, in which the women with a history of prolong infertility were less confident in their parenting skills and unable to establish a bedtime routine for their children (21). Similarly, an earlier study by Gibson et al. (22) demonstrated that IVF mothers reported lower level of self-esteem and less parenting competence than the control group, while they perceived that their children were more special and unique. We found no data on the level of parenting confidence of the mothers with a history of infertility who did not attend the Karitane RUs in order to compare their related level with those who attended the RUs. Notwithstanding, a previous research has shown that women conceiving through ART were at the risk of early parenting difficulties and likely to seek support and advice from professionals. The study by Fisher et al. (6) revealed that out of 153 women with ART pregnancies, 26 (17%) were admitted to the residential early parenting centers after delivery, indicating that this rate is 3.3 times greater than that of general population (5%). They also reported higher admission rate to such centers among women who were primiparous, did not receive adequate breastfeeding advice, had low level of confidence in caring for their children.

### Table 3: Mean scores of the KPCS before and after the intervention in the mothers with a history of infertility using paired t test

<table>
<thead>
<tr>
<th>Scale</th>
<th>Before the intervention</th>
<th>After the intervention</th>
<th>t (df=26)</th>
<th>d</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parenting confidence (KPCS)</td>
<td>36.9 ± 5.6</td>
<td>41.1 ± 3.4</td>
<td>-5.392</td>
<td>0.905</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

KPCS; Karitane Parenting Confidence Scale, df; Degree of freedom, d; (Effect size)≥0.08 or higher shows a large effect, P<0.05.

### Table 4: Scores of KPCS and EPDS at 0-6 months and 7-12 months after childbirth in the mothers with a history of infertility using independent samples t test

<table>
<thead>
<tr>
<th>Scale</th>
<th>Time</th>
<th>Mean ± SD</th>
<th>t (df=25)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPDS</td>
<td>0-6 months after childbirth (n=16)</td>
<td>7.4 ± 4.64</td>
<td>1.261</td>
<td>0.219</td>
</tr>
<tr>
<td>KPCS before the intervention</td>
<td>35.2 ± 6.08</td>
<td>39.4 ± 3.83</td>
<td>-2.026</td>
<td>0.054</td>
</tr>
</tbody>
</table>

KPCS; Karitane Parenting Confidence Scale, EPDS; Edinburgh Postnatal Depression Scale and df; Degree of freedom.
had a postpartum mood disorder (PPMD), had an unsettled infant and received insufficient support. In our study, the findings showed that half of the ART women were primiparous and sought parenting advice and support from a wide range of resources, indicating that our findings are similar to those of Fisher et al. (6). Our study also offers important insights about the impact of a residential intervention on low parenting confidence of the mothers with a history of infertility. Furthermore, our study showed that the parenting programs in the residential units were effective and there were significant changes in the women’s parenting confidence after receiving parenting education. This was confirmed by a large effect size, indicating remarkable improvements in the parenting confidence of the mothers with a history of infertility.

A study by Rowe and Fisher showed that 4-5 night stay in the Tweddle Child and Family Health Service resulted in significant improvements in the infant behaviours and maternal psychological functioning at one month after discharge, while these effects were sustained during the six-month follow-up period (23). In a study by Hayes et al. (24), they showed that the one-day intervention in a Melbourne metropolitan Day Stay Centre significantly improved parental confidence and infant’s behaviours at 6th week after discharge and lowered the levels of parental distress. Furthermore, parents reported that they were highly satisfied with the level of help they received from the nurses and social workers (85%), the involvement in individual care planning (83%) and the education provided in the parenting center (70%).

Our findings are consistent with those of Rowe and Fisher and Hayes et al., indicating positive outcomes, such as improvements in the maternal mental health and child’s behaviour, bedtime routine and feeding habits after receiving intervention, training and supports in the residential early parenting centers. Phillips et al. (10) reported that a five-day stay in the Karitane RUs resulted in statistically significant improvements in sleeping time, unsettled behaviour, night-time waking and maternal depression and anxiety at one month and three months after discharge. In another study by Treyvaud et al. (25), they demonstrated remarkable improvements in the maternal parenting behaviour during parent-child interaction, depression, anxiety and stress. They also reported an improvement in maternal reports of child’s difficult behaviour. Also, more than half of the mothers participating in their study achieved 80% of their parenting goals after attending the program. Attending the residential parenting program results in less unsettled behaviour in the children and helps the children become more content during wakefulness and sleep for longer period of time both during day and at night. These changes in the children’s behaviour after attending the residential parenting centers may contribute to the improvement of the mothers’ parenting confidence and their better mental health status. Even one-day parenting programs have remarkable effects on the improvement of parenting skills and children’s unsettled behaviours (26).

Results of our study showed that 14.8% of the mothers with a history of infertility reported symptoms of depression on the admission. Although the results of our study may not be generalized to the population due to the small sample size, these findings are in accordance with those of Klock (27) and Lee et al. (28), indicating that approximately 8-10% of the women with a history of infertility experienced symptoms of depression during postnatal period.

In our study, due to only presence of singleton mothers, we were unable to compare the level of parenting confidence between mothers of singletons and mothers of twins/triplets. Nevertheless, Baor and Soskolne have indicated that poor parental adjustment and parenting stress are higher in the infertile women conceiving twins or triplets through the ART. As compared with the mothers conceiving twins spontaneously, mothers of the ART twins report poorer coping resources and higher levels of maternal stress during postnatal period (29). The risk of parenting stress is 22% in the mothers of ART triplets, compared with 5% of the mothers of ART singletons and 9% of the mothers of naturally conceived singletons (30).

The present study had some limitations that need to be addressed. Firstly, due to use of previously recorded data of the client’s files, there was a lack of data on the paternal parenting confidence and depression. Secondly, due to lack of follow-up data on the maternal parenting confidence and depression post-discharge, this study failed to investigate the long-term effects of the intervention in this regard. Thirdly, this was a retrospective study...
in which we analyzed the previously recorded data of a small sample of mothers with a history of infertility who attended the Karitane RU’s (27 women). Therefore, our results may not be generalized to the larger population of postpartum women with a history of infertility. Last but not least, due to the access issues, we were not able to evaluate the parenting confidence of the mothers with a history of infertility who were not admitted to the Karitane RU’s. Future research with larger samples of women, from more varied backgrounds, with and without a history of infertility is needed to determine the extent to which the current findings are generalizable.

Conclusion

Results of this study demonstrated that 59.3% of the mothers with a history of infertility experienced low parenting confidence. These findings emphasized on the role of residential early parenting services in promoting the level of parenting confidence and highlighted the need for early recognition and referral of those who are at the risk to such centers. The mothers with a history of infertility need to be considered as a high-risk group and identified during the early stages of pregnancy and parenthood. Midwives, child and family health nurses, mental health clinicians and other health care providers that deal with the women who have babies after a period of infertility need to assess their mental health and parenting confidence before discharge from the hospital and follow them up to make sure they are supported throughout their journey.

Acknowledgments

The authors acknowledge in-kind support of Karitane Residential Units and Parenting Services for carrying out this research. We also would like to thank Grainne O’Loughlin, the Karitane CEO, for her support and the nursing unit managers and staff in the Karitane early parenting residential units for their assistance and contributions. The authors declare no conflict of interest.

References

Parenting Confidence in Mothers with A History of Infertility

Original Article

Psychometric Properties of The Persian Version of The Prenatal Attachment Inventory in Pregnant Iranian Women


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Abstract

Background: In 1993, Muller developed the Prenatal Attachment Inventory (PAI) which has been used widely in many studies and translated into several languages. The current study aimed to translate the PAI into Persian, assess the underlying structure of the PAI, and the appropriateness of the one-factor solution proposed by Muller.

Materials and Methods: In this cross-sectional study, we recruited a total of 322 primigravidae in their 27th to 34th gestational weeks that referred to private and governmental prenatal clinics in Tehran, Iran. All participants completed the Persian versions of the PAI and a demographic questionnaire. Participants were re-tested 2 weeks after the initial testing. The following psychometric properties of the PAI were investigated: construct validity by confirmatory factor analysis (CFA), internal consistency reliability with Cronbach’s alpha, and test-retest reliability according to the intraclass correlation coefficient (ICC).

Results: The CFA results indicated that a single-factor model provided good fit to the data, which confirmed the original model by its developer. The Cronbach’s alpha coefficient for PAI was 0.856 and the test-retest reliability with ICC was 0.784. Considering the duration between marriage and pregnancy, women with low duration scored significantly higher than women with high duration on PAI (P=0.043).

Conclusion: The Persian version of the PAI showed that one factor structure was adequate and could be used for measuring psychological affectionate attachment between Iranian mothers and their fetuses.

Keywords: Attachment, Pregnancy, Infertility


Introduction

In 1981, Cranley initially defined maternal-fetal attachment (MFA) as the extent to which “women engage in behaviors that represent an affiliation and interaction with their unborn child” (1). Then, Muller (2) presented another definition: “the unique and affectionate relationship that develops between a woman and her fetus”. Muller developed the Prenatal Attachment Inventory (PAI) (3) which has been continuously used as an instrument to measure psychological affectionate attachment between a mother and her fetus (4).

It is believed that the relationship between a mother and her child originates during pregnancy (5-8). Numerous conditions may affect the psychological status of a pregnant woman, resulting in change to the feto-maternal attachment. For example, there are reports that twin pregnancy, a history of infertility or infertility treatment, high risk pregnancy (9), maternal age (10), maternal mood (11-14), awareness of the fetus status by ultrasound (15), socio-economic levels (16), adequate prenatal care (17), pre-implantation genetic diagnosis (18), diet (19), a history of abor-
tion (20), and exercise (21) affect feto-maternal attachment. Attachment can be an indicator for certain pre- and post-natal psychological disorders in mothers (22).

PAI has been used in many prenatal studies worldwide in different languages and cultures (23-29). Each questionnaire and inventory must be adjusted with the country of the study, especially in terms of attitudes, beliefs, and emotions. Prior to research on the Iranian population, this inventory must be translated into Persian and evaluated prior to its use for research in Iran. Other studies have assessed and reported the PAI as single factor, three-factor, or five-factor structures. In a study by Pallant et al. (30), confirmatory factor analysis (CFA) of the original 21-item version of the PAI revealed poor fit to the model. These researchers supported a three-factor structure. The aim of the current study was to translate the PAI into Persian, primarily assess the underlying structure of the PAI, and the appropriateness of the one-factor solution previously proposed.

**Materials and Methods**

We used the forward-backward method to translate the PAI into Persian. The original inventory (3) was first translated from English to Persian, then from Persian to English, and again from English to Persian. Each translation was performed by a separate independent health staff member proficient in the English language. Cultural changes were as follows. In the 5th question: “I let other people put their hands on my tummy to feel the baby move”. In Islamic contexts, another person is not permitted to touch a woman’s body except her intimates “maharem”. Therefore, we have changed the question to: “I let my intimates put their hands on my tummy to feel the baby’s movement”. In the 8th question: “I tell others what the baby does inside me”. In Iran, most females are modest and shy, particularly with regards to issues related to reproduction and sexuality. They normally do not discuss these issues with others, especially those who reside in smaller towns and villages. We have changed this item to: “I tell my friends and relatives what the baby does inside of me”.

**Content validity**

After adjusting the questionnaire according to cultural, social, and religious ideas to prevent any bias from opposing beliefs, a group of sociologists, gynecologist, psychologist, clergies, and law experts carefully reviewed the questionnaire and exchanged their ideas in a group meeting. All group members were well familiar with reproductive health.

**Face validity**

After the final editing and best design of the questionnaire, we distributed it among 22 first-time pregnant women in the 27th to 34th gestational week of pregnancy. An expert midwife with adequate education to avoid bias conducted the questionnaire via one-on-one interviews. After reviewing the results of the interview, we develop another edition and corrected the structure of the questionnaire according to the Persian language.

**Prenatal attachment inventory**

The PAI is a self-reporting instrument that consists of 21 items. Each item is scored on a 4-point Likert scale where 1=almost never, 2=sometimes, 3=often, and 4=almost always. Examples of items The PAI is a self-reporting instrument that consists of 21 items. Each item is scored on a 4-point Likert scale where 1=almost never, 2=sometimes, 3=often, and 4=almost always. Examples of items on the scale include: “I wonder what the baby looks like”, “I know when my baby is asleep”, and “I try to imagine what the baby is up to.” Total scores can range from 21 to 84, with higher scores indicative of higher levels of prenatal attachment.

**Demographic characteristics**

The demographic information questionnaire included age, duration from marriage to pregnancy, education level, occupation, and type of pregnancy (wanted or unwanted).

**Participants**

In this cross-sectional study, we assessed the reliability of the PAI by administering this questionnaire to 322 first-time pregnant women in their 27th to 32nd gestational weeks. The women referred to private and governmental prenatal clinics in Tehran, Iran. The questionnaire was administered to these women again after 10-12 days. Inclusion criteria were: being able to read
and write Persian, over 18 years of age, low-risk pregnancy, gestational age of over 25 weeks, and no previous abortions. We excluded women younger than 18 years of age because they presumably have experienced stress which could influence maternal attachment. We also excluded high-risk pregnancy and abortion because these events might lead to a different type of attachment to the fetus.

**Ethical consideration**

The Ethics Committee at Royan Institute approved this study. All participants received information about the purpose of this study and gave their verbal informed consent to participate.

**Statistical analysis**

CFA was used to examine the factor structure of the PAI. The fit indices we have employed to test the model fit included: chi-square ($\chi^2$), relative chi-square [$\chi^2$/degree of freedom (df)], comparative fit index (CFI), root mean square error of approximation (RMSEA), and the standardized root mean square residual (SRMR). A non-significant $\chi^2$ statistic indicates a good model fit (P>0.05). Unfortunately, the $\chi^2$ statistic is highly sensitive to sample size, especially if the observations are greater than 200. An alternate evaluation of the $\chi^2$ statistic is to examine the $\chi^2$/df for the model. A $\chi^2$/df ratio of 3 or less is indicative of a good model fit. Values of CFI>0.9, SRMR<0.08, and RMSEA<0.08 indicate good fit with the data. Internal consistency of the PAI was examined using Cronbach’s alpha coefficient and test-retest reliability of the scale by ICC.

All statistical analyses were performed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA), except for the CFA, which was performed using Lisrel 8.80 (Scientific Software International, Inc., Lincolnwood, IL, USA). All statistical tests were two-tailed and a P value<0.05 was considered statistically significant.

**Results**

**Participants’ characteristics**

Table 1 lists the socio-demographic characteristics of the participants. Participants had a mean age of 28.57 ± 4.13 years (range: 18 to 43 years). Of participants, the majority were housewives (63.8%), 54.4% had college or university degrees, and 93.1% wanted to become pregnant. The mean duration from marriage to pregnancy was 4.31 ± 2.75 years.

<table>
<thead>
<tr>
<th>Table 1: Socio-demographic characteristics of the participants</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Y)</td>
<td>28.57 ± 4.13</td>
</tr>
<tr>
<td>Duration from marriage to pregnancy (Y)</td>
<td>4.31 ± 2.75</td>
</tr>
<tr>
<td>Education level</td>
<td>n (%)</td>
</tr>
<tr>
<td>Elementary</td>
<td>14 (6.0)</td>
</tr>
<tr>
<td>Secondary</td>
<td>92 (39.6)</td>
</tr>
<tr>
<td>University</td>
<td>126 (54.4)</td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
</tr>
<tr>
<td>Employed</td>
<td>79 (34.1)</td>
</tr>
<tr>
<td>Housewife</td>
<td>148 (63.8)</td>
</tr>
<tr>
<td>Student</td>
<td>5 (2.1)</td>
</tr>
<tr>
<td>Type of pregnancy</td>
<td></td>
</tr>
<tr>
<td>Wanted</td>
<td>216 (93.1)</td>
</tr>
<tr>
<td>Unwanted</td>
<td>16 (6.9)</td>
</tr>
</tbody>
</table>

**Reliability analysis**

Cronbach’s alpha coefficient for assessing internal consistency of the PAI was 0.856. The 2-week test-retest reliability with ICC was 0.784.

**Confirmatory factor analysis**

The CFA was performed to determine the fit of the previously identified one-factor model. The goodness of fit indices revealed that the single-factor model was a good fit to the data ($\chi^2=532.36$, df=189, P<0.001, $\chi^2$/df=2.82, CFI=0.90, RMSEA=0.089, and SRMR=0.078). All standardized factor loadings were significant, in the expected direction, and ranged from 0.29 to 0.64 (data not shown).

**Comparison of the Prenatal Attachment Inventory by type of pregnancy and duration from marriage to pregnancy**

We used the independent samples t test to examine the differences between PAI, type of pregnancy, and duration from marriage to pregnancy. There was no significant difference be-
between groups of wanted pregnancies and unwanted pregnancies on the PAI (P=0.945). The results indicated that women with low duration (64.14 ± 9.12) scored significantly higher than women with high duration (61.68 ± 9.24) between marriage and pregnancy on the PAI (P=0.043) (Table 2).

Table 2: Comparison of the Prenatal Attachment Inventory (PAI) by type of pregnancy and duration from marriage to pregnancy

<table>
<thead>
<tr>
<th>Type of pregnancy</th>
<th>n</th>
<th>Mean (SD)</th>
<th>t</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wanted</td>
<td>216</td>
<td>62.83 (9.20)</td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>Unwanted</td>
<td>16</td>
<td>63.00 (10.15)</td>
<td></td>
<td>0.945</td>
</tr>
<tr>
<td>Duration from marriage to pregnancy</td>
<td>2.03</td>
<td>0.043</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;4 years</td>
<td>110</td>
<td>64.14 (9.12)</td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td>≥4 years</td>
<td>122</td>
<td>61.68 (9.24)</td>
<td></td>
<td>0.945</td>
</tr>
</tbody>
</table>

Discussion

This is the first study to assess psychometric properties of the PAI in pregnant Iranian women. PAI is a well-known questionnaire for measurement of feto-maternal attachment. This questionnaire has been translated into several languages and used in numerous countries (24-29). The PAI has been used to produce new questionnaires (31-36). Culture and beliefs of a society may impact attachment between a mother and her infant (31), and attitude towards the unborn child is different in various parts of the world. Therefore, it is important to conduct research in order to prove any relation between demographic variables, education, and socioeconomic levels to prenatal attachment (9, 10, 32).

The current study demonstrated that the one-factor structure of the questionnaire had adequate psychometric properties. CFA results showed that the one-factor structure of the PAI had good psychometric properties with adequate internal consistency. Pallant et al. (30) reported that the CFA of a single-factor was a poor fit to the model and the three-factor solution was the most appropriate to represent the PAI items. Cronbach’s alpha above 0.70 showed appropriate internal consistency among the questions so that it could be used in the Iranian context as a good inventory to measure attachment between a mother and her fetus. It would explain the psychological connection between a pregnant woman and her unborn child. Another study has reported a variation in the behavior of the individual PAI subscales during both the prenatal and postnatal periods. The reliability of the total PAI scale reported was acceptable (Cronbach alpha=0.86) (36). In this study confirmed the external validity of tool by test-retest reliability. An ICC equal to 0.784 showed a very good correlation in repeating the test during the time interval. Pallant et al. (30) demonstrated that the three-factor inventory had adequate internal consistency and reliability (above 0.7).

The results of the independent samples t-test showed a significantly high prenatal attachment relationship in women who had a slight time difference between their marriage and pregnancy.

As mentioned before, prenatal attachment may predict future relations between a mother and her child (30). Thus, it would be of benefit to determine factors that affect this relationship and discover methods to decrease prenatal attachment reducing factors to help the future of a mother and child. It has also been reported that factors such as genetic screening (37-39), twin pregnancy (23, 40), trauma (41), maternal age (10), maternal mood (11-14), and miscarriage (20) affect the MFA. There may be a correlation between prenatal and postnatal attachment (24). A growing number of studies report the impact of prenatal attachment on subsequent postnatal bonding (36), however further studies are necessary to better understand its effect on the mother’s adjustment to the parenting role, the mother-child relationship, and the development and well-being of the child. There should be additional studies that pertain to influencing factors in different parts of the world, particularly Middle Eastern countries.

Conclusion

The Persian version of the PAI showed that one factor structure is adequate and can be used for measuring psychological affectionate attachment between Iranian mothers and their fetuses.

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and writing of the paper. This project financially supported by Royan institute. The authors report no conflicts of interest.

References


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Orexin Decreases Aromatase Gene Expression in The Hypothalamus of Androgenized Female Rats

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Abstract

Background: Orexin is a hypothalamic orexigenic neuropeptide, which third cerebral injection of it mainly exerts inhibitory effects on reproductive functions. It increases significantly the Aromatase (Cyp19) gene expression in the hypothalamus of male rats. Aromatase is an enzyme which converts androgens to estradiol in the hypothalamus of rats. Prenatal or neonatal exposure of females to testosterone masculinizes the pattern of Cyp19 mRNA levels in adulthood. In the present study the effects of central injections of orexin-A on hypothalamic Cyp19 gene expression of adult female rats were investigated, while they had been androgenized on third day of postnatal life.

Materials and Methods: In this experimental study, twenty female Wistar rats received subcutaneous injections of testosterone propionate (50 µg/100 µl) on their third day of postnatal life. Adult androgenized rats weighing 180-220 g, received either 3 µl saline or one of 2, 4 or 8 µg/3 µl concentration of orexin via third cerebral ventricle. Five non-androgenized rats, as control group, received intra cerebral ventricle (ICV) injection of 3 µl saline. The hypothalamuses were dissected out and mean Cyp19 mRNA levels were determined by semi-quantitative real time-polymerase chain reaction (PCR) method. Data were analyzed by unpaired t test and one-way ANOVA using SPSS software, version 16.

Results: Mean relative Cyp19 mRNA level was significantly increased in the hypothalamus of androgenized compared to non-androgenized female rats. Central injections of 2, 4 or 8 µg/3 µl orexin decreased significantly the hypothalamic Cyp19 mRNA level of androgenized rats compared to androgenized-control groups.

Conclusion: The results suggested that the orexin may exert inhibitory effects on the gene expression of Cyp19 in the hypothalamus of neonatal androgenized female rats in adulthood.

Keywords: Orexin, Cyp19, Female Rats

Introduction

In mammals, a complex network of central and peripheral signals controls the hypothalamus-pituitary-gonadal (HPG) axis. Among the peptides involved in the control of energy balance and reproduction, orexin neuropeptides are important factors for regulation of the reproductive axis. Orexin-A is a 33 amino-acids orexigenic neuropeptide (1, 2). It is mainly synthesized in the lateral hypothalamus and the fibers project to the hypothalamic nuclei to regulate the reproductive functions (3-5). The mechanism, whereby orexin affects HPG axis is not completely clear yet and both stimulatory and inhibitory effects of orexin-A or -B were observed on pulse frequency and pulsatile secretion of gonadotropin-releasing hormone/luteinizing hormone (GnRH/LH) release in female rats (6-12). Aromatase cytochrome P450 is an enzyme coded by Cyp19 gene (also known as Cyp19A1 or P-450AR-OM). While the highest activity is observed in the
hypothalamic nuclei including median preoptic area, this enzyme converts androgens (e.g. testosterone) to estradiol in peripheral tissues and brain (13-15). Testosterone is an important regulator of the Cyp19 gene expression in the hypothalamus of rats (13). Owing to distinct circulating androgen levels in different sexes, the expression of Cyp19 gene in the male rat hypothalamus is greater than in females (13-15). It has been demonstrated that prenatal or neonatal females, receiving exogenous injection of testosterone propionate (TP) during some crucial developmental stages, could partially exhibit infertility in adulthood due to the Cyp19 gene expression increase (16-19). Considering that elevation of androgen could lead to many abnormalities, like hyperandrogenic disorders and anovulation, study the pathophysiological effects of this hormone aberration appears to be very critical (18). Investigations show that aromatase could regulate not only the masculine sexual behaviour in males, but also the cyclic ovulatory LH surge in females (13). Curiously, it has been demonstrated while aromatase applies a prohibition effect on the ovulation procedure by blocking gonadotropin surges, aromatase inhibitors could exert a stimulatory effect on this procedure (13, 20). It has been reported that hypothalamic interneurons -including neuropeptide Y (NPY), pre-opiomelanocortin (POMC) or ghrelin- may play a role in mediating the inhibitory effects of orexin on HPG axis (21-23). We have previously shown that central injection of orexin significantly increased the Cyp19 gene expression and estradiol hormone levels in the hypothalamus of male rats (24). The purpose of present study was to investigate the effects of orexin central injection on hypothalamic Cyp19 gene expression levels in androgenic rat model.

Materials and Methods

Animals

In the present experimental study, twenty neonatal female Wistar rats (provided by Neurophysiology Research Center of Shahid Beheshti University, Tehran, Iran) received subcutaneous injection of TP (50 µg/100 µl) on the third day of postnatal life, as with previous studies (25-32). Also, five non-androgenized female rats were used as control group. Control and androgenized pups were housed with their mothers in cages under conventional control of temperature (22 ± 2°C) and light (12 hours light/dark cycle, light on 07:00 hours). Animals had free access to food and water all the time. All animal procedures were performed in accordance with ethical committee of Shahid Beheshti University. The procedures was designed consistent with previous investigations whereby the injection of TP single dose into neonatal female rats led to persistent adulthood infertility, 100 days after birth (25-32).

Intra cerebral ventricle cannulation and injection

Animal surgery procedures and handling were carried out as previously described (33). Adult control and androgenized rats with 100 days of age (26-32) and 180-220 g body-weight (BW) were anesthetized using intraperitoneal (IP) injection of a ketamine and xylezine mixture (ketamine 80 mg/kg BW+xylezine 10 mg/kg BW). For central injections, a 22-gauge stainless cannula was implanted into the third cerebral ventricle according to coordinates of Paxinos and Watson Atlas (anterior-pstterior (AP)=−2.3, mid line (ML)=0.0, dorsal-ventricle (DV)=6.5). The cannula was secured to the skull with three stainless steel screws and dental cement. The animals were kept in individual cages. After one week recovery period, twenty androgenized rats in four groups (five rats in each group) received either 3 µl saline or one of the 2, 4 or 8 µg/3 µl orexin-A via third cerebral ventricle.

Five non-androgenized rats, as control group, received ICV injection of 3 µl saline in estrous phase of estrous cycle at the 100 days of age. The appropriate doses of orexin were selected with regards to our previous studies, implicating on the stimulatory and inhibitory effects of orexin on Cyp19 gene expression in the hypothalamus and HPG axis of male rats (12, 16). Orexin-A (Ana spec Co., USA) was dissolved in saline and injected Intra-cerebroventricularly by a 27-gauge stainless steel injector (protruded 0.5 mm beyond the cannula), connected to Hamilton micro-syringe by polyethylene (PE-20) tubing between 09:00 and 10:00 a.m. For subcutaneous injection, TP was dissolved in olive oil and injected by an insulin syringe. At the end of the experiment, these rats were anesthetized, sacrificed by decapitation and subsequently the brains were quickly collected. The hypothamaluses were dissected out as previously described (34). The samples were immediately frozen in liquid nitrogen and stored at -80°C.
RNA isolation and semi-quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from individual frozen hypothalamus. Total RNA was extracted using pureZol RNA isolation reagent according to manufacturer instruction (BioRAD, USA). The quantity of each RNA sample was performed by measuring absorbance at 260 nm. Regarding that β-Actin (Actb) transcription is consistently expressed within different tissues, including brain, it was considered as housekeeping gene to normalize the other gene mRNA expression levels, using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) technique. For that, the first cDNA strand was synthesized from 5 μg of total RNA according to manufacturer instruction (RT-PCR kit, vivantis, Malaysia). Subsequently, Cyp19 and β-Actin genes fragment were respectively amplified on 34 cycles (94°C for 30 seconds, 61°C for 30 seconds, 72°C for 30 seconds) and 35 cycles (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds) at a final volume of 50 μl containing cDNA template (2 µl), 10X PCR buffer (5 µl), 50 mM MgCl2 (1.5 µl), 10 mM dNTP Mix (1 µl), 100 µM sense and antisense primers (1 µl of each one) and 500 U Taq-DNA Polymerase (0.5 µl) as well as sterile water (38 µl) according to manufacturer instruction (PCR kit, vivantis, Malaysia). PCR amplification produced a 511 base pairs (bp) fragment using β-Actin-F: 5′-GAAATCGTGCGTGA-3′ and β-Actin-R: 5′-GCTAGGCATTTGCGGTGGA-3′ primers (35, 36), or a 289 bp fragment using Cyp19-F: 5′-GCTTCTCATTGCAAGAAG-3′ and Cyp19-R: 5′-AGGTTAATTCTTGGCGG-3′ primers (37). The RT-PCR products were analysed by 1% agarose gel electrophoresis. Band intensities were compared by imaging safe view staining and quantified using ImageJ software program.

Statistical analysis

Differences between androgenized and non-androgenized control groups were assessed using student unpaired t test. Significant differences of orexin-treated groups were determined by one-way ANOVA followed by post hoc Dunnet test, using SPSS software version 16. A P value of 0.05 was considered as significant threshold. Data presented as the mean value with SEM of independent experiments.

Results

To study the effect of Cyp19, transcription of this gene was semi-quantitatively compared between hypothalamus of the five androgenized- and five control-saline-treated rats. Qualitative results showed that relative Cyp19 mRNA levels in hypothalamus of the androgenized rats are significantly higher than controls (Fig.1A). A further analysis was applied to relatively determine the semi-quantitative levels of Cyp19 mRNA using qualitative results densitometry scanning. Findings demonstrated that mean hypothalamic Cyp19 mRNA levels were significantly increased in the hypothalamus of androgenized saline-treated rats to 83%, in comparison with control saline-treated rats (Fig.1B).

We subsequently investigated the effect of different orexin-A concentrations on Cyp19 mRNA expression level in hypotalamos of the presented groups. In terms of the quality, data analyses of central injection showed that injection of 2, 4 or 8 µg orexin-A led to reduction of hypothalamic Cyp19 mRNA levels compared to androgenized control group (Fig.2A). Figure 2B provides a semi-quantitative analysis of the data determined by densitometry scanning (obtained from 5 animals used in experiments for each treatment). These results also showed that injections of 2, 4 or 8 µg orexin-A decreased hypothalamic Cyp19 mRNA levels by 20, 48 or 16% compared to androgenized-saline group. In all three groups, this decrease in the mean Cyp19 mRNA levels was statistically significant compared to androgenized-saline group (Fig.2B).
Discussion

The result of the present study showed that relative mean Cyp19 gene expression was significantly increased in the hypothalamus of neonatal androgenized female rats (with the age of 100 days of life) compared to non-androgenized adult control rats (in estrous phase of estrous cycle, with the age of 100 days of life). This result is consistent with the previous studies which reported that neonatal exposure of female rats to testosterone masculinizes the pattern of hypothalamic Cyp19 gene expressions in adulthood (16-19, 26-32). It is well known that existence of androgens during critical differentiation period of brain sexual regions (late gestation and continues into ten days of postnatal life) can permanently alter the gender-specific capacity for aromatization in the hypothalamus (38, 39). Previous studies showed that high level of Cyp19 gene expression in the hypothalamus of androgenized female rats may be a possible mechanism whereby androgen induces sterility and lack of cyclic ovulatory discharge of LH in adulthood.

Orexin is a hypothalamic neuropeptide which exerts mostly inhibitory effects on reproductive axis (6-12, 40). It has interestingly been established that aromatase exert an inhibitory effect on ovulation via inhibiting gonadotropin surges (13, 20). So that aromatase levels were low during estrous phase of estrous cycle (32). In the present study, the potential effects of central injection of orexin were investigated on Cyp19 gene expression in androgenized female rats. The results showed that hypothalamic Cyp19 mRNA levels were significantly decreased in orexin-treated androgenized rats compared to androgenized control group. In the present study, for the first time we determined the effects of orexin on Cyp19 gene expressions in the hypothalamus of androgenized female rats. So far, there is not any report to indicate the exact mechanism leading to reducing Cyp19 gene expression in adult neonatal androgenized female rats, upon induction of orexin. Never the less, it has been revealed that the levels of Cyp19 gene expression in the hypothalamus of TP-treated perinatal or neonatal females are not significantly different from those in the hypothalamus of normal male rats (38, 39). We have also previously reported that central injection of orexin significantly increased the mean Cyp19 mRNA levels and mean estradiol.
concentrations in the hypothalamus of wild type male rats (24). In regard to these results, we initially estimated to observe similar stimulatory effects of orexin on Cyp19 gene expression in neonatal androgenized female rats. Although, the reason of this controversy between the later female and wild type male rats is not clear. One possible difference between the Cyp19 gene expression patterns in these groups of rats may be due to blood testosterone levels in adulthood. Roselli and Klosterman (14) reported that exposure of the brain to steroid hormones appears to be necessary for sexual differentiation of Cyp19 expression during prenatal life, although gonadal hormones might also be able to exert additional effects during puberty and adulthood. On the other hand, it is possible that plasma testosterone concentration in adult male rats affects the orexin influence on Cyp19 mRNA level in a different manner compared to androgenized adult female rats. Although further studies are required to better understand the exact effects of orexin on Cyp19 gene expression pattern in female rats, a side specific effect of orexin on GnRH/LH release could be another cause of obtaining present results (11).

It has been shown that the brain areas-involved in the controlling HPG axis including rostral preoptic area (rPOA), medial POA (mPOA) and arcuate nucleus/median eminence (ARC/ME) is innervated by orexin neurons, leading to effect differently on the LH release in female rats. So that, orexin enhances LH levels after injection into the rPOA, while it inhibits LH release after injection into the mPOA or ARC/ME (11). In this study, the effect of orexin on hypothalamic Cyp19 gene expression was determined by injection into the third cerebral ventricular injection of the androgenized female rats. Like LH release, injection of orexin into HPG axis specific nuclei might differently affect Cyp19 gene expression. Therefore, to discuss the exact effect of orexin on Cyp19 gene expression in neonatal androgenized adult females, it is recommended to investigate the effect of orexin on Cyp19 gene expression of rPOA, mPOA or ARC.

Conclusion

This paper demonstrated that mean Cyp19 mRNA level was significantly increased in the hypothalamus of three days-old androgenized control rats compared to non-androgenized control ones. As a novel finding, it was also reported that injection of orexin significantly decreased the mean hypothalamic Cyp19 mRNA level in androgenzed female rats compared to androgenized control group.

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References

Orexin Negatively Affects Aromatase in Androgenized Rats

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Assessment of **DPY19L2** Deletion in Familial and Non-Familial Individuals with Globozoospermia and **DPY19L2** Genotyping

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Abstract

**Background:** Globozoospermia is a rare syndrome with an incidence of less than 0.1% among infertile men. Researchers have recently identified a large deletion, about 200 kbp, encompassing the whole length of **DPY19L2** or mutations in **SPATA16** and **PICK1** genes associated with globozoospermia. The aim of this study was to analyze the **DPY19L2** gene deletion using polymerase chain reaction technique for the exons 1, 4-8, 11 and 22 as well as break point (BP) “a” in globozoospermic men.

**Materials and Methods:** In this experimental study, genome samples were collected from 27 men with globozoospermia (cases) and 36 fertile individuals (controls), and genomic analysis was carried out on each sample.

**Results:** Deletion of **DPY19L2** gene accounted for 74% of individuals with globozoospermia. **DPY19L2** gene deletion was considered as the molecular pathogenic factor for the onset of globozoospermia in infertile men. By quantitative real-time polymerase chain reaction (qPCR), we genotyped **DPY19L2** deletion and identified carriers within the population.

**Conclusion:** This technique may be considered as a method for family counseling and has the potential to be used as a pre-implantation genetic diagnosis, especially in ethnic community with high rate of consanguineous marriages.

**Keywords:** Gene Expression, Genotyping, Globozoospermia


Introduction

Globozoospermia is a rare autosomal recessive genetic syndrome with an incidence of less than 0.1%. In this syndrome, due to defect in the process of acrosome biogenesis, the sperm contains a round head shape, consequently leading to no penetration into the oocyte during fertilization. Thus, direct intracytoplasmic sperm insemination (ICSI) along with artificial oocyte activation is the only solution to gain pregnancy at couples suffering this abnormality (1). Genetic pedigree assessment of these individuals indicates the congenital origin of globozoospermia. To define molecular defects involved in this disorder, several autosomal genes have been identified in knockout mice models including: **Csnk2a2**, **Hrb**, **Gopc**, **Pick1**, **Gba2**, **Vps54**, **Zphp1** and...
Defect of these genes in mouse models represented phenotypically similar abnormalities to human globozoospermia. However, among the aforementioned genes, only *PICK1* mutation was yet detected in human. *PICK1* protein is involved in subcellular trafficking in brain, pancreas and testis. The respective gene is located on human chromosome 22, and contains 13 exons. In spermatogenesis, *PICK1* is involved in trafficking of pro-acrosomal vesicles from golgi apparatus to acrosome. Liu et al. (10) showed a homozygous missense mutation (G198A) at the C-terminal domain of *PICK1* which disrupted *PvuII* site, culminating in formation of sperms with round head shape in human. Other human autosomal genes involved in globozoospermia are *SPATA16* and *DPY19L2* (11-13). *SPATA16* is a testis specific gene, translating a protein which is localized in the golgi apparatus and plays a role in the transportation of pro-acrosomal vesicles from golgi to the acrosome in the round and elongated spermatids (14). Dam et al. (11) found a homozygous sequence variation in the last nucleotide of exon 4 (G848A) of this gene which impaired *NciI* or *HpaII* recognition site, in three infertile brothers of a Jewish family with globozoospermia. However, the most likely considered gene to have a pivotal role in globozoospermia is *DPY19L2*. This gene is expressed primarily in spermatids with a specific localization limited to the inner nuclear membrane, facing the acrosomal vesicle. Lack of the relevant protein causes instability of acrosome vesicles and thereby loss of acrosome (15). It has been demonstrated that complete deletion of *DPY19L2* by non-allelic homologous recombination (NAHR) results in globozoospermia (12, 13). Recent studies have revealed that *DPY19L2* gene function could be eliminated at nine possible breakpoints covering three regions, known as breakpoints “a, b and c” in two low copy flanking repeats (LCRs) of *DPY19L2* gene. High incidence (96.5%) of LCR sequences facilitates the occurrence of NAHR in this region (16).

Considering the role of aforementioned genes in globozoospermia and in line with our previous study (16), the aim of this study was to evaluate the prevalence of missense mutations, G848A, in exon 4 of *SPATA16* gene and G198A in exon 13 of *PICK1*, as well as *DPY19L2* deletion in Iranian infertile individuals with globozoospermia referring to Isfahan fertility and infertility center (IFIC). Herein, we observed complete deletion of *DPY19L2* gene in 20 out of 27 globozoospermic individuals, but no mutation was detected in *SPATA16* or *PICK1* gene. We also performed quantitative real-time polymerase chain reaction (qPCR) assay to identify individuals with homo/hemizygous deletion of *DPY19L2* gene.

**Materials and Methods**

**Mutational analysis of *SPATA16*, *PICK1* and *DPY19L2* genes**

This experimental study was approved by Institutional Review Board (IRB) of Royan Institute. In this case-control study, 27 male with globozoospermia from Iranian population were contributed. An arbitrary number was assigned to each globozoospermic individual (G1 to G29), out of whole two individuals, G11 and G17, were omitted due to missing. We assessed the mutations for *SPATA16* and *PICK1* genes and provided pedigrees for two families with complete deletion of *DPY19L2* and one family with deletion of exon 5, 6 and 7 in *DPY19L2* gene.

In this process, blood samples were taken from 27 individuals, with globozoospermia with round-headed spermatozoa who referred to IFIC, as well as their family members after completing a consent form. Two out of 27 persons with more than 50% acrosomeless spermatozoa in their normal and round-headed sperm samples were considered to have partial globozoospermia, while the rest of individuals were suffering from total globozoospermia. Peripheral blood samples were also taken from 30 fertile men as well as the parents of three individuals with globozoospermia (G8, 14 and 21). In the sample group, except three brothers (G21, 22, and 23) and two cases of five (G5, 6, 20, 26, and 27) and two (G9 and 29) cousins subjects, the remaining 17 individuals with globozoospermia belonged to unrelated families (Table 1).
Genomic DNA was extracted from individuals’ peripheral blood samples using standard salting out procedure and kept at -20°C until usage (17). Spe-
cific primers for identification of G848A, in exon 4 of SPATA16 gene and G198A in exon 13 of PICK1 gene were designed by oligo7 primer designing software (Molecular Biology Insights, CO, USA) according to the respective sequences obtained from National Center for Biotechnology Information (NCBI) database, whereas primer sequences (Table 2) for assessment of DPY19L2 deletion were ordered according to previous report (16). Missense mutations of SPATA16 and PICK1 genes were assessed using Restriction Fragment Length Polymorphism PCR (RFLP-PCR) assay, due to ability of their PCR products digestion by NciI and PvuII restriction enzymes, respectively. Indeed, G848A nucleotide variation in SPATA16 gene causes disruption of NciI site in this location. Thus, a partial PCR product (635 bp) of this gene encompassing G848A could not be cut to produce 283 and 352 bp fragments. Similarly, mutation of G198A region in PICK1 gene disrupts one of two PvuII restric-
tion sites located in this 548 bp PCR product. Thus, G198A mutation produces two bands after PvuII cut, lack of which could cause production of three bands after PvuII digestion. In this study, we did not evaluate the other mutations in these two genes. Following identification of three exons (5, 6 and 7) deletion in one of the affected Iranian individual (G9) which was previously reported by Elinati et al. (16), and due to the history of infertility in his family, blood samples of several volunteer family members were obtained and the target of interest was analyzed in their DNA samples. For detection of DPY19L2 deletion, a multiplex PCR assay was performed for exons 1, 5, 6, 7, 11 and 22 of this gene, together with a part of β-ACTIN or PROTAMIN1 genes, as internal control using specific primers (Table 2). Lack of amplification for all or some DPY19L2 exons indicates respectively total or partial deletion of this gene in the studied cases. To confirm complete deletion of this gene, specific breakpoint “a” amplification was performed in the samples with lack of amplification for all DPY19L2 exons.

Table 1: Features of 27 individuals with globozoospermia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Type of globozoospermia</th>
<th>Consanguinity</th>
<th>Deficiency in DPY19L2 gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Complete Partial</td>
<td></td>
<td>No deletion Complete deletion Partial deletion</td>
<td></td>
</tr>
<tr>
<td>G1, 4, 7, 12, 13, 15</td>
<td>✓</td>
<td>Non-familial</td>
<td>✓</td>
<td>(16)</td>
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<tr>
<td>G2, 8, 10, 14,16</td>
<td>✓</td>
<td>Non-familial</td>
<td>✓</td>
<td>(16)</td>
</tr>
<tr>
<td>G3, 18</td>
<td>✓</td>
<td>Non-familial</td>
<td>✓</td>
<td>(16)</td>
</tr>
<tr>
<td>G19</td>
<td>✓</td>
<td>Non-familial</td>
<td>✓</td>
<td>(16)</td>
</tr>
<tr>
<td>G5, 6, 20</td>
<td>✓</td>
<td>Familial</td>
<td>✓</td>
<td>(16)</td>
</tr>
<tr>
<td>G9</td>
<td>✓</td>
<td>Familial (G29)</td>
<td>✓</td>
<td>(16)</td>
</tr>
<tr>
<td>G21, 22, 23</td>
<td>✓</td>
<td>Familial</td>
<td>✓</td>
<td>Current study</td>
</tr>
<tr>
<td>G24</td>
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<td>✓</td>
<td>Current study</td>
</tr>
<tr>
<td>G25, 28</td>
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<td>✓</td>
<td>Current study</td>
</tr>
<tr>
<td>G26, 27</td>
<td>✓</td>
<td>Familial (G5)</td>
<td>✓</td>
<td>Current study</td>
</tr>
<tr>
<td>G29</td>
<td>✓</td>
<td>Familial (G9)</td>
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<td>Current study</td>
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</tbody>
</table>

### Table 2: List of primers used for polymerase chain reaction and real time PCR analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Amplified sequences</th>
<th>Primer sequence (5’→3’)</th>
<th>Annealing temperature (°C)</th>
<th>Product length (bp)</th>
</tr>
</thead>
</table>
| Conventional PCR | β-ACTIN             | F: CGTGACATTAAGGAGAAGCTGTGC  
                            R: CTCAAGGAGGAGCAGAAGATCTTCTGAT | 55                          | 375                |
| DPY19L2        | Exon 1              | F: GGCAAACTTCTTCTACTCGGAC  
                            R: GACCCAGCTCCACGACCATCTCCTT  | 65                          | 504                |
|                | Exon 4              | F: CAAAATAGCGAAGAATGATTAG  
                            R: TTCTACTCAACTAAGGATAAACAC  | 54                          | 414                |
|                | Exon 5              | F: AGCTCTCATCAGTGCACCTAT  
                            R: AGCTACTCCTGAAACTATTTT     | 60                          | 432                |
|                | Exon 6              | F: GGGTAATAATTAACACAGCA  
                            R: AAAACACAGAACTAAGGATG     | 57                          | 462                |
|                | Exon 7              | F: AATTTTACGTACACTTGTAGATA  
                            R: ATTTAAACACTTCTCAACATG     | 55                          | 420                |
|                | Exon 8              | F: TGGGAGCTGTAATTTGCTG  
                            R: TCCAAAAGTGTGAATTTGGA     | 55                          | 371                |
|                | Exon 11             | F: AACCTCCTCAAGTGACTTAG  
                            R: TTGGCCAAGAGTGCTATT      | 53                          | 516                |
|                | Exon 22             | F: GTGTCTGTATTTAAAGCTGTG  
                            R: ATTTAGCTAGACGACATACAT    | 59                          | 313                |
| Break point “a” |                    | F: ATGCCATGTTGCTGCT      
                            R: TCTTTCTGGGAAAGGTATTAGGTA | 62                          | 1700               |
| SPATA16        | Exon 4              | F: AATCTTTGGCATTTAGTATATC  
                            R: GGTCAAGGAGGCTTTATATTAC  | 58                          | 635                |
| PICK1          | Exon 13             | F: TGGGCTGCCATCCCAGTCAT  
                            R: GCCCCAGGCTGGTCACCTC      | 66                          | 568                |
| PROTAMINE1     |                     | F: CCCCTGCCATCTATAACAGGAGCCGC  
                            R: TCAAGAACAAGGAGAAAGAGTAGG  | 60                          | 530                |
| Real-time PCR  | β-ACTIN             | F: AGATGGCGTGTGTTACAGAGAAG  
                            R: TTGTTGCACTGGAAGGAGAGGAG  | 60                          | 92                 |
| DPY19L2        |                     | F: GCACCCAGCTCCACATCCTCTT  
                            R: TCCCATCTCCTCTCTACCTCGCG  | 60                          | 144                |

**Quantitative assessment of mutated DPY19L2 alleles**

qPCR was implemented by two alternative methods, to analyze the genotyping of DPY19L2 gene for the family members of three cases (G8, 14 and 21) in terms of homo/hemizygosity deletion or normal state of DPY19L2 gene. Specific DPY19L2 and β-ACTIN primer pairs were designed to quantify both the target and reference genes (Table 2). Of note, primer efficiencies for target gene (DPY19L2) and reference gene (β-ACTIN) were almost equal (Fig.1).

In the first method, samples were quantified absolutely, using a control blood sample obtained from a healthy fertile donor, who voluntarily participated in this study. After genomic DNA extraction by standard salting out procedure, 60 ng of standard genomic DNA was used as a template for further serial dilution preparations. Different amounts of DNA (60, 12, 2.5, 0.5, 0.1 ng) from this fertile donor were used as template in each PCR reaction in three set of PCR to draw a standard threshold cycle (Ct) curve (red squares shown in the Fig.1). Then, 60 ng of sample tests were subjected to PCR reactions (blue squares shown in the Fig.1). The quantity of the target gene (DPY19L2, lower curve shown in the Fig.1) and the reference gene (β-ACTIN, upper curve shown in the Fig.1) of each subject was calculated based on their Ct in the standard curve which was drawn.
with different amounts of DNA from the fertile (control) sample in ABI step one plus real-time PCR system (Life Technologies, CA, USA). Proportion of PCR products of \(DPY19L2\) to \(\beta\text{-ACTIN}\) quantities was considered for further analyses. This proportion for fertile was considered between 0.8-1, for carrier and patient cases was approximately 0.5 (ranged 0.3-0.7) and 0 respectively, as reported earlier (18). Additionally, to assess the accuracy of this method, equal volume of DNA extracts from blood samples taken from the fertile individual (control sample) and a patient with globozoospermia (G14) were mixed and the resulting mixture was used as a heterozygous (hetero) sample.

In the second method, conventional relative quantification (RQ, using \(2^{-\Delta\Delta C_{t}}\) equation) method was used with the same samples, utilizing 60 ng of DNA templates to quantify PCR-products of \(DPY19L2\) relative to \(\beta\text{-ACTIN}\). In this study, RQ level was considered 0.8-1 for normal cases, while this level was approximately 0.5 (ranged 0.3-0.7) and 0 in carrier and patients respectively, as previously reported (18). All PCR reactions contained 5 µl SYBR Green (TaKaRa, Japan), 0.2 µl Rox and 5 µM of each specific primer (0.2 µl) for \(DPY19L2\) or for \(\beta\text{-ACTIN}\) (0.5 µl) in a 10 µl final volume of PCR reaction.

**Results**

**Clinical characteristics of the patients with globozoospermia**

Clinical parameters of the patients who participated in this study are depicted in the Table 3. Analyses showed lower sperm motility of the patients, compared to the highlighted standard criteria by World Health Organization (WHO). Regarding the round-headed shape of the sperms, in this study, ICSI technique was used to obtain successful fertilization culminated in three healthy births (Table 3). In this survey, three pedigree members that suffered from globozoospermia were further studied.
Table 3: Clinical parameters of patients with globozoospermia

<table>
<thead>
<tr>
<th>Patient</th>
<th>Consanguinity of the parents</th>
<th>Round-headed sperm (%)</th>
<th>Sperm parameters</th>
<th>ICSI attempts and results</th>
<th>Live delivery (Sexuality)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Volume (mL)</td>
<td>Sperm concentration (10^6/mL)</td>
<td>Progressive motility (%)</td>
<td>Number of ICSI (ET cycles)</td>
</tr>
<tr>
<td>G1</td>
<td>Non-familial</td>
<td>100</td>
<td>3</td>
<td>80</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td>NA</td>
<td>100</td>
<td>3</td>
<td>80</td>
<td>30</td>
</tr>
<tr>
<td>G3</td>
<td>NA</td>
<td>100</td>
<td>4</td>
<td>64</td>
<td>15</td>
</tr>
<tr>
<td>G4</td>
<td>Familial</td>
<td>100</td>
<td>3</td>
<td>20</td>
<td>5</td>
</tr>
<tr>
<td>G5</td>
<td>Familial</td>
<td>100</td>
<td>3.5</td>
<td>40</td>
<td>2</td>
</tr>
<tr>
<td>G6</td>
<td>Familial</td>
<td>100</td>
<td>4</td>
<td>66</td>
<td>10</td>
</tr>
<tr>
<td>G7</td>
<td>Non-familial</td>
<td>100</td>
<td>1</td>
<td>65</td>
<td>25</td>
</tr>
<tr>
<td>G8</td>
<td>Non-familial</td>
<td>100</td>
<td>4</td>
<td>66</td>
<td>10</td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G9</td>
<td>Familial</td>
<td>100</td>
<td>4</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>G10</td>
<td>Familial</td>
<td>100</td>
<td>2.5</td>
<td>40</td>
<td>20</td>
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<td></td>
</tr>
<tr>
<td>G12</td>
<td>Non-familial</td>
<td>100</td>
<td>1.5</td>
<td>70</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>G13</td>
<td>Non-familial</td>
<td>100</td>
<td>0.5</td>
<td>67</td>
<td>25</td>
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<tr>
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<tr>
<td>G14</td>
<td>Familial</td>
<td>100</td>
<td>1</td>
<td>2</td>
<td>25</td>
</tr>
<tr>
<td>G15</td>
<td>Non-familial</td>
<td>100</td>
<td>2</td>
<td>74</td>
<td>0</td>
</tr>
<tr>
<td>G16</td>
<td>Non-familial</td>
<td>100</td>
<td>3</td>
<td>30</td>
<td>15</td>
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<td></td>
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<td>NA</td>
<td>100</td>
<td>4</td>
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<tr>
<td>G19</td>
<td>NA</td>
<td>98</td>
<td>4</td>
<td>80</td>
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<tr>
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<td></td>
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<td></td>
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<tr>
<td>G20</td>
<td>Familial</td>
<td>100</td>
<td>3</td>
<td>40</td>
<td>15</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>G21</td>
<td>Non-familial</td>
<td>100</td>
<td>3.1</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>G22</td>
<td>Non-familial</td>
<td>100</td>
<td>6.7</td>
<td>60</td>
<td>35</td>
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<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>G23’</td>
<td>Non-familial</td>
<td>100</td>
<td>2.1</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>G24</td>
<td>Familial</td>
<td>100</td>
<td>2</td>
<td>18</td>
<td>5</td>
</tr>
<tr>
<td>G25</td>
<td>Familial</td>
<td>96</td>
<td>2.9</td>
<td>90</td>
<td>40</td>
</tr>
<tr>
<td>G26</td>
<td>Familial</td>
<td>100</td>
<td>2.3</td>
<td>40</td>
<td>10</td>
</tr>
<tr>
<td>G27</td>
<td>Familial</td>
<td>100</td>
<td>1</td>
<td>40</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G28</td>
<td>Familial</td>
<td>98</td>
<td>2.3</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>G29</td>
<td>Familial</td>
<td>100</td>
<td>3</td>
<td>45</td>
<td>40</td>
</tr>
</tbody>
</table>

Two samples of G11 and G17 were lost, thus they were deleted. ET; Freeze-thawed embryo transfer, ICSI; Intra - cytoplasmic sperm in- seminat, NA; Not assigned, ND; Not done, +; Stands for successful pregnancy, -; Stands for abortion, and ‘; Globo 23 is single and not married.
Mutational analysis in \textit{SPATA16} and \textit{PICK1} genes

In this study, 27 cases with globozoospermia and 30 fertile men as control group were analyzed for detection of nucleotide variation (Table 1). In our first screening, regarding that digestion of the PCR products resulted in similar pattern to the fertile cases (data not shown), we did identify missense mutations of neither G848A in exon 4 of \textit{SPATA16} gene (Fig.2, left panel) nor G198A in exon 13 of \textit{PICK1} gene in the studied cases (Fig.2, right panel).

Analysis of \textit{DPY19L2} deletion in the cases with globozoospermia

We have previously reported that \textit{DPY19L2} gene deletion leads to globozoospermia (16). In this study, further to 14 (out of 18) individuals who had shown some deletion in \textit{DPY19L2} gene, six (out of nine) new cases with globozoospermia, missed the entire length of \textit{DPY19L2} gene (G21, 22, 23, 24, 26 and 27, Table 1). One of these six individuals was unrelated (G24), while the remaining individuals were originated from two different pedigrees (Table 1).

This experiment was carried out by several multiplex PCR on exon 1 (Fig.3A), exon 11 (Fig.3B) and exon 22 of \textit{DPY19L2} gene (Fig.3C) with a part of \(\beta\)-\textit{ACTIN} or \textit{PROTAMINE1} gene. Two new individuals (G25 and 28), with partially globozoospermia demonstrations, showed a wild-type condition for \textit{DPY19L2} gene (Table 1). Furthermore, data indicated the presence of breakpoint “a” (BPa) in most of the new cases (five out of six) with entire \textit{DPY19L2} gene deletion (Fig.3D, respective lanes for G21 and 26).

Moreover, \textit{DPY19L2} gene hemizygosity (complete deletion of one \textit{DPY19L2} gene allele) was evaluated in parents of one case (G8), who has previously been recognized to suffer from deletion of entire length of \textit{DPY19L2} gene (16). Here we confirmed hemizygosity of \textit{DPY19L2} for both parents of G8, by amplification of BPa (Fig.4A) and exon 11 (Fig.4B). Of note that other siblings of this family were fertile.
Evaluation of familial globozoospermia

In this experiment study, two cases (G5 and 9) were selected for sibling analysis. As shown in the Figure 5, the genetic pedigree belongs to family of G5 (with the history of reproductive failure and miscarriage) revealed that all of five members (G5, 6, 20, 26, and 27) had globozoospermia associated with complete deletion of \(DPY19L2\) gene. We have previously demonstrated (16) a partial deletion of \(DPY19L2\) including exons 5, 6 and 7 in one case (G9, Table 1). Due to infertility history of his family (Fig.6) and access to DNA samples of all family members, multiplex PCR of the aforementioned exons was performed. Curiously, we determined similar mutations pattern of \(DPY19L2\) gene to G9 patient, in the cousin with complete globozoospermia (G29). Indeed, detection of exons 4 and 8 by PCR confirmed this partial deletion (data not shown).

---

**Fig.5**: Pedigree of one case with globozoospermia (G5) and repeated pregnancy loss. There is more consanguineous marriage in this family but for simplicity detailed data are not depicted in the pedigree. Polymerase chain reaction (PCR) analysis showed G26, 27, 5, 6 and 20 suffering from globozoospermia due to complete deletion of \(DPY19L2\) gene. II2, III11 and III15 are infertile individuals with performing no genetic analysis. The inset numbers which are shown in the squares/circles represent the numbers of healthy (fertile) siblings who were not shown in this pedigree.
To set a reliable method for homo/hemizygosity state of DPY19L2 deletion, we performed qPCR based genotyping analysis for family members of one case who showed whole DPY19L2 gene deletion, G21. The DPY19L2 deletion consanguineous pedigree for G21 patient is shown in the Figure 7. Data analyses demonstrated whole DPY19L2 gene deletion of one allele for the G21 parents. Quantity proportion values of DPY19L2 to β-ACTIN for carriers of DPY19L2 deletion were almost between 0.4-0.6, while in normal cases, it ranged between 0.8-1 based on two calculated methods (quantities ratio and 2^{ΔΔCt} ratio presented in the Table 3). In addition, no amplification plot was detected for G21, 22 and 23 indicating lack of the mentioned gene in these subjects. In addition, detection of BPa in hemizygote individuals of the family confirmed the outcomes of qPCR assay (Fig.3D, where P1 and P2 are parents of G21 patient, B1 is the hemizygote fertile brother. G21 is proband person) (Table 4).

### Table 4: DPY19L2 genotype achieved by quantitative real-time PCR for the family members of Globo 21 and Globo 22

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Quantities ratio</th>
<th>2^{ΔΔCt} ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father (P1)</td>
<td>0.522924</td>
<td>0.4</td>
</tr>
<tr>
<td>Mother (P2)</td>
<td>0.408959</td>
<td>0.4</td>
</tr>
<tr>
<td>Brother-1 (B1)*</td>
<td>1.006982</td>
<td>1</td>
</tr>
<tr>
<td>B1 sibling</td>
<td>0.957183</td>
<td>0.9</td>
</tr>
<tr>
<td>Brother-2 (B2)*</td>
<td>0.499332</td>
<td>0.4</td>
</tr>
<tr>
<td>B2 wife</td>
<td>0.414017</td>
<td>0.4</td>
</tr>
<tr>
<td>Globo22 wife</td>
<td>1.037303</td>
<td>1</td>
</tr>
<tr>
<td>Globo21 wife</td>
<td>0.488268</td>
<td>0.4</td>
</tr>
</tbody>
</table>

*; B1 and B2 are 2 fertile patient’s (Globo21) brothers, Ct; Threshold cycle, and PCR; Polymerase chain reaction.
To extend the application of previously suggested method (quantities ratio) for identification of gene homo/hemizygosity at different individuals, we performed further analyses on the G8 and 14 patients’ parents, besides of the hemizygote sample (hetero) as notified in materials and methods. Data affirmed the hemizygote status of the parents and hetero case by two alternatively implicated calculation methods (Table 5).

**Discussion**

In the recent years, there have been an increasing amounts of literatures proposing the molecular mechanisms of globozoospermia (7, 9-13, 15, 16, 19-21). Our previous studies have described DPY19L2 gene as a basic factor required for development of normal acrosome biogenesis. Partial or complete deletion of the DPY19L2 gene is pivotal factor in globozoospermia (16).

Therefore, we investigated complete deletion of DPY19L2 gene effects to reaffirm the potential association of DPY19L2 gene and globozoospermia. In addition, deletion of this gene was evaluated in the family members of three globozoospermic individuals. Thus, deletion analysis of DPY19L2 gene (12q14.2) was carried out in three exons 1, 11, 22 of DPY19L2 gene, using multiplex PCR, compared to β-ACTIN or PROTAMINE1 genes as internal controls. Briefly, all of three assessed exons of DPY19L2 gene (1, 11, 22) were missed in 20 out of 27 cases (74%) suggesting total absence of DPY19L2 gene in these cases.

It should be noted that identification of total deletion of DPY19L2 gene with BPa in 18 cases, out of 27, has previously been reported by Elinati et al. (16). Overall, six out of nine new individuals showed complete deletion of DPY19L2 gene, five of whom carried BPa and the remaining may have unknown BP. Also, one new patient (G29) harbored a partial deletion of this gene and two others (G25 and G28) with partial globozoospermia had two wild type alleles. Previous studies have also demonstrated molecular mutations in DPY19L2 gene (19, 21). Deletion of the DPY19L2 gene is a common genomic rearrangement that occurs due to LCRs flanking the gene by NAHR. Concurrent with the cases, family members of three globozoospermic patients were investigated in this study. In this regard, two pedigrees (G5 and 9 pedigrees) from different geographically accommodation regions, similar ethnicity and high rate of consanguineous marriages showed the history of reproductive failure due to globozoospermia. Regarding high incidence of this rare abnormality among tribal races, diagnosis of carrier individuals could help them, in terms of genetic management, for future family planning.

Several studies have previously detected heterozygosity of the other genes, like SMN1 and DYSTROPHIN, through quantitative real-time PCR based on comparative Ct method (18, 22, 23). In this article, we identified the carriers in one pedigree (G21 pedigree) by this method and also proposed a modified method, quantities ratio. Thus, we designed qPCR assay for family members of G21. Analyses were performed based on proportion of DPY19L2 to β-ACTIN quantities. After providing the standard curve based on serial diluted DNA samples of a fertile man, quantities of the reference and target gene were estimated. Quantitative analysis of DPY19L2 gene for G21

<table>
<thead>
<tr>
<th>Subject</th>
<th>Quantities ratio</th>
<th>2^-∆∆Ct ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Globo 14 father</td>
<td>0.58377</td>
<td>0.5</td>
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<tr>
<td>Globo 14 mother</td>
<td>0.40623</td>
<td>0.4</td>
</tr>
<tr>
<td>Globo 8 father (P1)</td>
<td>0.47476</td>
<td>0.5</td>
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<tr>
<td>Globo 8 mother (P2)</td>
<td>0.424836</td>
<td>0.4</td>
</tr>
<tr>
<td>Hetero</td>
<td>0.560374</td>
<td>0.4</td>
</tr>
<tr>
<td>Control-1</td>
<td>0.973175</td>
<td>0.9</td>
</tr>
<tr>
<td>Control-2</td>
<td>1.381863</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Ct; Threshold cycle and PCR; Polymerase chain reaction.

**Table 5: **DPY19L2 genotype achieved by quantitative real-time PCR
family members led us to identify individuals with hemizygosity at this gene. We determined that parents with a quantity ratio ranging between 0.4-0.6 are carrier. One of the fertile brothers (B2) as well as partners of G21 and B2, were hemizygote for deletion of DPY19L2 gene. Quantity ratio for normal cases, consisting one of the fertile brother, the grand daughter and partner of G22, were ranging from 0.95-1.3. These results were similar to previously reported threshold cycle method verifying our conclusion to determine the individuals with no gene deletion or carriers (18, 22). Considering non-consangunuity of parents, the incidence of the abnormality in this family could be attributed to their accommodation in the same geographical area. To validate our calculation method on the allele hemizygosity, we extended experiments on the G8 and G14 patients’ parents who kindly accepted to participate voluntarily in this survey. These findings are in agreement with previous studies, indicating a strong relationship between DPY19L2 gene and globozoospermia. However, molecular cause of few cases remains yet unclear, requiring further investigations to identify genetic defect(s) in the other gene(s) affecting globozoospermia.

Regarding the other genes, in the present study mutation screening of the SPATA16 and PICK1 genes were also carried out on 27 cases with globozoospermia and 30 fertile men. Our data revealed that PICK1 and SPATA16 genes were intact in all of studied individuals.

Conclusion

Our result revealed that qPCR analysis can be used for genotyping of DPY19L2 deletion and this may help genetic consolers in family planning. In future, it might also help prevent occurrence of this syndrome in carrier families through pre-implantation genetic diagnosis, especially in ethnic community with high consanguineous marriages.

Acknowledgements

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References


Original Article

Thyroxin Is Useful to Improve Sperm Motility

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Abstract

Background: The aim of this study was to evaluate the non-genomic action of thyroxin on sperm kinetic and its probable use to improve sperm recovery after applying an enrichment method like “swim-up” in comparison with the available one, pentoxifylline.

Materials and Methods: This is an experimental study. A total of 50 patients were recruited, followed by infertility consultation. Conventional sperm assays were performed according to World Health Organization criteria-2010 (WHO-2010). A Computer Aided Semen Analysis System was employed to assess kinetic parameters and concentrations. Number of the motile sperm recovered after preparation technique was calculated.

Results: Addition of T4 (0.002 µg/ml) to semen samples increased hypermotility at 20 minutes (control: 14.18 ± 5.1% vs. 17.66 ± 8.88%, P<0.03, data expressed as mean ± SD) and remained unchanged after 40 minutes. Significant differences were found in the motile sperm recovered after swim-up (control: 8.93×10^6 ± 9.52×10^6 vs. 17.20×10^6 ± 21.16×10^6, P<0.03), achieving all of the tested samples a desirable threshold value for artificial insemination outcome, while adding pentoxifylline increased the number of recovered sperm after swim-up in 60% of the studied cases. No synergism between two treatments could be determined.

Conclusion: We propose a new physiological tool to artificially improve insemination. The discussion opens windows to investigate unknown pathways involved in sperm capacitation and gives innovative arguments to better understand infertility mechanisms.

Keywords: Sperm Motility, Thyroxin, Pentoxifylline, Artificial Insemination


Introduction

Assisted reproductive technology has been grown by leaps and bounds in the last few years. It is now being increasingly available to infertile couples in both developed and developing countries. In the “Tenth World Report” on assisted reproductive technology, a total of 954743 initiated cycles resulted in an estimated 237809 babies born in 2004. Of all cycles, 60.6% were intracytoplasmic sperm injection (ICSI) (1). The intrauterine insemination (IUI) with husband’s sperm is another assisted reproductive technology, generally believed to be the first choice of treatment rather than more invasive and expensive techniques particularly in the case of cervical infertility, moderate male infertility, dysovulation, mild or moderate endometriosis or unexplained infertility. In the last three indications, ovarian stimulation is necessary. Higher rates of pregnancy obtained by IUI with husband’s sperm and lower risk of multiple pregnancy are related to couple demographic characteristics (age of partners, lifestyle and duration of infertility) and the etiology of infertility (ovarian reserve, uterus, and spermogram). Pregnancy rates were observed ranging from 8 to 20% per cycle according to indications (2). In spite of the huge heterogeneity of patient groups and IUI treat-
ment strategies, it is generally accepted that inseminating motile count after washing should be between 0.8 and 5 million; IUI pregnancy outcome is significantly improved above the latter count, while below the former, a successful outcome is unlikely (3). A recent publication confirmed that total motile spermatozoa inseminated is a predictor of pregnancy rate as well as maternal age and emphasized the impact of the time interval from the end of sperm preparation to IUI on the outcome (4), a variable that probably prevents the deleterious effect of oxidative stress. Adding pentoxifylline, as an inhibitor of cAMP phosphodiesterase enzyme, in semen preparation for IUI improves sperm motility in asthenozoospermic samples due to its capacity to favor increasing intracellular cAMP concentration. Applying this inhibitor has been demonstrated to exert higher and significantly different pregnancy rates in couples classified infertile for male factor (5).

Asthenozoospermia is probably the main feature in male infertility and has multifactorial origins (6). Although the prevalence of hypothyroidism is higher in females than in males, we wonder about the role of thyronines in sperm motility, considering that they act on nearly every cell in the body increasing the basal metabolic rate. In an experimental design hypothyroid rats showed alterations in the motility of sperm recovered from their epididymis. Transmission electron microscopy technique revealed changes in epididymis epithelial cell’s mitochondria associated with incipient apoptosis, while modifications in proliferative capacity could not be evidenced by argyrophilic proteins of the nucleolar organizer region (7). This technique detects, using silver salts, argyrophilic proteins of the nucleolar organizer region (AgNOR). The number and size of NOR reflect cell activity, proliferation and transformation. Classically thyroid hormones were thought to act in a genomic way. The discovery of a non-genomic action of T4 hormone on sperm motility and its probable use to improve sperm recovery after an enrichment method like “swim-up” in comparison with the available one, pentoxifylline.

Materials and Methods

Patients

A total of 50 patients (age: 30-50 years old) consulting for infertility were recruited; males with abnormal thyroid function or using thyroid medication were excluded (clinical history). The present study was conducted according to the guidelines laid down in the “Declaration of Helsinki” and approved by the Institutional Review Board of The Clinical Hospital “José de San Martin”; all the participants received information on the project and gave written informed consent.

Design

This is an experimental study and was designed as a prospective, analytical and intervention investigation without randomization.

Sperm assays

Conventional sperm assay was performed according to World Health Organization criteria- 2010 (WHO-2010) (9). A Computer Aided Semen Analysis system (SCA Microptic SL Barcelona, Spain) was employed to assess kinetic parameters and sperm count. The basic components of the system were: a bright field microscope with phase contrast microscopy to visualize the sample (Nikon E- 200, Japan), a digital camera to capture images (Basler A312 Inc., Vision-Technology, Germany) and a computer with the installed SCA® software. Samples were laid on a thermostatic plate at 37°C. A minimum of 400 sperm cell tracks were captured and 25 digitized images per second were analyzed for each sample. The assays were conducted in accordance with instrument’s standardization and mRNA translation. Furthermore, they influence the regulation of actin polymerization affecting cytoskeleton dynamics. They also modulate mitochondrial respiration (8).

The aim of this study was to evaluate the non-genomic action of T4 hormone on sperm kinetic and its probable use to improve sperm recovery after an enrichment method like “swim-up” in comparison with the available one, pentoxifylline.
validation (10), using a Leja chamber 10 (10 µm in depth). A qualified operator validated each analyzed image.

Data from individual motile spermatozoa, defined by 8 kinematic parameters [curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR), mean amplitude of lateral head displacement (ALH), wobble (WOB) a measure of oscillation of the actual path about the average path and beat cross frequency (BCF)] were assessed and ratios were then calculated relating the different speeds (LIN=VSL/VCL, SRT=VSL/VAP, WOB=VAP/VCL). The criteria for detecting hyperactivated spermatozoa was VCL>35 μm/s, ALH>2.5 μm, STR>85%. This CASA criteria for hyperactivation was established by the SCA manufacturer and corresponded to the “transitional activated pattern” (11).

**Sperm preparation technique: “swim-up”**

By the swim up technique, the sperms were selected on their motility and the capability to swim out of the seminal plasma. This is the most prevalently used technique in “in vitro fertilization” laboratories and will be preferred if the semen sample is normozoospermic (12). The swim-up technique generally produces less reactive oxygen species (ROS) than the other commonly used enrichment technique by using the density-gradient centrifugation, thus generating less sperm DNA damage (13). Briefly after the fluidification of the sample, the semen (well mixed) was divided in fractions of 0.5 ml and transferred into centrifuge tubes. Then, 1.5 ml of culture medium was placed over the semen with extreme attention in each tube, leading to form two phases. The tubes were put in the incubator, inclined at an angle around 45°C and incubated at 37°C for 60 minutes. By inclining the tubes at 45°C, we increased the surface between the medium and the semen, improving the capability of the sperms to swim out of the semen and to reach the medium. After that, the tubes were turned back the vertical position and the upper phases of each one were gently aspirated and collected into one tube which was subsequently centrifuged at 600 g for 15 minutes and its volume was adjusted to 0.5 ml. If IUI was performed, 0.3-0.4 ml of spermatozoa suspended in sterile medium would be required.

Before and after treatment of the seminal fluid, the following parameters were evaluated in line with the WHO Manual 2010: volume (ml), concentration (millions/ml) and motility (progressive motility) (9). The concentration of the progressive spermatozoa (PS) is calculated by multiplying the percentage of the progressive sperms (PS%) and the concentration of the sperms (S) in the final preparation. Total number of the PS (TPS) is calculated by multiplying (PS) and the volume (V) in the final preparation.

\[
[PS]=\text{PS}\times[S]\text{final}
\]

\[
TPS=[PS]\times V_{\text{final}}
\]

The total number of the progressive sperms in the preparation, before IUI, might be defined as a threshold value in predicting IUI outcome. The accepted cut-off value is five millions (3).

Capacitating medium was composed of HAM F-10 1x (+25 mM HEPES and L-glutamine, GIBCO-Life Technology, USA) supplemented with penicillin-streptomycin (10000 U/ml penicillin, 10 ml µg/ml streptomycin, both obtained from GIBCO-Life Technology, USA) and serum substitute supplement (Irvine Scientific, California, USA).

**Improving sperm enrichment technique**

Following the experimental design proposed by Nassar et al. (14), we compared the effect of pentoxifylline and T4 (Levotiroxina Montpellier 200 µg/ml) on sperm recovery after swim-up.

The recovery rate was calculated as follows:

\[
\%\text{ Recovery rate}=\%\text{ PS}_{\text{final}}\times V_{\text{final}}\times[S]_{\text{final}}/\%\text{ PS}_{\text{initial}}\times V_{\text{initial}}\times[S]_{\text{initial}}\times 100
\]

In this equation, initial and final means sperm parameters before and after the “swim -up”, respectively.

**Statistical analysis**

Evaluation results after supplementation was based on Student t test (MedCal). P values of less than 0.05 were considered statistically significant.
Results

Choosing an optimal dose of T4

We used a semen sample with exceeding the normal cut-off value established by WHO, showing sperm concentration of \(>179.6 \times 10^6\) sperm/ml, sperm progressive motility of 63.9\%, sperm morphology of 23\%, sperm vitality of 85\% and sperm volume of 4.8 ml. The sample was diluted 1/10 to achieve an optimal concentration for kinetic studies. Kinetic parameters were evaluated after the adding T4 (Levotiroxina Montpellier, 200 µg/ml). Hormone dilutions ranging from particular serum concentration (0.1 µg/ml) to the expected seminal plasma concentration (0.001 µg/ml) were tested [final concentration of 0.2 µg/ml (Dil.: 1), 0.002 µg/ml (Dil.: 2), 0.000002 µg/ml (Dil.: 3), 0.00000002 µg/ml (Dil.: 4). The undiluted hormone showed cytotoxicity for the sperm, represented by complete immotility and necrozoospermia (Vital test). In our system, Ga was defined with VCL>35 µm/s. Lineality>50\% and Straightness>80\%. Hormone dilution of 2 (Dil.: 2) was determined to improve Ga sperm motility, suggesting eligibility of this T4 concentration (basal n=307: 35.2\%; Dil. 1. n=335: 35.2\%; Dil. 2. n=357: 44.5\%; Dil 3. n=427: 34.9%; Dil 4. n=423: 34\%).

Non genomic effect of T4 on sperm kinetic

Semen samples were fractioned in two aliquots of 0.5ml each (n: 17). Hormone dilution was prepared at that moment preventing oxygen and light effect [0.02 µg/ml in phosphate-buffered saline (PBS) with pH=7.2-7.4]. Working in sterility, to avoid contamination, 50 µl of the freshly prepared hormone was added to the first aliquot. No hormone was added to the second aliquot, as the control of test group. Significant differences were found in the number of motile sperms recovered after procedure in the studied groups (control: \(2.16 \times 10^6 \pm 2.55 \times 10^6\) vs. \(3.00 \times 10^6 \pm 2.6 \times 10^6\), \(P<0.03\), data expressed as mean ± SD).

Improving the chances for intrauterine insemination outcome

Total number of the progressive sperms in the preparation before the IUI could be defined as a threshold value in predicting outcome in IUI. The accepted cut-off value is five millions (3).

Table 1 shows projection of the results evaluated in the former experiment, in case of using complete sample (i.e. whole volume of the ejaculate, as a procedure currently performed in andrology laboratories prior to IUI). Significant differences were found in the number of motile sperm recovered after this procedure in the studied groups (control: \(8.93 \times 10^6 \pm 9.52 \times 10^6\) vs. \(17.20 \times 10^6 \pm 21.16 \times 10^6\), \(P<0.03\), data expressed as mean ± SD).

From 17 tested semen samples, 14 increased the number of recovered sperms, reaching the desired threshold value for IUI outcome. Notably, only in three cases a negative difference was observed, due to outstanding semen smears, whereby no needs of improvement methods are required.

Thyroxine and Sperm Motility
Comparing the effect of the proposed T4 method with pentoxifylline

Semen samples were fractioned in four aliquots (n: 10). The first aliquot was served as the control group. T4, with final concentration of 0.002 µg/ml, was added to the second aliquot. pentoxifylline (final concentration of 1 mg/ml) was added to the third aliquot and the synergism between these two treatments was evaluated in the fourth aliquot. Swim-up method was then performed and the recovery rate was calculated. Findings showed that T4 treatment was statistically superior to the control (control: 15.31 ± 8.46% vs. 25.71 ± 11.46%, P<0.01, data expressed as mean ± SD). No significant difference was observed by comparing the control with treated pentoxifylline group (control: 15.31 ± 8.46% vs. 24.85 ± 17.56%, data expressed as mean ± SD). In addition, we did not find any significant difference between control group and the treated samples with both T4 and pentoxifylline (control: 15.31 ± 8.46% vs. 21.47 ± 20.51%). Out of ten, the recovery rate was improved in eight samples by using T4 treatment (80%), while this improvement was observed only in six samples (60%) by treating with pentoxifylline (Fig.1). No synergism was detected between these types of treatment in our experiments.

### Table 1: Differences in the total motile sperm recovered with/without T4 hormone in each patient, by using the whole ejaculate

<table>
<thead>
<tr>
<th>Total motile sperm without T4 (×10⁶)</th>
<th>Total motile sperm with T4 (×10⁶)</th>
<th>Outcome differences between procedures (×10⁶)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.61</td>
<td>10.71</td>
<td>7.09</td>
</tr>
<tr>
<td>3.61</td>
<td>4.46</td>
<td>0.86</td>
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<td>3.04</td>
<td>6.27</td>
<td>3.22</td>
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<tr>
<td>1.91</td>
<td>4.84</td>
<td>2.93</td>
</tr>
<tr>
<td>7.12</td>
<td>8.93</td>
<td>1.81</td>
</tr>
<tr>
<td>19.82</td>
<td>15.76</td>
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<tr>
<td>36.64</td>
<td>86.17</td>
<td>49.53</td>
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<td>15.56</td>
<td>17.19</td>
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<tr>
<td>71.13</td>
<td>50.95</td>
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<td>8.09</td>
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<tr>
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<td>5.72</td>
<td>2.07</td>
</tr>
</tbody>
</table>

**Discussion**

In this study, treating semen samples with T4 generated a significant increase in the percentage of hyperactive sperm. The effect of this hormone appears to happen rapidly, since there was no change between data on the objective motility after 20 and 40 minutes. The immediate action of this hormone oriented us to propose a non-genomic action of T4, as a novel concept which might change the current...
investigation approaches in fertility. More beyond that and aligned with previous studies from our group, demonstrating the positive effect of pentoxifylline induction on the hyperactivity and quantity of progressive motile sperms after swim-up (15), at basal time, encouraged us toward investigating the potential effect of T4, as a novel agent, on optimization of the sperm preparation for IUI in the fertilization laboratories. In this experiment, we demonstrated that our hypothesis was correct, evidenced by the improvement of sperm count after swim-up. Thus, a patent was then presented in our country for this finding on April 13th 2013. titled: “Método in vitro para la capacidad de espermatozoides. Instituto Nacional de la Propiedad Industrial. Administración Nacional de Patentes. INPI Exp: 20130101645. Inventores: Gabriela R. Mendeluk; Mónica Rosales; Mercedes N. Pugliese; Patricia H. Chenlo”.

In this study, we did not detect any synergism between pentoxifylline and T4. Similar to the previous study, treatment with pentoxifylline increased the number of recovered sperms after swim-up in 60% of the cases, from a mathematical point of view. Focusing on a program of assisted reproduction, at least 5 million progressive motile sperms are needed for IUI outcome. From a clinical point of view, all of the studied samples overcame this barrier by employing T4. Interestingly, overcoming the cut-off value is the only negative result obtained by comparing swim-up outcome treated with or without the hormone, suggesting that there is an upper motility limit, above which adding the hormone has no effect. Despite the proposed method is reasonable for subfertile sperms, it could not be applicable for severely impaired semen samples, as an alternative beyond ICSI. This is in accordance with findings obtained from the target group which has been benefited by this treatment.

Thyroid hormones can exert their actions at different cellular levels: within the cell nucleus, at the plasma membrane, in cytoplasm, and at the mitochondrion. Regarding the fact that sperm nucleus is compacted, the first option was discarded in this study. Actions of thyroid hormone that are not initiated by liganding hormone to intranuclear thyroid receptors are termed nongenomic. Nongenomic actions are independent from protein synthesis, thus inducing short time responses. They may trigger plasma membrane receptors like αVβ3 integrin, activating signal transduction via kinase pathways or modifying ion fluxes and membrane potential. They can also act by specific binding sites located in the cytosol. This mechanism is related to cytoskeleton dynamic, since they promote actin polymerization (16).

In 1996, Fusi et al. (17) reported that expression of specific integrin chains were altered with the functional state of spermatozoa. The cells displaying beta 3 integrin were correlated with a proportion of spermatozoa which had undergone an acrosome reaction, following calcium ionophore exposure. Boissonnas et al. (18) demonstrated the presence of αVβ3 integrin by performing Western blot and immunofluorescence techniques on the sperm membrane. Using the previous statements, we can infer that there is a non-genomic receptor for T4 hormone, at least in sperm membrane. The molecular mechanisms of the effect of this hormone on sperm kinetic are still difficult to evaluate. Our hypothesis, based on what have yet been determined about the non-genomic effect of T4 on ion channels, is that it increases Ca^{2+} intake (19) which in the HCO3^{-} environment stimulates atypical sperm adenyl cyclase. Finally, generation of cAMP could increase protein kinase A (PKA) activity at the molecular level leading to vigorous flagellar movement, called “hyperactivation” (20, 21). Pentoxifylline, as a phosphodiesterase inhibitor, potentiate cAMP lifespan by preventing degradation of this molecule. Indeed, it acts in the last part of this cascade, while T4 is involved on the upstream having a major role in the phenomenon. More beyond that, and in this context, thyroid hormones also act upstream of phospholipase C (PLC), forming diacylglycerol (DAG) and inositol 3.4.5-phosphate (IP3), while the latter molecule has a capacity to release Ca^{2+} from intracellular stores feeding back the process. Consistent to this, we could explain the non-synergism and probable complementation between T4 and pentoxifylline, as well as inferior recovery rates obtained by using the latter agent.

In females, hypothyroidism is mainly associated with oligomenorrhea. Several adverse reproduction outcomes are related to overt hypothyroidism, such as increased incidence of spontaneous abortions, placental abruption, fetal distress in labor, premature delivery and/or low birth weight, as well as gestation-induced hypertension (22). Thyroid dysfunction has also been linked to reduced fertility. Hence, it should be further investigated the fact whether adding T4, as with in vitro, could be effectively supplied in vivo. It is proposed that by lack of
this agent administration, the sperm would have less chance to acquire hyperactivated status, as a physiological and mechanical requirement to improve fertilization outcome. In our opinion, the hormonal thyroid status, either in male or female, should be considered while evaluating the predictive factors for pregnancy in case of IUI administration (23).

It has been proposed that IUI is less expensive, less invasive, and comparatively effective for selected patients, as a first-line treatment for couples (24). We are currently suggesting a new physiological tool to improve this technique which requires further evaluation to validate its potentially effective application in reproduction. The discussion opens our minds to think in unknown pathways involved in sperm capacitation and gives innovative arguments to better understand infertility mechanisms.

Conclusion

Our report is the first, to our knowledge to envisage a non-genomic action of thyroxine on sperm with direct impact on clinical.

Acknowledgments

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References

Human Sperm Quality and Metal Toxicants: Protective Effects of some Flavonoids on Male Reproductive Function

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Abstract
Background: Metals can cause male infertility through affection of spermatogenesis and sperm quality. Strong evidences confirm that male infertility in metal-exposed humans is mediated via various mechanisms such as production of reactive oxygen species (ROS). Flavonoids have antioxidant and metal chelating properties which make them suitable candidates for neutralizing adverse effects of metals on semen quality. In the current study, we have evaluated the effects of five types of flavonoids (rutin, naringin, kaempferol, quercetin, and catechin) on recovery of sperm motility and prevention of membrane oxidative damage from aluminum chloride (AlCl₃), cadmium chloride (CdCl₂), and lead chloride (PbCl₂).

Materials and Methods: In this experimental study, motility and lipid peroxidation of metal-exposed sperm was investigated in the presence of different concentrations of five kinds of flavonoids. Malondialdehyde (MDA) production was assessed as a lipid peroxidation marker.

Results: Aluminum chloride (AlCl₃), cadmium chloride (CdCl₂), and lead chloride (PbCl₂) diminished sperm motility. Treatment of metal-exposed sperm with rutin, naringin, and kaempferol attenuated the negative effects of the metals on sperm motility. Quercetin and catechin decreased the motility of metal-exposed sperm.

Conclusion: Based on the MDA production results, only AlCl₃ significantly induced lipid peroxidation. Treatment with rutin, naringin, and kaempferol significantly decreased MDA production.

Keywords: Metal Toxicity, Sperm Motility, Lipid Peroxidation, Flavonoids, Semen Quality

Introduction
Metals are one of the main constituents of an industrialized lifestyle that have a wide range of applications. Metals such as lead (Pb), aluminum (Al) and cadmium (Cd) induce toxicity in humans and other living organisms by impacting enzyme activity and generation of free radical production. However, in terms of their unique characteristics, their applications are expansive, even in medical and drug industries (1, 2).

Metals can affect male and female fertility by induction of reactive oxygen species (ROS) production. Therefore, antioxidant therapy that inhibits metal-induced toxicity is under active investigation (3). Flavonoids are a broad group of natural antioxidant compounds with flavan nucleus and a benzo-γ-pyrene structure. These compounds are low molecular weight polyphenols ubiquitously synthesized by green plants that may show various pharmacological attributes according to their chemical structures (4). Direct antioxidant effects and the ability of flavonoids to chelate metal ions have been previously researched (5-7). Researchers report the existence of a cardioprotective role (8, 9) and free radical scavenging potential of flavonoids (10). Until now, over 4000 natural flavonoids have been identified in leaves, seeds, barks, and flowers.
of different plants (11). Protection against ultraviolet (UV) light, pathogens, herbivores, and the attraction of pollinating insects are major proposed roles for flavonoids in various plants (12-14). Flavonoids can occur both in the free form and as glycosides. Their structure is composed of a basic C$_6$-C$_3$-C$_6$ phenyl-benzopyran backbone (Fig.1). The position of the phenyl ring relative to the benzopyran moiety, oxidation of central ring, hydroxylation profile, and degree of polymerization determine chemical properties of a flavonoid (15).

Fig.1: Chemical structure of flavonoids. A. Basic structure of a flavonoid with two benzene rings and a heterocyclic pyran ring as the linker. Chemical structures of: B. Rutin, C. Naringin, D. Kaempferol, E. Quercetin, and F. Catechin.

ROS induce cellular membrane instability (16), destruction of DNA structures, and promotion of transformation, (17) ultimately resulting in cellular aging (18), mutagenesis (17), carcinogenesis (19), induction of coronary heart disease (CHD) (4), and infertility (20). In addition to ROS, nitrogen reactive species (NOS) can cause cardiovascular diseases (CVD) through oxidation of LDL particles (21, 22) and increased release of matrix metalloproteinase-2 (MMP-2) in the coronary effluent (23). Based on the scientific findings, a flavonoid-rich diet is highly recommended to decrease CVD and other ROS-/NOS-induced myocardial injuries (4).

Recent interest in flavonoids arises from the potential health benefits attributed to the antioxidant activities of these polyphenolic compounds. Functional hydroxyl groups in flavonoids mediate their antioxidant effects by scavenging free radicals and/or by chelating metal ions (4, 11). The chelating of metals can be crucial in prevention of radical generation which damage target biomolecules (11). In the current study, we have evaluated the effects of five types of flavonoids (rutin, naringin, kaempferol, quercetin, and catechin) on recovery of sperm motility and prevention of membrane oxidative damage from aluminum chloride (AlCl$_3$), cadmium chloride (CdCl$_2$), and lead chloride (PbCl$_2$).

**Materials and Methods**

**Materials**

For this experimental study, AlCl$_3$, CdCl$_2$, PbCl$_2$, naringin, kaempferol, and quercetin were obtained from Merck (Darmstadt, Germany). Rutin, catechin and the remainder of chemicals and reagents used in this research were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**Sample collection and preparation of sperm suspension**

Sperm samples considered compatible to the world health organization (WHO) reference value for human semen (volume ≥3.0, sperm concentration/ml ≥50×10$^6$, forward motility ≥60%, and atypical forms ≤40%) (24) were collected and pooled from 40 healthy, non-smoking volunteers, that resided in Ahvaz, Khuzestan Province, Iran. We compared the effects of flavonoids on motility and lipid peroxidation of metal-exposed sperms using laboratory studies. The Institutional Ethics Committee of Ahvaz University of Medical Sciences reviewed and approved the protocol. All participants in the current study signed informed consents. Collected sperm samples were separated from semen plasma for assessment of clinical attributes by washing three times with an equal volume of M$_6$ solution and subsequent centrifugation for 10 minutes at 1600 g (25). M$_6$ solution contained (per liter, pH=7.4): 0.55% NaCl, 0.03% KCl, 0.019% CaCl$_2$, 0.016% K$_3$PO$_4$, 0.029% MgSO$_4$, 0.031% NaHCO$_3$, 0.496% HEPES, 0.26% sodium lactate, 36×10$^{-4}$% sodium pyruvate, 0.11% glucose, 0.4% bovine serum albumin, 60×10$^{-4}$% penicillin, and 50×10$^{-4}$% streptomycin. Separated pellets were suspended in M$_6$ solution at a density of 100 million sperm/ml and freshly were used. Sperm counts were performed by a MMC-SK Sperm Counting Chamber (Saint Petersburg, Russia).

**Incubation of sperm samples with aluminum chloride, cadmium chloride, and lead chloride**

We evaluated the effects of AlCl$_3$, CdCl$_2$, and PbCl$_2$ on sperm motility and lipid peroxidation of sperm cells at different concentrations (125 µM,
250 μM, 500 μM, 1 mM, and 5 mM) of the metal salts. The metal salt solutions were prepared in M₉ solution. Sperm samples were incubated in the presence of defined concentrations of these metals for 2 hours at 37°C. From the examined concentrations of metals, we selected those that significantly impacted sperm motility for additional experiments with the flavonoids (P≤0.05).

Effects of flavonoids on the motility of metal-exposed sperm

Sperm samples were treated for 2 hours at 37°C with AlCl₃ (1.0 mM), CdCl₂ (500 μM) or PbCl₂ (250 μM) in the presence of various concentrations (25, 50, 100, 200, 500, and 1000 μM) of rutin, naringin, kaempferol, quercetin, and catechin. Subsequently, we assessed sperm mobility by MMC Sperm. In order to increase solubility, all flavonoids were solvated in a 1:1 (v/v) of Dimethyl sulfoxide (DMSO): M₉ solution prior to their treatment of the sperm cells.

Effects of flavonoids on lipid peroxidation of metal-exposed sperm

Induction of lipid peroxidation was evaluated in sperm samples in the presence of various concentrations of AlCl₃, CdCl₂, and PbCl₂. Between treated groups, sperm samples treated with 20 mM of AlCl₃ were simultaneously incubated with 25 μM, 50 μM, 100 μM, 200 μM, 500 μM, and 1 mM each of rutin, naringin, kaempferol, quercetin, and catechin for 2 hours at 37°C. After incubation, we assessed for lipid peroxidation of the sperm cells according to the indicated approach.

Analytical methods

Assessment of sperm motility

Evaluation of sperm motility was performed by MMC Sperm (MultiMedia Catalog Sperm). MMC Sperm is an automated image analysis software package for sperm quality analysis according to parameters recommended by the WHO laboratory manual (26).

Measurement of lipid peroxidation

Lipid peroxidation was measured using malondialdehyde (MDA) and thiobarbituric acid-reactivity (27, 28). Briefly, 50 μl of 0.2% butylated hydroxytoluene (dissolved in ethanol) and 1.0 ml of 15% aqueous trichloroacetic acid were successively added to 2.0×10⁷ sperm. The mixture was then centrifuged at 4000 g for 15 minutes at 4°C. An aliquot of 500 μl of the deproteinized supernatant was added to 1.0 ml thiobarbituric acid (0.375% in 0.25 M HCl) and the mixture was heated at 100°C for 20 minutes. After cooling, the solution was analyzed by a spectrophotometer at 532 nm.

Statistical analysis

All treatments were performed in triplicate. Each experiment was run at least three times. Results were expressed as mean ± SE. Significance of difference between treatment groups was determined by the student’s t test. P<0.05 was considered statistically significant.

Results

Effects of aluminum chloride, cadmium chloride, and lead chloride on sperm motility

AlCl₃ is an abundant metal in the earth which has toxic effects. High concentrations of AlCl₃ induce free radical-mediated cytotoxicity and can be toxic for the male reproductive system (29, 30). In previous studies, it has been shown that treatment with AlCl₃ could decrease ejaculate volume, sperm concentration, and sperm motility (31). CdCl₂ is a well-known nephrotoxin and carcinogen (32, 33) that can induce ROS production. Exposure to CdCl₂ may result in decreased sperm concentration, diminished sperm motility, creation of abnormal forms of sperm following long-term exposure to CdCl₂ (3, 34), and infertility in treated male mice (35). PbCl₂ poisoning can result in decreased sperm motility. A number of reports discuss DNA fragmentation in sperm cells exposed to this metal in vitro (36). Our in vitro studies have confirmed the above mentioned findings where different concentrations of AlCl₃, CdCl₂, and PbCl₂ significantly decreased sperm motility (P≤0.05, Fig.2). Mean sperm motility after a 2-hour incubation period in the presence of 5.0 mM AlCl₃, CdCl₂, and PbCl₂ were 93% (AlCl₃), 75% (CdCl₂), and 41% (PbCl₂) less than the control groups. As seen in Figure 2, the effect of Pb on sperm motility was higher at the same concentrations of the three tested metals AlCl₃, at the 1.0 mM concentration, significantly affected sperm motility (P≤0.0013). The 500 μM concentration of CdCl₂ significantly affected sperm motility (P≤0.032), whereas PbCl₂ significantly affected motility at the 250 μM (P≤0.0005) concentration (Fig.2). The adverse effects of all three metals on sperm motility were completely dose-dependent.
Effects of flavonoids on motility of aluminum chloride-exposed sperm

Previous studies reported an in vitro protective effect of ascorbic acid (vitamin C) and tocopherol (vitamin E) on AlCl₃-treated sperm (31, 37). As seen in Figure 2, 1000 µM of AlCl₃ significantly decreased sperm motility by 15% (P≤0.0013). Therefore, we used this concentration for additional studies with flavonoids. We used different concentrations of rutin, naringin, kaempferol, quercetin, and catechin for motility recovery of AlCl₃-exposed sperm. Compared to the untreated control group, rutin increased sperm motility by 9% at the 50 µM concentration and 18% at the 200 µM concentration. Naringin, at a final concentration of 100 µM, significantly increased sperm motility by 9% (P≤0.038). There was a gradual increase in recovery of sperm motility when the concentration of naringin increased to 500 µM (Fig.3). Kaempferol showed the most protective effect of all the tested flavonoids. There was 10% recovery of sperm motility at the kaempferol concentration of 25 µM. On the other hand, effects of quercetin and catechin on the sperm mobility completely differed from the other tested flavonoids - rutin, naringin, and kaempferol. The antioxidants, quercetin and catechin did not protect sperm cells from heavy metal-mediated damages; rather, they showed inhibitory effects on sperm motility. When we increased the concentrations of quercetin and catechin from 0 to 1000 µM, there was a gradual decrease in sperm motility compared to the untreated control group. Mean motility of AlCl₃-exposed sperm after a 2 hours incubation period in the presence of 1000 µM quercetin was 22% and for catechin, it was 28%.

Effects of flavonoids on motility of cadmium chloride-exposed sperm

Previous studies by El-Demerdash et al. (3) in male rats showed beneficial effects of vitamin E and β-carotene in reducing the toxic effects of CdCl₂ on the male reproductive system. In the current study, we observed that treatment with rutin, naringin, and kaempferol resulted in recovery of motility in CdCl₂-exposed sperm cells. Our results showed that rutin, naringin, and kaempferol at 25-500 µM significantly increased (P≤0.05) motility of CdCl₂-exposed sperm cells in a dose-dependent manner (Fig.4). In contrast, quercetin and catechin did not induce any protective effect against CdCl₂ toxicity; they reduced the motility of CdCl₂-exposed sperm compared to the untreated control samples (Fig.4). These results disagreed with an in vivo study by Farombi et al. (38) about the antioxidative nature of quercetin. They showed that administration of the biflavonoid, kolaviron, or quercetin prevented Cd-mediated decreased sperm motility in adult male rats. Other researchers reported the positive effects of quercetin on sperm capacity under both in vitro and in vivo conditions (39). Supplementation of quercetin restored the decrease in glutathione (GSH) level, and superoxide dismutase (SOD) and GSH peroxidase activities in Cd-exposed mice. This discrepancy between in vitro and in vivo results might be attributed to the difference in quercetin exposure time or to in situ metabolic alteration of quercetin (40).
Effects of flavonoids on motility of lead chloride-exposed sperm

Toxic effects of PbCl$_4$ on sperm quality, motility, DNA fragmentation, and acrosome reaction have been investigated extensively in mice and humans (36, 41-44). According to our results (Fig.2), PbCl$_4$ compared to AlCl$_3$ and CdCl$_2$ had more adverse effects on sperm motility at the 0.125 to 5.0 mM concentrations. We used the 250 µM concentration of PbCl$_4$ for additional experiments with flavonoids. Quercetin and catechin decreased motility of PbCl$_4$-exposed sperm cells in a dose-dependent manner. However, as seen in Figure 5, the 500 µM concentration of rutin, naringin, and kaempferol significantly increased sperm motility to 65% (rutin), 60% (naringin) and 63% (kaempferol). Rutin was more efficient in fortifying sperm cells against PbCl$_4$-induced harmful attacks.

Sperm lipid peroxidation in the presence of aluminum chloride, cadmium chloride and lead chloride

Sperm membranes are rich in polyunsaturated fatty acids (PUFAs) (45). Previous in vivo studies have demonstrated that Al could increase peroxidation of PUFAs in sperm samples (31, 46). The presence of a high level of PUFA in the sperm plasma membrane is required for membrane fusion events associated with fertilization. Loss of fluidity as a result of lipid peroxidation can diminish the rates of sperm-oocyte fusion (47). Our in vitro studies have shown that AlCl$_3$ at concentrations higher than 0.5 mM significantly induced MDA production after 1 hour of incubation (P≤0.0008, Fig.6). MDA is an end-product of enzymatic and oxygen radical-initiated oxidative decomposition of PUFAs and most frequently used as an indicator of lipid peroxidation. We have shown that the effect of AlCl$_3$ on sperm lipid peroxidation was dose- and time-dependent (Fig.6). There were no significant changes in sperm MDA formation observed following incubation with 0.5-30 mM of CdCl$_2$ or PbCl$_4$ (data not shown). Therefore, we only investigated the effects of flavonoids on MDA formation in AlCl$_3$-exposed sperm cells.

Effects of flavonoids on lipid peroxidation of aluminum chloride-exposed sperm

Researchers previously reported the protective effect of ascorbic acid as an antioxidant against induction of lipid peroxidation by AlCl$_3$ in sperm cells (46). However, to the best of our knowledge there was no report about the protective effect
of flavonoids against lipid peroxidation in Al-exposed sperm cells. Moretti et al. showed that quercetin, rutin, and, to a lesser extent, naringenin, significantly decreased tert-butyl hydroperoxide induced lipid peroxidation in human sperm (48). Their studies indicated that epicatechin was not efficacious as an antioxidant to protect sperm cells against oxidants. Our investigations showed that kaempferol was the most effective amongst the tested products in protection of sperm cells against AlCl$_3$-induced lipid peroxidation (Fig. 7). Kaempferol, at a concentration of 100 µM, reduced MDA production from 250 nmol/ml (in untreated cells) to approximately 80 nmol/ml. Naringin and rutin were less effective in protection of AlCl$_3$-exposed sperm cells against lipid peroxidation compared to kaempferol. We observed that quercetin and catechin did not protect sperm. Quercetin, as an antioxidant, did not protect sperm cells against lipid peroxidation; rather, it had inhibitory effects on sperm motility. Khanduja et al. (49) have reported a significant decrease in sperm Ca$^{2+}$-ATPase activity following quercetin treatment. Ca$^{2+}$-ATPase is the responsible enzyme that provides energy for progressive movement of sperm cells. Inhibition of Ca$^{2+}$-ATPase activity has been shown to result in Ca$^{2+}$ accumulation in the cells and blockage of the sperm motility apparatus (50).

**Discussion**

The impact of heavy metal toxicity, even at low concentrations, on the male reproductive system has been extensively investigated and confirmed (51-54). Sperm motility depends on the synchronized actions of proteins, sugars, ions, and small organic molecules. It is one of the main factors that facilitates the journey of sperm toward the egg and the subsequent fertilization process (55). Defects in sperm motility are a common reason for infertility in humans (56). In the current study we have shown that AlCl$_3$, CdCl$_2$, and PbCl$_2$ significantly affected sperm motility. PbCl$_2$ had the most toxic effect.

Infertility due to metal toxicity usually occurs as a result of ROS induction (57). Therefore, antioxidant therapy is a promising strategy for treatment of individuals with heavy metal poisoning (58). Among natural antioxidants, flavonoids are more likely to exert protective activities against metal toxicity compared to carotenoids and vitamin E (37, 59). Based on our results, three flavonoids, rutin, naringin, and kaempferol have been shown to restore motility of AlCl$_3$-, CdCl$_2$-, and PbCl$_2$-exposed sperm cells. The other two flavonoids, catechin and quercetin, had no positive effects on motility of metal-exposed sperm; rather, they decreased sperm motility compared to untreated control samples.

We conducted additional research on the protective effects of flavonoids as antioxidant agents against heavy metal-induced lipid peroxidation. MDA formation was assessed in AlCl$_3$-exposed sperm cells treated with the five above mentioned flavonoids. Among flavonoids, quercetin due to its free radical scavenging and metal chelating abilities has been extensively investigated (60). However, according to the obtained results, quercetin and catechin did not protect sperm cells from ROS-mediated damages. They adversely affected sperm motility. Inhibition of sperm motility without considerable effects on peroxidation of PUFAs would indicate involvement of other inhibitory mechanisms. In contrast, increased motility of Al-exposed sperm cells treated with rutin, naringin and kaempferol was accompanied by decreased levels of MDA formation. We have concluded that antioxidant or chelating properties were not sufficient to protect sperm cells against the harmful damages of heavy metals. Flavonoids, as naturally occurring compounds may have some inhibitory effects on enzyme activities (49) or exert their growth inhibitory activities through binding to human receptors (61). Therefore, it is essential to...
know the exact mechanisms of metal-induced toxicity and the properties of flavonoids before prescribing medications to combat the adverse effects of heavy metals on infertility.

Acknowledgments

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Flavonoids Effects on Metal-Exposed Human Sperm

Effect of Long-Term Fish Oil Supplementation on Semen Quality and Serum Testosterone Concentrations in Male Dogs

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Abstract

Background: Manipulating the dietary fatty acid (FA) content can alter FA profiles of reproductive tissues. Numerous researchers have evaluated the effect of fish oil (FO) supplementation on reproductive characteristics in domestic animals, but reliable information concerning dietary FO effects on semen quality and testosterone concentrations in dogs has not been reported. Therefore, this study evaluated the effects of dietary FO on semen quality and serum testosterone concentrations in dogs.

Materials and Methods: In this cross-over experimental study, 5 male dogs consumed either a control diet or the same diet supplemented with 54 mg FO/kg metabolic body weight (BW) for 120 days. After the 120-day wash-out period, control (C) dogs received FO and FO-fed dogs consumed the control diet. In the first period, 2 dogs were allocated to the FO group and 3 to the C group. In the second period, 3 dogs were allocated to the FO group and 2 to the C group. Semen samples collected on days 0, 60, 90 and 120 were evaluated by standard methods. Day 120 semen samples were analyzed for FA profiles. Blood samples were collected on days 0, 30, 60, 90 and 120 to measure serum testosterone concentrations. Data were analyzed by analysis of variance with repeated measures using the Mixed Models procedure of SAS (version 9.0, SAS Institute Inc., Cary, NC, USA). Animals and period of time (first or second 120 days) were random variables; and treatment, time, and the treatment by time interaction were considered fixed effects.

Results: FO supplementation increased the percentage of motile sperm (P=0.02), total sperm count (P<0.01), total sperm viability (P<0.01), and total morphologically normal sperm (P<0.01). Supplementation decreased the percentage of viable sperm (P=0.03) and serum testosterone concentration (P<0.01). FO supplementation also increased the percentage of arachidonic acid, eicosapentaenoic acid, (EPA) and total n-3 in semen samples (P<0.05).

Conclusion: These results are consistent with the concept that long-term FO supplementation influences semen quality and testosterone concentrations in dogs by altering semen FA profiles.

Keywords: Canine, Fatty Acids, Semen, Testosterone

Introduction

The fatty acid (FA) content in diets can be manipulated to alter FA profiles of reproductive tissues. Fish oils (FO) contain omega 3 FA (n-3) and are a major source of eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA, C22:6 n-3) (1). Numerous researchers have evaluated the effect of FO supplementation on repro-

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Productive parameters in domestic animals. They reported improved sperm motility and decreased morphologically abnormal sperm in boars (2), increased fertility in turkeys (3), and attenuated seasonal declines in semen quality and increased total sperm count in rams (4). However, other studies conducted in boars showed that dietary FO had no effect on semen quality (5, 6). FO supplementation in turkeys had no effect on sperm motility and sperm viability compared with the control group (7). Other studies reported changes in sperm FA profiles by FO supplementation in boars (5) and rams (4).

Testosterone is a hormone essential for spermatogenesis and male fertility (8). This hormone acts synergistically with both luteinizing hormone and follicle stimulating hormone to increase spermatogenesis efficiency and fertility. Reports vary on the effect of FO supplementation on testosterone concentrations in different species. FO supplementation has been shown to increase testosterone concentrations in rats (9) and rams but decreased testosterone concentrations in boars (10).

To our knowledge, there are no studies that have specifically evaluated sperm parameters and serum testosterone concentrations in dogs that received FO supplements. Therefore, the present study evaluated the effects of dietary FO that contained n-3 FA on semen quality and serum testosterone concentrations in dogs. We hypothesized that FO supplementation would improve semen quality and increase serum testosterone in dogs.

Materials and Methods

Animals and treatments

The study was approved by the Institutional Animal Care and Use Committee (IACUC, Number 34-1-13) of the School of Veterinary Sciences, National University of La Plata, Buenos Aires, Argentina.

All dogs started the study at the same time. One month before the study, the dogs consumed commercial balanced food (Table 1) and were trained for semen collection, performed by manual stimulation twice per week. We chose the study dogs according to age, body condition and a complete medical record (history, clinical visit and clinical examination). As complementary methods, the dogs underwent routine blood and chemical tests, along with semen evaluations.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Dry matter (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>30.04</td>
</tr>
<tr>
<td>Fat</td>
<td>15.02</td>
</tr>
<tr>
<td>Fiber</td>
<td>1.72</td>
</tr>
<tr>
<td>Ash</td>
<td>7.83</td>
</tr>
<tr>
<td>Calcium</td>
<td>1.50</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>1.07</td>
</tr>
<tr>
<td>Mineral and vitamins Mix*</td>
<td>0.048</td>
</tr>
</tbody>
</table>

*; Concentration according to manufacturer information: contains 12.39% Vitamin E, 0.20% Vitamin K, 0.82% Vitamin B1, 0.82% Vitamin B2, 0.82% Vitamin B6, 0.0004 Vitamin B12, 0.12% Acid Folc, 0.10% Acid Nicotinic, 2.06% Calcium Pantothenate, 0.02% Biotin, 82.61% Coline, 0.01% copper, 0.01% iron, 0.02% zinc, 0.003% iodine, 0.01% manganese and 0.0002% of selenium in the mineral and vitamin nucleus.

This cross-over, controlled experimental trial studied 5 healthy privately owned mixed-breed dogs, 2 to 5 years of age, that weighed 17.9 ± 3.10 kg, with body condition scores of 3 in on a 1 to 5 scale (11). Before the study, semen characteristics of all dogs were within normal limits with sperm concentration>200×10^6/ml, sperm motility>70%, sperm progressive motility>70%, sperm viability>80%, and morphologically abnormal sperm<20% (12). Dogs were randomly assigned to two groups, control (C) and FO. The C group only received a control diet for 120 days, whereas the FO group received the control diet plus 54 mg FO/kg of metabolic body weight (BW^0.75) for 120 days. This dose was supplemented orally within an enteric-coated capsule. The duration of each period was to comprise two complete cycles of spermatogenesis (13). In the first period, 2 dogs were allocated to the FO group and 3 to the C group. In the second period, 3 dogs were allocated to the FO group and 2 to the C group. Finally, to avoid any carry-over effects, we included a 120-day wash-out period between treatments during which all dogs received the control diet. This time allowed for a complete FA wash-out as described previously by Cao et al. (14).

The dogs were kept in the owners’ homes during the study. The owners signed a written consent before the experiment and agreed to feed either the commercial balanced food provided (C) or the commercial balanced food with the supplement (FO).
Water was allowed on an ad libitum basis. Daily rations were controlled according to the daily maintenance requirements. Maintenance energy requirements (MERs) were calculated based on the formula: 

\[ \text{MER} = [130 \times (\text{BW})^{0.75}] \] (15).

**Semen collection and evaluation**

Semen was collected by manual stimulation. After collection, semen volume was determined in the spermatic fraction using a graduated tube. Sperm concentration \((10^6/\text{ml})\) was determined using a Neubauer chamber, followed by a calculation of the total sperm count \((\text{volume} \times \text{concentration})\). Subjective vigor, defined as the linearity and quality of spermatic movement, was evaluated with a 0 to 5 score as described previously by da Rocha et al. (16). The percentages of motile sperm and progressive motility were evaluated on a drop of semen placed between a slide and cover glass on a heated stage and visualized by light microscopy at ×400 magnification in 10 fields. Sperm with normal morphology were evaluated in samples stained with Rose Bengal under optical microscopy at ×1000 (17); afterwards, we calculated the number of total morphologically normal sperm \((\text{total sperm count} \times \% \text{of sperm with normal morphology})\). All evaluations were performed in duplicate and we counted 200 sperm cells.

**Testosterone measurement**

We obtained blood samples by peripheral venipuncture at 0, 20, 40, 60, 80, 100 and 120 minutes, beginning at 09:00 on days 0, 30, 60, 90 and 120 of each period. This 2 hour window for each day was used to buffer the changes in serum testosterone concentrations. The average serum testosterone concentration for this 2 hour window was used in the statistical analysis. To reduce potential damage in successive blood extractions, we used different veins (cephalic, saphenous and right and left jugular). Blood was centrifuged at 1400 x g for 5 minutes and serum was harvested and stored frozen at -18°C until analysis. Testosterone was determined by electrochemiluminescence (Elecsys®, Cobas®, West Sussex, England), as previously validated in dogs by Garcia Romero et al. (18).

**Feed analysis**

Feed was pooled and analyzed for dry matter (80°C for 48 hours), neutral detergent fiber (NDF, Ankom200 Fiber Analyzer, ANKOM Technology, Fairport, NY), crude protein (CP, Kjeldahl N x 6.25), lipids (Ether extract, XT101 ANKOM Technology Method 2) and ash (Table 1) (19).

**Lipid analysis of feed, fish oil and spermatic fraction**

We analyzed a pool of feed and supplemental FO to evaluate the FA profile (Table 2). Semen samples for lipid analysis were collected on days 120 of each period and stored at -80°C until analysis.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Commercial feed</th>
<th>Fish oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetradecanoic (14:0)</td>
<td>0.8</td>
<td>5.8</td>
</tr>
<tr>
<td>Palmitic (16:0)</td>
<td>19.6</td>
<td>24</td>
</tr>
<tr>
<td>Palmitoleic (16:1)</td>
<td>2.9</td>
<td>10.1</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td>4.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Oleic (18:1 n-9)</td>
<td>25.7</td>
<td>22.3</td>
</tr>
<tr>
<td>Vaccenic (18:1 n-7)</td>
<td>-</td>
<td>3.3</td>
</tr>
<tr>
<td>Linoleic (18:2 n-6)</td>
<td>38.1</td>
<td>2</td>
</tr>
<tr>
<td>Gamma-linolenic (18:3 n-6)</td>
<td>1.4</td>
<td>-</td>
</tr>
<tr>
<td>Alpha-linolenic (18:3 n-3)</td>
<td>4.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Eicosanoic (20:1 n-9)</td>
<td>0.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Dihomo-gamma-linolenic</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>(20:3 n-6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eicosatrienoic (20:3 n-3)</td>
<td>0.4</td>
<td>-</td>
</tr>
<tr>
<td>Arachidonic (20:4 n-6)</td>
<td>1.4</td>
<td>1</td>
</tr>
<tr>
<td>Eicosatetraenoic (20:4 n-3)</td>
<td>-</td>
<td>0.8</td>
</tr>
<tr>
<td>Eicosapentaenoic (20:5 n-3)</td>
<td>0.1</td>
<td>7.6</td>
</tr>
<tr>
<td>Docosapentaenoic (22:5 n-6)</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>Docosapentaenoic (22:5 n-3)</td>
<td>0.2</td>
<td>1.2</td>
</tr>
<tr>
<td>Docosahexaenoic (22:6 n-3)</td>
<td>0.2</td>
<td>14.9</td>
</tr>
<tr>
<td>Σ SFA</td>
<td>25.1</td>
<td>33.2</td>
</tr>
<tr>
<td>Σ MUFA</td>
<td>28.7</td>
<td>38.2</td>
</tr>
<tr>
<td>Σ PUFA</td>
<td>46.2</td>
<td>28.6</td>
</tr>
<tr>
<td>Σ n-6</td>
<td>41.3</td>
<td>3</td>
</tr>
<tr>
<td>Σ n-3</td>
<td>4.9</td>
<td>25.6</td>
</tr>
</tbody>
</table>

SFA; Saturated fatty acids, MUFA; Monounsaturated fatty acids, PUFA; Polyunsaturated fatty acids, n-6; Omega 6, and n-3; Omega 3.

Samples for lipid extraction were analyzed following the method described by Folch et al. (20).
Once lipids were obtained, they were saponified with potassium hydroxide dissolved in ethanol for 45 minutes at 80°C, then acidified with a 0.5 ml hydrochloric acid concentrate. The acids were esterified with boron trifluoride at 64°C for 1.5 hours. FA composition was determined by gas liquid chromatography with a 30 mm capillary column (Omega Wax 250, Supelco, Bellefonte, PA, USA). Temperature was programmed for a linear increase of 3°C per minute from 175 to 230°C. The chromatographed peaks were identified by comparing their retention times with standards.

**Statistical analysis**

We used a cross-over design with repeated measures in time. Each individual dog was considered an experimental unit. Data were analyzed with the PROC MIXED of SAS (version 9.0, SAS Institute Inc., Cary, NC, USA). The linear mixed model included the random effect of dogs, the period of the cross-over design, the fixed effect of time (0 vs. 30 vs. 60 vs. 90 vs. 120 days), treatment (C vs. FO), and time by treatment interaction. The interaction between treatment and period of the cross-over design was also included. The slice option of SAS was used to detect the time points when significant differences in the time by treatment interaction occurred when the interaction was significant. For semen FA profiles, we used only one time point. Therefore, time effect and interaction with treatment were removed from the model.

Data are represented as least square means (LSM) ± the standard error of the means (SEM). For the main effects (time or treatment) the alpha level of significance was set at P<0.05 and P<0.1 was considered a tendency. For the interaction of the main effect (time by treatment) P<0.1 was considered statistically significant (21, 22).

**Results**

**Semen evaluation**

FO supplementation increased the percentage of motile sperm (P=0.02), total sperm count (P<0.01), total sperm viability (P<0.01), and total morphologically normal sperm (P<0.01). FO supplementation decreased the percentage of sperm viability (P=0.03). There was a trend (P=0.09) for an increase in the percentage of morphologically normal sperm (Table 3).

<p>| Table 3: Effect of fish oil supplementation for 120 days on semen characteristics in five male dogs in the control (C, n=5) and fish oil (FO, n=5) groups |
|---------------------------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 60</th>
<th>Day 90</th>
<th>Day 120</th>
<th>SEM</th>
<th>P value</th>
<th>Time</th>
<th>FO x time</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Semen volume (ml)</strong></td>
<td>1.42</td>
<td>1.40</td>
<td>1.32</td>
<td>1.68</td>
<td>1.70</td>
<td>1.50</td>
<td>1.90</td>
<td>0.32</td>
</tr>
<tr>
<td>Motile sperm (%)</td>
<td>88.00</td>
<td>90.50</td>
<td>90.00</td>
<td>93.00</td>
<td>88.00</td>
<td>93.33</td>
<td>89.00</td>
<td>92.00</td>
</tr>
<tr>
<td>Sperm progressive motility (%)</td>
<td>86.00</td>
<td>83.00</td>
<td>89.00</td>
<td>89.00</td>
<td>86.00</td>
<td>91.66</td>
<td>85.00</td>
<td>92.00</td>
</tr>
<tr>
<td>Vigor</td>
<td>4.5</td>
<td>4.3</td>
<td>4.5</td>
<td>4.4</td>
<td>4.5</td>
<td>4.4</td>
<td>4.5</td>
<td>4.4</td>
</tr>
<tr>
<td>Total sperm count (10^6)</td>
<td>399.80</td>
<td>439.60</td>
<td>404.90</td>
<td>1105.20</td>
<td>649.00</td>
<td>1212.00</td>
<td>511.40</td>
<td>1361.00</td>
</tr>
<tr>
<td>Total sperm viability (10^6)</td>
<td>338.22</td>
<td>379.38</td>
<td>322.43</td>
<td>806.64</td>
<td>549.10</td>
<td>904.40</td>
<td>419.30</td>
<td>963.23</td>
</tr>
<tr>
<td>Sperm viability (%)</td>
<td>83.60</td>
<td>84.60</td>
<td>82.00</td>
<td>76.00</td>
<td>83.00</td>
<td>72.13</td>
<td>81.00</td>
<td>71.40</td>
</tr>
<tr>
<td>Total morphologically normal sperm (10^6)</td>
<td>311.94</td>
<td>376.72</td>
<td>295.00</td>
<td>843.25</td>
<td>541.67</td>
<td>1042.56</td>
<td>433.80</td>
<td>1099.60</td>
</tr>
<tr>
<td>Morphologically normal sperm (%)</td>
<td>79.40</td>
<td>84.00</td>
<td>73.00</td>
<td>78.60</td>
<td>83.00</td>
<td>87.25</td>
<td>81.00</td>
<td>81.00</td>
</tr>
</tbody>
</table>

C: Receiving only the control diet for 120 days and FO: Receiving 13.8 mg of n-3 from fish oil/kg of metabolic weight for 120 days.
There was a time by treatment effect on total sperm count \( (P=0.02, \text{Fig. 1}) \), total sperm viability \( (P=0.076, \text{trend, Fig. 2}) \), and total morphologically normal sperm \( (P=0.02, \text{Fig. 3}) \). Conversely, we observed no effects of FO supplementation on semen volume, sperm progressive motility, and vigor \( (P>0.10, \text{Table 3}) \).
Testosterone concentration

There was a time by treatment effect on serum testosterone concentrations (P<0.01, Fig.4). Both groups had similar serum testosterone concentrations on day 0 (4.8 ± 0.3 ng/ml). In C dogs, these concentrations did not change with time, whereas in FO dogs they decreased on day 30 (1.31 ± 0.3 ng/ml) and remained low on days 60 (1.84 ± 0.5 ng/ml), 90 (1.20 ± 0.3), and 120 (0.89 ± 0.3).

Fatty acid profiles of semen samples

Dogs supplemented with FO had greater semen FA concentration of arachidonic acid (AA, AA 20:4 n-6, P=0.01), EPA (20:5 n-3, P=0.02) and total n-3 (P=0.05, Table 4). Despite the increase of arachidonic acid (AA 20:4 n-6) in FO dogs, the ratio n-6/n-3 tended (P=0.09) to be smaller compared with C dogs. FO dogs tended to have higher DHA (22:6 n-3, P=0.09) and palmitoleic acid (16:1 n-7, P=0.07) but lower bosseopentaenoic acid (20:5 n-6, P=0.06) levels than C dogs.

![Fig.4: Effect of fish oil supplementation for 120 days on serum testosterone concentration in five male dogs in the control (C, n=5) and fish oil groups (FO, n=5).](image)

Table 4: Fatty acid composition (LSM ± SEM) of semen samples from five male dogs in the control (C, n=5) and fish oil (FO, n=5) groups at the end (120 days) of the experiment

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Day 120</th>
<th>C</th>
<th>FO</th>
<th>SEM</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetradecanoic (14:0)</td>
<td></td>
<td>1.55</td>
<td>1.81</td>
<td>0.11</td>
<td>0.155</td>
</tr>
<tr>
<td>Palmitic (16:0)</td>
<td></td>
<td>32.80</td>
<td>31.27</td>
<td>0.67</td>
<td>0.15</td>
</tr>
<tr>
<td>Palmitoleic (16:1 n-7)</td>
<td></td>
<td>1.29</td>
<td>2.00</td>
<td>0.23</td>
<td>0.07</td>
</tr>
<tr>
<td>Stearic (18:0)</td>
<td></td>
<td>13.85</td>
<td>13.95</td>
<td>0.61</td>
<td>0.90</td>
</tr>
<tr>
<td>Oleic (18:1 n-9)</td>
<td></td>
<td>7.34</td>
<td>7.92</td>
<td>0.90</td>
<td>0.66</td>
</tr>
<tr>
<td>Vaccenic (18:1 n-7)</td>
<td></td>
<td>3.42</td>
<td>3.39</td>
<td>0.32</td>
<td>0.93</td>
</tr>
<tr>
<td>Linoleic (18:2 n-6)</td>
<td></td>
<td>3.95</td>
<td>5.23</td>
<td>0.64</td>
<td>0.21</td>
</tr>
<tr>
<td>Eicosenoic (20:1 n-9)</td>
<td></td>
<td>0.99</td>
<td>0.45</td>
<td>0.46</td>
<td>0.44</td>
</tr>
<tr>
<td>Eicosatrienoic (20:3 n-6)</td>
<td></td>
<td>2.08</td>
<td>2.12</td>
<td>0.20</td>
<td>0.89</td>
</tr>
<tr>
<td>Arachidonic (20:4 n-6)</td>
<td></td>
<td>5.02</td>
<td>7.37</td>
<td>0.51</td>
<td>0.01</td>
</tr>
<tr>
<td>Eicosapentaenoic (20:5 n-3)</td>
<td></td>
<td>0.36</td>
<td>0.51</td>
<td>0.03</td>
<td>0.02</td>
</tr>
<tr>
<td>Bosseopentaenoic (20:5 n-6)</td>
<td></td>
<td>3.01</td>
<td>2.53</td>
<td>0.15</td>
<td>0.06</td>
</tr>
<tr>
<td>Docosapentaenoic (22:5 n-6)</td>
<td></td>
<td>20.20</td>
<td>16.53</td>
<td>1.44</td>
<td>0.11</td>
</tr>
<tr>
<td>Docosahexaenoic (22:6 n-3)</td>
<td></td>
<td>3.90</td>
<td>4.78</td>
<td>0.32</td>
<td>0.09</td>
</tr>
<tr>
<td>Σ SFA</td>
<td></td>
<td>47.79</td>
<td>47.25</td>
<td>0.92</td>
<td>0.69</td>
</tr>
<tr>
<td>Σ MUFA</td>
<td></td>
<td>13.36</td>
<td>13.91</td>
<td>1.44</td>
<td>0.79</td>
</tr>
<tr>
<td>Σ PUFA</td>
<td></td>
<td>38.66</td>
<td>38.83</td>
<td>1.38</td>
<td>0.93</td>
</tr>
<tr>
<td>Σ n-6</td>
<td></td>
<td>34.20</td>
<td>33.53</td>
<td>1.28</td>
<td>0.71</td>
</tr>
<tr>
<td>Σ n-3</td>
<td></td>
<td>4.28</td>
<td>5.30</td>
<td>0.32</td>
<td>0.05</td>
</tr>
<tr>
<td>n-6/n-3 ratio</td>
<td></td>
<td>8.26</td>
<td>6.41</td>
<td>0.68</td>
<td>0.09</td>
</tr>
</tbody>
</table>

LSM; Least squares means, SFA; Saturated fatty acids, MUFA; Monounsaturated fatty acids, PUFA; Polyunsaturated fatty acid, C; Control, and FO; Fish oil.
Discussion

The present study assessed the effects of FO supplementation on semen quality and serum testosterone concentrations in dogs. We hypothesized that FO supplementation improved semen quality and increased serum testosterone in dogs. In agreement with our hypothesis, FO supplementation improved their semen characteristics. This finding supported results from previous studies conducted in other species such as pigs (2), turkeys (3) and rams (4), in which FO supplementation improved semen quality.

This improvement could be due to changes in the sperm phospholipid FA profile (2) due to an increase in n-3 FAs (23). The higher flexibility in the sperm membrane produced by the increase in n-3 FAs might improve the characteristic flagellar motion of sperm (24). Samadian et al. (4) also mentioned that dietary FO supplementation produced changes in the EPA precursors that might be relevant to the alterations in semen quality. In a previous report on dogs by da Rocha et al. (16), daily supplementation with a blend of FAs (n-3, omega 6 and omega 9) together with vitamin E, increased sperm concentration, volume and vigor, and decreased morphologically abnormal sperm. In the current study, we found no effects of FO on semen volume or vigor. This discrepancy might be due to the FA blend and the doses of omega and vitamin E used in the former work. In our study, the effect of FO supplementation on semen characteristics could be attributed to the changes in the semen FA profile. Furthermore, the absence of antioxidant supplementation in FO dogs could have influenced the results. Despite the greater number of viable sperm in FO dogs, we observed a higher percentage of viable sperm in C dogs. The decrease in percentage of viable sperm might be due to the lack of extra antioxidants in FO dogs.

A previous report (10) has stated that the inclusion of vitamin E as an antioxidant may be needed in conjunction with polyunsaturated fatty acids (PUFAs) supplementation in the diet. If antioxidants are not present in an adequate concentration, cellular membrane PUFAs can be oxidized and produce free radicals (25). On the other hand, in our study we have detected a time by treatment effect on total sperm count and total morphologically normal sperm, along with a trend on total sperm viability on day 60 and during the length of the study. The increase in total sperm count might be due to the effect of FO supplementation. According to evidence in rams, FO, as a source of n-3, increased sperm concentration. This increase was due to an acceleration of spermatogenesis. It has been suggested that n-3 FA play an important role on the development of functional sperm (4). Conversely, some studies in boars showed no effects of FO on semen quality or fertility (10).

As mentioned above, differences among studies could be due to the species, breed, sources of FA supplements, supplementation period, season, husbandry practices or semen handling procedures (10, 26, 27). In the current experiment, we evaluated the total FA in semen. FO supplementation increased total n-3 FA in semen samples of FO dogs. The increase in semen EPA concentration might be due to the EPA concentration in FO. During the C and the FO treatment periods, dogs received on average 8.84 mg and 16.72 mg DHA per kg BW0.75, respectively (8.84 mg dietary and 7.88 mg from the supplement). Although FO dogs received almost a double amount of DHA compared to C dogs, there were no significant DHA differences in semen samples in either group, even though we observed a tendency to increase in FO dogs. This non-significant numerical difference might be due to the high variability of DHA in semen or to changes in the efficiency of absorption and utilization of DHA; however, a clear conclusion could not be drawn from the current experiment. Although an unexpected increase occurred in semen AA in FO dogs, we could not explain the physiological mechanism for such an increase, mainly because the AA on the supplement was only 1%.

Contrary to our expectations, FO supplementation decreased serum testosterone concentrations. To our knowledge, this was the first study which described the effect of supplementation with FO that contained n-3 on serum testosterone in dogs. Previous findings in rats (9), humans (28) and boars (10) showed some discrepancies on the effect of n-3 FA supplementation on testosterone concentration. In rats, an association existed between the increase in testosterone concentration after FO supplementation with changes in the number of luteinizing hormone receptors due to the modifi-
cation of the lipid composition of the Leydig cell membrane (9). Conversely, in humans (28), boars (10) and fish (29), the decrease in testosterone concentrations was related to changes in the synthesis of prostaglandin series 2 to series 3 and the inhibition of prostaglandin formation from AA by EPA/DHA treatment. On the other hand PUFAs might alter the function of transcription factors that control gene expression such as the peroxisome proliferator activated receptors and affect cellular concentrations of enzymes that regulate both prostaglandin and synthesis of steroids pathways (30). Castellano et al. (10) suggested that variations in the testosterone response to n-3 supplementation might be related to large variations in the type and quantity of n-3 FAs in the diet, to the duration of the supplementation period, and to the animal model used. Based on our results and those from Castellano et al. (10), we assumed that differences could also be due to the total dietary amount of EPA or DHA. In the current experiment, the lack of additional antioxidants in FO dogs could have altered the serum concentration of testosterone due to alterations in the synthesis of steroids (31).

Further studies are needed to understand the mechanism whereby FO (EPA or DHA) decreases serum testosterone concentration in dogs. Also, there is a need for studies that compare and explain the biology of different responses to FO supplementation among species.

Conclusion
Long-term FO supplementation for 120 days might influence semen quality and serum testosterone concentrations in dogs by altering the semen FA profile.

Acknowledgements
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References
Sperm Parameters and Serum Testosterone in Dogs

Attenuation of Methotrexate-Induced Embryotoxicity and Oxidative Stress by Ethyl Pyruvate

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Abstract

Background: Methotrexate (MTX), as an anti-folate agent, is widely used in the treatment of rheumatic disorders and malignant tumors, however it damages reproductive system in mice. The aim of this research was to study the effects of ethyl pyruvate (EP) on embryo development and oxidative stress changes in the testis of mice treated with MTX.

Materials and Methods: In this experimental study, thirty-two adult male Naval Medical Research Institute mice, with average weight of 26 ± 2 g, were divided into four groups. The first group (control) received distilled water (0.1 ml/mice/day), while the second group was intraperitoneally (IP) treated with 20 mg/kg MTX once per week. The third group was IP treated with 40 mg/kg/day EP, and the fourth group was IP treated with both 20 mg/kg MTX and 40 mg/kg/day EP for 30 days. At the end of treatment fertilization rate and embryonic development were evaluated. Differences between these groups were assessed by ANOVA using the SPSS software package for Windows with a Tukey-Kramer multiple post-hoc comparison test.

Results: MTX treatment caused significant (P<0.05) increase in malondialdehyde (MDA) and reduced catalase (CAT), as well as leading to in vitro fertilization (IVF) and embryonic development. The improved effects of EP on the IVF were determined by the reduced level of MDA (index of oxidative stress) and significant increased level of CAT (a key antioxidant). We observed significant increase in fertilization rate and embryonic development in the treated group with both MTX and EP.

Conclusion: It is suggested that EP can be useful in ameliorating testicular damages and embryotoxicity induced by MTX. These effects could be attributed to its antioxidant properties.

Keywords: Ethyl Pyruvate, In Vitro Fertilization, Methotrexate, Testis

Introduction

Infertility has been one of the most controversial medical and social issues. It was previously proposed as a punishment in some civilizations, while it is an illness (1). In addition, lack of the knowledge on medicine is generally the main factor to always consider female as responsible for reproductive system failure. Several investigations within the last decade help scientists and clinicians distinguish crucial parameters involved in couple’s infertility, one of which is sperm malfunction in male (2). About 5-15% of couples are infertile, out of whom about 50% of infertility is caused by male disorders (3).

Methotrexate (MTX) is an anti-neoplastic agent which is used alone or in combination with other agents to treat severe psoriasis and rheumatoid arthritis (4). It is also used against a broad range of neoplastic disorders including acute lymphoblastic leukemia, non-Hodgkin’s lymphoma, breast cancer and testicular tumors (5, 6).

It is well demonstrated that cancer chemotherapeutic drugs could cause acute toxic side-effects in multiple organs such as gastrointestinal tract, lung, kidney, liver, testes and skin. Several currently available anti-cancer drugs, specifically MTX,
generally function by destruction of particular proliferating cells with both normal and malignant origins (7, 8). MTX might also cause primary infertility by affecting hypothalamic-pituitary-gonadal axis or gonads, directly (9). This drug is bound to dihydrofolate-reductase enzyme, which inhibits DNA replication and prevents the synthesis of purines, thymidilate as well as glycine, eventually leading to cell death (10). It has been manifested that MTX, as a cytotoxic agent, should be considered a potential occupational reproductive hazard which is harmful for the fetus, and could potentially cause human carcinogenesis (11). Study of this drug in animal models showed cytotoxicity, altered spermatogenesis, degeneration of spermatocytes in sertoli and leydig cells (12, 13).

On the other hand, pyruvate is a key intermediate metabolite of glucose, playing role as a potent anti-oxidant and free scavenger. It has been demonstrated that pyruvate has also an anti-inflammatory effect, both in vitro and in vivo (14). While being a good scavenger for hydrogen peroxide and superoxide radicals (15), the efficiency of pyruvate as a therapeutic agent is limited by its instability (16). In comparison, ethyl pyruvate (EP), a simple aliphatic ester derived from pyruvic acid, is safer and more stable than pyruvate (17). Similar to pyruvate, EP can rapidly and stoichiometrically scavenge hydrogen peroxide. Investigations showed that treatment with EP reduces oxidative stress both in vitro and in vivo models of ischemia/reperfusion injury (15).

In vitro fertilization (IVF), is a safe and effective way and the last option for the infertile couples who have tried and failed to conceive using standard treatments such as surgery, fertility drugs and artificial insemination (18). The aim of this research was to study the effect of EP on embryo development and oxidative stress changes in mice testis treated with MTX.

Materials and Methods

Animals

This was an experimental study on thirty two adult male Naval Medical Research Institute mice with 8-10 weeks of age and 26 ± 2 g average weight. The mice were purchased from animal house of Science Faculty of Urmia University (Urmia, Iran). All animals were kept under standard environmental conditions, including 22 ± 2°C room temperature, relative humidity of 50 ± 10% and a dark/light photoperiod of 12/12 hours, respectively. The animals had open access to standard diet pellets, water and libitum. This study was performed according to Ethical Committee Guidance for Research at Laboratory Animals of Urmia University.

Experimental design

Animals were randomly divided into four equal number groups and treated for 30 days. Group 1 (control) received 0.1 ml/mice/day distilled water intraperitoneally (IP). Group 2 mice were IP administered once per week with 20 mg/kg MTX. Group 3 was IP treated with 40 mg/kg/day EP. Group 4 received both MTX and EP treatment, with similar dosages to the individual treatments.

Oocyte collection

To perform assisted reproductive technique (ART) procedure, the oviduct ampullas was initially collected. Briefly, each male mouse (total number: 32) was mated with three female mice (total number: 96). Superovulation was induced in 6-7 weeks old female mice by IP injection of 10 IU pregnant mare serum gonadotropin (PMSG, Boxmeer, Netherlands), in addition to IP injection of 10 IU human chorionic gonadotropin (hCG, Folligon, Netherlands) after 48 hours. 12-14 hours after hCG injection, female mice were sacrificed by cervical vertebrae dislocation. The oviduct ampullas were removed and transferred to a petri-dish containing human tubular fluid (HTF, Sigma, St. Louis, USA) medium+4 mg/ml bovine serum albumin (BSA, Sigma, St. Louis, USA). The ampulla portions were distinguished by stereo microscope (Model TL2, Olympus Co., Japan), and oocytes were dissected out.

Preparation of culture media for in vitro fertilization

Required fertilization media was prepared and incubated for 12 hours in a gas mixture of 5% CO₂ at 37°C, one day before conception. For each group, separate dishes were considered. Conception dishes with HTF medium were combined with 4 mg/ml BSA. Droplets of fertilization medium and washing HTF medium was prepared for the IVF dishes, followed by covering them with mineral oil (Sigma) and incubation at 37°C for overnight.
Sperm preparation and insemination

24 hours after the last treatment, male mice were sacrificed by cervical dislocation. Using a ventral midline skin incision the epididymides were separated from the testicles. Afterward, caudal epididymis (cut into 2-3 pieces) was placed in 1 ml HTF medium with a combination of 4 mg/ml BSA and incubated at 37°C for 60 minutes in a 5% CO₂ incubator. Obtained spermatozoa were capacitated by incubation at 37°C and 5% CO₂ for 1 hour.

In addition, 12-14 hours after injection of HCG, female mice were sacrificed by cervical dislocation. The ampules of fallopian tubes were removed, put in the HTF medium with 37°C temperature. The oocytes were subsequently removed with dissecting techniques, followed by washing with the HTF and transferring to the fertilization droplets under mineral oil containing HTF and 4 mg/ml BSA. As soon as overtaking capacitation step of the sperms, they were added to the medium, in a concentration of 1×10⁶ sperms/ml culture medium.

Assessment of fertilization and embryonic development

Using an inverted microscope (Model TL2, Olympus Co., Japan), fertilized oocytes were evaluated by appearance of the pronuclei (female and male) under magnitude of ×200. After denuding of granulosa cells and washing with HTF medium (100 µl), zygotes were transferred into pre-equilibrated fresh medium and cultured for five days. After 24 hours of zygote culture, the rate of two-cell embryos was assessed. Embryonic development was evaluated by inverted microscope after 120 hours culture in vitro. Intact, fragmented and/or lysed embryos, which have not been developed, were considered as "arrested embryos".

Catalase assay

Catalase (CAT) activity, based on the ability to decompose H₂O₂ in homogenized testicular tissue, was determined by Aebi method (19). By reducing the wavelength absorption at 240 nm in the absorption spectrum, decomposition of H₂O₂ was detectable. For this purpose, 30 mM hydrogen peroxide was used as substrate, followed by 50 mM phosphate buffer (pH=7) as an alternative substrate in the blank solution. The assay solution contained 2 ml tissue homogenate and 1 ml hydrogen peroxide. The reaction was started by adding H₂O₂ and using a spectrophotometer (pharmacian novaspec, and biochrom, England) at a wavelength of 240 nm. Decrease in absorbance was evaluated for 30 seconds and the values were expressed in terms of (µg)/g testis tissue.

Lipid peroxidation assay (Malondialdehyde)

Malondialdehyde (MDA) is a product of lipid peroxidation. This experiment is widely used as an oxidative stress index. 300 µl of 10% trichloroacetic acid was added to 150 µl of the sample and centrifuged at 1000 rpm for 10 minutes at 4°C. 300 µl of the supernatant was subsequently transferred to a test tube containing 300 µl of 67% thiobarbituric acid. The mixture was incubated for 25 minutes at 100°C. After cooling the solution for 5 minutes, a pink color was appeared due to the reaction between MDA and TBA and absorbance was measured using a spectrophotometer at 535 nm wavelength (20).

Statistical analysis

SPSS-21 software was used for statistical analysis. The results were compared by one-way ANOVA, supplemented with a Tukey-Kramer multiple comparison test. Values of less than 0.05 were considered statistically significant. All results were expressed as means ± SEM.

Results

Fertilization and embryonic development

In this study, after 30 days, treated mice group with 20 mg/kg of MTX showed significantly reduced rate of fertilization as well as percentage of blastocysts (Table 1, Fig.1). Compared to this group, there was a significant increase in fertilization rate and blastocysts percentage of mice treated with both MTX and EP (Table 1, Fig.2). Findings also demonstrated that the range of two-cell embryos was reduced in zygotes obtained from the MTX treated mice, although this change was not significant (Table 1, Fig.1). In addition, no significant difference was observed in two-cell embryos among the all groups. Furthermore, percentage of the arrested embryos was enhanced after MTX treatment compared to the control group, after 30 days treatment (P<0.05). Analysis of the mice treated with EP showed a significant decrease in the percentage of arrested embryos in comparison with MTX group (Table 1, Fig.3).
EP Ameliorates MTX Induced-Embryo Toxicity

### Table 1: Effect of MTX and EP on fertilization and embryonic development of the mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>MTX</th>
<th>EP</th>
<th>MTX+EP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of oocytes</td>
<td>52</td>
<td>67</td>
<td>39</td>
<td>32</td>
</tr>
<tr>
<td>Fertilized oocytes (%)</td>
<td>92.11 ± 0.70</td>
<td>66.68 ± 2.29&lt;sup&gt;a&lt;/sup&gt;</td>
<td>89.28 ± 1.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>75.99 ± 2.70&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>2-cell embryos (%)</td>
<td>83.93 ± 1.64</td>
<td>74.28 ± 2.47</td>
<td>81.90 ± 2.38</td>
<td>83.28 ± 3.67</td>
</tr>
<tr>
<td>Blastocysts (%)</td>
<td>82.46 ± 2.24</td>
<td>36.66 ± 7.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.13 ± 4.75&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.04 ± 7.48&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Arrested embryos (%)</td>
<td>9.65 ± 1.54</td>
<td>30.02 ± 5.40&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>15.81 ± 1.77&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.21 ± 1.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All the values are expressed as mean ± SEM (n=8).

<sup>a</sup>; Significant differences (P<0.05) compared to control group,  
<sup>b</sup>; Significant differences (P<0.05) compared to MTX group, MTX; Methotrexate, and EP; Ethyl pyruvate.

![Fig.1](image1.jpg)  
**Fig.1:** Representative image of blastocysts embryo (arrow number 1) and hatching embryo (arrow number 2) in the control group. The image was captured with ×200 microscope magnitude.

![Fig.2](image2.jpg)  
**Fig.2:** Representative image of blastocysts embryo (arrow number 1) and unfertilized oocytes (arrow number 2) in the Methotrexate (MTX) treated group. The image was captured with ×200 microscope magnitude.

### Table 2: Effect of MTX and EP on CAT and MDA levels in the adult male mice

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>MTX</th>
<th>EP</th>
<th>MTX+EP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT (µ/g tissue)</td>
<td>0.54 ± 0.02</td>
<td>0.22 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.53 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.45 ± 0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>MDA (µmol/g tissue)</td>
<td>165.66 ± 4.63</td>
<td>357.33 ± 4.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>187.00 ± 9.53&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>203.66 ± 20.4&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All the values are expressed as Mean±SEM (n=8).

<sup>a</sup>; Significant differences (P<0.05) compared to control group,  
<sup>b</sup>; Significant differences (P<0.05) compared to MTX group, MTX; Methotrexate, EP; Ethyl pyruvate, CAT; Catalase, and MDA; Malondialdehyde.
Biochemical analysis

MTX administration for 30 days led to significant decrease in CAT level and increase in the level of MDA compared to the control. By the end of treatment period, EP administered groups showed significant increase in CAT and decrease in the MDA level compared to the MTX (Table 2).

Discussion

MTX, as a well-known anti-cancer agent with a narrow therapeutic window, is used for the treatment of malignant and non-malignant conditions (21). Although the cytotoxic effects of this agent has been determined in various organs (22). Several investigations implicated the potential negative effects of MTX on gonads. Thus, it is currently well known that administration of MTX can lead to reproductive system damages, including decreased epididymal and testicular weights, and reduced epididymal sperm counts and fertility rate (21). Sperm counts are a crude measure of fertility (23, 24) and decrease in the count of sperm could often result from drug interference in the process of spermatogenesis and omission of sperm cells during different developmental stages (25). This agent also inhibits the synthesis of thymidylate, serine, and methionine, leading to disruption of DNA, RNA as well as protein function and consequently cell death (26). The sperm chromatin structure assay (SCSA) is an independent predictor of successful pregnancy (27). According to the results obtained from SCSA, Atashfaraz et al. (28) recently showed that MTX caused an increase in DNA fragmentation.

A single exposure study, using IP route, indicated the transmissibility potential of MTX, substantiating its teratogenicity and embryo-lethality effects (29, 30). The toxic effect of MTX on gonads was clearly determined by study on the histological evaluation of testes. Several symptoms, including increased disorganization, vacuolization, decreased spermatogonial and spermatid counts in the seminiferous tubules, indicate that MTX interferes in the process of spermatogenesis (21). In addition, similar studies have been conducted, reporting damages in spermatogonia as well as spermatocytes after repeated treatments with MTX in rats (8, 31). In terms of male fertility, further to the counts, motility of sperms is also an important factor. Significant increase in lipid peroxidation along with sperm motility reduction might be a consequence of oxidative stress status observed by MTX administration (32, 33). It has also been demonstrated that MTX can reduce the fertilization rate, percentage of blastocysts while increasing percentage of arrested embryos, probably due to the effect of this agent on oxidative stress induction. Recent medical advances have indicated that oxygen radicals and hydrogen peroxides are in association with the undesired adverse effects of several anti-tumor drugs (34).

Free radicals are proposed to play a crucial role in MTX induced toxicity. In present study, we determined that level of MDA in the MTX-treated mice was significantly higher than the control group. This finding was consistent with several reports implicating that MTX induces oxidative stress in tissues by increasing MDA levels (35, 34). The product of lipid peroxidation, MDA, and its level is widely used as an index of oxidative stress (36). Recent evidence has implicated oxidative stress as an etiological factor in the development of male infertility (37, 38). Generally, men with significantly higher seminal reactive oxygen species (ROS) levels and lower antioxidant potential are diag-
nosed with idiopathic infertility (39). Zorn et al. (40) found that high seminal plasma ROS levels contribute to impaired sperm fertilization ability and lower pregnancy rates after IVF.

CAT is an hemoprotein, catalyzing H$_2$O$_2$ reduction and protects tissues from ROS and hydroxyl radical levels growth (41). CAT, acting as a preventative antioxidant, plays an important role in protection against the deleterious effects of lipid peroxidation (42). In this study, we showed that MTX-treatment significantly reduced CAT level in testicular tissue. On the other hand, EP induced a reduction in MDA value and significantly increased CAT level in the mice treated with both MTX and EP. Given the close similarity of EP to an endogenous metabolite, the safety profile in animals and common application of this agent as a food supplement (43), it is unlikely to be harmful to humans. EP, a marker for oxidative stress both in vitro (44) and in vivo (45), inhibits lipid peroxidation. Tsung et al. (46) has recently indicated that it also decreases lipid peroxidation in liver tissue. Moreover, EP can react with ROS via both oxidative carboxylation and formation of hydroxylated adducts at the 3-carbon (47).

Ultimately, we found that EP increased several fertility-related parameters, including fertilization rate and blastocysts percentage, and also reduced percentage of the arrested embryos probably due to its effect on the total antioxidant capacity and H$_2$O$_2$ scavengers.

Conclusion

The present study gives evidences of MTX-induced fertility damage and shows the capability of EP in preventing this damage by inhibiting the lipid peroxidation and improving the activity of antioxidant enzymes. The results of this study evaluated the efficacy of EP as a protective agent against the side-effects of chemotherapeutic agents.

Acknowledgements

We are grateful for the help and financial support of Urmia University in this study. There is no conflict of interest to declare.

References

Effect of *Withania somnifera* (L.) Dunal on Sex Hormone and Gonadotropin Levels in Addicted Male Rats

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Abstract

**Background:** Opioid consumption has been widely increasing across the globe; however, it can cause adverse effects on the body. Morphine, an opioid, can reduce sex hormones and fertility. *Withania somnifera* (WS) is a traditional herb used to improve sexual activities. This study strives to investigate the effect of WS on sex hormones and gonadotropins in addicted male rats.

**Materials and Methods:** In this experimental study, forty-eight male National Maritime Research Institute (NMRI) rats were randomly divided into four groups: i. Control group, ii. WS-treated control group, iii. Addicted group, and iv. WS-treated addicted group. Water-soluble morphine was given to rats for 21 days to induce addiction, concurrently the treated groups (2 and 4) also received WS plant-mixed pelleted food (6.25%). At the end of the treatment, the sex hormone and gonadotropin levels of the rats’ sera were determined in all the groups.

**Results:** Except for follicle-stimulating hormone (FSH), morphine reduced most of the gonadotropin and sex hormone levels. Whereas WS caused a considerable increase in the hormones in the treated addicted group, there was only a slight increase in the treated control group.

**Conclusion:** WS increased sex hormones and gonadotropins-especially testosterone, estrogen, and luteinizing hormone-in the addicted male rats and even increased the progesterone level, a stimulant of most sex hormones in addicted male rats.

**Keywords:** Morphine, *Withania somnifera*, Gonadotropins, Estrogen, Testosterone

Introduction

Recent years have witnessed a rise in the use of opioids and the concomitant increase in their adverse effects such as addiction.

Morphine, one of the strongest opioids, is routinely used to alleviate acute and chronic pain (1). However, it is accompanied by numerous side effects such as peripheral edema, immune suppression, hyperalgesia, sleep apnea, and complications in the digestive, nervous, and genitourinary systems (2, 3). Morphine can adversely affect sexual hormones and fertility in humankind. Laboratory studies have also shown the side effects of this opioid on the sexual hormones of male and female rats (4). Decreased libido, increased rates of still birth, and genetic defects are some of the side effects of morphine use in rats (5). Studies on humans also suggest an inhibitory effect of endogenous opioids on testicular function and testosterone production (6).

Chronic use of Morphine can cause not only hormonal and sexual disorders but also behavioral disorders in humans (7). Use of chemical drugs to treat these disorders has had an insignificant posi-
tive effect and numerous complications. According to the World Health Organization (WHO), about three-quarters of the world population relies upon traditional remedies (mainly herbs) for the health care of its people (8). Scientists, therefore, seek to identify new drugs among herbal medicines that would have fewer complications.

*Withania somnifera* (WS), also known as ashwangandha and winter cherry, has been an important traditional herbal medicine for over 3000 years (9). WS is a densely pubescent shrub up to 1-m tall belonging to the family of Solanaceae. Chemical compounds including large quantities of antioxidants, tannis, flavonoids, and phenolic compounds have been identified in WS (10). It has been reported that WS possesses anti-inflammatory and antioxidant effects and can be useful in the treatment of endocrine and neural disorders (11).

The medicinal properties of WS are attributed to specific secondary metabolites such as alkaloids and withasteroids-withanolides (12). WS produces the largest number of withanolides in diversified functional groups, some of which possess specific therapeutic significance (13). It has been shown that withanolide A may induce neurite development and be useful in the treatment of Alzheimer's disease, Parkinson's disease, and other neurological disorders (14).

One study demonstrated the positive effects of WS on male rats insofar as it raised the luteinizing hormone (LH) level in adult male rats (15). Studies also indicate that the aqueous extract of WS induces some changes in hypophyseal gonadotropins accompanied by an increase in folliculogenesis in immature female rats (16). Another study reported that WS increased libido in adult rats (17). A study on 150 adult men showed that WS treatment led to an increase in testosterone and LH and a decrease in follicle-stimulating hormone (FSH) and serum prolactin. The same study also reported that WS increased antioxidants and decreased oxidative agents, thereby reducing oxidative stresses (18).

With regard to the effect of WS on sexual hormones and gonadotropins and sexual disorders in opioid users, this study sought to investigate the effect of this herb on sexual hormones and gonadotropins in addicted male rats.

**Materials and Methods**

**Experimental animals**

In this experimental study, 48 male National Maritime Research Institute (NMRI) rats (Razi Institute, Iran) weighing an average of 250 g were randomly divided in to four groups: i. Control group, ii. WS-treated control group, iii. Addicted group, and iv. WS-treated addicted group. The rats were kept in cages at a temperature of 24 ± 2°C and were given access to food and water. Twelve-hour light-dark cycling was set.

**Study protocol**

In order to induce morphine addiction in groups 3 and 4, water-soluble morphine was given for 21 days at the same time the treated groups (2 and 4) also received WS plant-mixed pelleted food.

The morphine solution was given to rats in doses of 0.1, 0.2, and 0.3 mg/mL; each dose was administered for 48 hours and then a dose of 4 mg/mL was given for the remaining 15 days. Also, 3% sucrose was added to the solution to omit the bitterness of morphine. Addiction was verified by injecting 2 mg/kg of naloxone intraperitoneally and observing withdrawal symptoms (19).

The methods were confirmed by the Ethics Committee of Shahid Beheshti University of Medical Sciences, and the laboratory animals were afforded due care in accordance with the regulations of the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA).

**Preparation of Withania somnifera**

WS was obtained and approved for the study by the Department of Botany, Shahid Beheshti University of Medical Sciences. Its roots were separated and ground and then combined with pelleted food at a weight ratio of 6.25%. For this purpose, 1000 g of ground rat chow pellet was macerated with 62.5 g of dried WS powder. Next, the new miscellaneous compound was subjected to a pellet-maker device for a new mixed-pellet production. The treated groups (2 and 4) received WS mixed pelleted food for 21 days (20). Given a daily pellet weight consumption (5 /kg/d) in the rats and 6.25% WS in the new pellet, each animal received 0.3 ± 0.01 g/kg WS in each day.
Blood sampling

At the end of treatment, blood samples (3-5 cc) were obtained from the heart of all the rats. The sera were separated via centrifuge (Sigma 4-10, USA) at 2000 rpm for 15 minutes and stored at -70°C in a freezer for hormone analysis.

Plasma analysis

The plasma levels of the sex hormones and gonadotropins were verified using kits manufactured by Monobind Inc., USA, as well as Elisa kits (Lab-systems Ltd., Finland). The measurements were done in the central laboratory of Shahid Mostafa Khomeini Hospital.

Statistical analysis

The data were analyzed with the one-way analysis of variance, followed by post-hoc Tukey test. A P<0.05 was considered statistically significant.

Results

As is shown in Figure 1, morphine addiction reduced the estrogen level (22.76 pg/mL) compared to the control group (50.56 pg/mL). Whereas WS treatment in the control group caused a significant increase (P<0.05) in the estrogen level (86.89 pg/mL), it had no significant effect on the estrogen level of the addicted male rats.

The progesterone levels in all the experimental groups are illustrated in Figure 2. The progesterone level was increased in the WS-treated group (66.28 pmol/mL) and decreased in the addicted group (43.21 pmol/mL) insignificantly compared to the control group (54.24 pmol/mL).

The data on FSH are depicted in Figure 4, which shows that FSH had a statistically significant rise (P<0.05) only in the WS-treated control group (0.422 mIU/mL) compared to the control group (0.299 mIU/mL).

The data on LH levels in all the groups (Fig.5) revealed that there was a significant decrease in the LH level in the addicted male rats (0.0125 mIU/...
mL) compared to the control group (0.191 mIU/mL). However, WS administration inhibited a decrease in the LH levels in the addicted rats significantly.

Discussion

In our male rats, morphine addiction significantly decreased testosterone and LH secretion, but not progesterone and FSH levels compared to the control group. Clinical studies have suggested that opiates may interfere with sex hormone secretion. Heroin use in men resulted in acute suppression of LH release from the pituitary followed by a secondary drop in plasma testosterone levels (21). Also, epidemiological studies have examined a possible link between hypogonadism and opioid use, in both patients and drug addicts. Statistically significant decreases in plasma hormone concentrations were found with lower testosterone and LH levels in men and lower estradiol, progesterone, LH, and FSH levels in women. Animal studies have provided consistent results (22). In male rats, chronic administration of morphine significantly decreased serum testosterone and LH levels, but not FSH release (23). The results of the present study are in agreement with the mentioned reports in human male addicts and male rats.

It is suspected that opioids affect testosterone release through the hypothalamic-pituitary axis and inhibition of LH secretion (21-23). Opioids may decrease gonadotropins both by amending the sex hormone-hypothalamic feedback process and by interfering with the pituitary release of gonadotropins. Moreover, opioids can directly reduce testosterone secretion and testicular interstitial fluid by their negative effect on the testes (24). In the present study in addicted male rats, it seems that morphine caused suppression of LH release from the pituitary followed by a secondary drop in plasma testosterone levels. Therefore, it seems that morphine affects sex hormones and gonadotropins in male and female rats differently.

In our animals-morphine decreased the estrogen level significantly, while the estrogen level was increased significantly in the WS-treated control group. In contrast, in the WS-treated addicted group, there was no decrease in the hormone. This finding chimes in with the result of another study in which the estrogen level was increased following the administration of WS (20). The same study also reported that WS might increase sexual steroids such as estrogen and progesterone via the stimulation of the hypothalamus-hypophysis axis.

In the present study, morphine did not change the level of progesterone significantly. Therefore, in the morphine addicted groups, the WS root exerted no significant effect on progesterone levels.

Our findings also revealed that a dietary intake of the WS root antagonized the reductive effect of morphine on testosterone and LH. Some studies have reported that some of the central effects of morphine are counteracted by the administration of the methanolic extract of the root of WS (25). Also, was shown that repeated administration of WS for 9
days attenuated the development of tolerance to the analgesic effect of morphine. WS also suppressed morphine-withdrawal jumps, a sign of the development of dependence on opiates (26). Accordingly, it seems that WS counters morphine-induced LH suppression release from the pituitary and subsequently enhances plasma testosterone levels. Elevated plasma testosterone levels by WS caused a secondary increase in estrogen levels. Since morphine did not change plasma FSH levels, WS also did not affect FSH levels in addicted male rats. On the other hand while LH and testosterone levels did not exhibit a significant change, FSH and estrogen levels increased significantly in the WS-treated control group. Therefore, it seems that WS affected sex hormones and gonadotropins differently depending on the treatment of the animals. Histological examinations have revealed an apparent increase in the diameter of seminiferous tubules and the number of seminiferous tubular cell layers in the testes of WS-treated control immature rats (15).

WS has been described in traditional medicine as an aphrodisiac that can be used to treat male sex dysfunction and infertility (27). It may, therefore, be suggested that in control normal rats, WS causes normal potentiation of the function of the testes by elevating FSH and its tropic effect on the Sertoli cells (15), without affecting testosterone as the principal male sex hormone. On the other hand, in addicted male rats with sex hormone and gonadotropin deficiency, WS recovered plasma LH and testosterone (23). Long-term treatment with the WS root extract resulted in a higher level of testosterone and LH among infertile men having suboptimal testosterone levels before therapy (27). Our findings in addicted male rats are concordant with the results obtained from infertile men having suboptimal testosterone levels.

Conclusion

In generally, sexual hormones and gonadotropin levels especially LH, testosterone, and estrogen were decreased by morphine administration. Nevertheless, WS administration in the addicted rats prevented the decrease in testosterone and LH.

Acknowledgments

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References


Curcumin Inhibits The Adverse Effects of Sodium Arsenite in Mouse Epididymal Sperm

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Abstract

Background: The aim of this study was to investigate the effects of curcumin on epididymal sperm parameters in adult male Navel Medical Research Institute (NMRI) mice exposed to sodium arsenite.

Materials and Methods: In this experimental study, we divided the animals into four groups: control, sodium arsenite (5 mg/kg), curcumin (100 mg/kg) and curcumin+sodium arsenite. Exposures were performed by intraperitoneal injections for a 5-week period. After the exposure period, we recorded the animals' body and left testes weights. The left caudal epididymis was used to count the sperm number and analyze motility, viability, morphological abnormalities, acrosome reaction, DNA integrity, and histone-protamine replacement in the spermatozoa. One-way analysis of variance (ANOVA) followed by the Tukey’s test was used to assess the statistical significance of the data with SPSS 16.0. P<0.05 was considered significant.

Results: Mice exposed to sodium arsenite showed a significant decrease in the number, motility, viability, normal sperm morphology and acrosome integrity of spermatozoa compared to the control group. In the curcumin+sodium arsenite group, curcumin significantly reversed these adverse effects to the point where they approximated the control. In addition, the application of curcumin alone had no significant difference in these parameters compared to the control and curcumin+sodium arsenite groups. However, we observed no significant differences in the body and the testis weight as well as the DNA integrity and histone-protamine replacement in the spermatozoa of the four groups.

Conclusion: Curcumin compensated for the toxic effects of sodium arsenite on a number of sperm parameters in adult mice.

Keywords: Curcumin, Natural Antioxidant, Sodium Arsenite

Introduction

Numerous evidence exists that today’s men have considerably lower sperm counts compared to those who lived 50 years ago (1). Aspects of male reproductive health may undergo changes and induce infertility in men such as alterations in sperm production, maturation and fertilizing ability. Male reproductive abnormalities may be attributed to exposure to environmental toxicants such as arsenic (2). The toxic metalloid arsenic is released into the environment through industrial activities such as smelting of metals as well as coal burning (3) which contaminates drinking water (4). In addition, arsenic is used in food preservatives, herbicides, and insecticides (3). Drugs also contain arsenic (5). Therefore humans are exposed to this toxicant via different ways. Arsenic can exert adverse effects on the male reproductive system by altering reproductive hormones (6), sperm parameters (2, 6), testicular enzymes, and testis histopathology (2, 7). An increasing body of evidence suggests that oxidative stress is a
possible mechanism in which arsenic damages organs (8, 9). In this context, arsenic generates reactive oxygen species (ROS) which affects testicular function (8). In acute conditions, this will result in male infertility.

Oxidative stress is associated with risk factors for infertility. Therefore, numerous studies have attempted to ameliorate the adverse effects of oxidative stress using antioxidant therapy to improve the endogenous antioxidant defense system. In this regard, application of antioxidants derived from plants can be an effective strategy for alleviating infertility caused by oxidative stress. Curcumin, a yellow phenolic pigment and active component of the rhizomes of *Curcuma longa* (turmeric), has a wide range of pharmacological and biological properties that include anti-inflammatory and anticancer effects (10-12). Curcumin is an antioxidant (13, 14) that protects against oxidative damage in lipids (15), proteins (16), and DNA (17). The antioxidant property of curcumin is proposed to be related to its phenolic hydroxyl and methoxyl group on the phenyl ring. The phenolic hydroxyl group is located at the ortho position with respect to the methoxy group, which substantially increases the antioxidant activities of curcumin (18). Curcumin is reported to protect the male reproductive tract against the damaging effects of lipid peroxidation induced by oxidative stress (19-21).

Previous studies reported adverse effects of sodium arsenite on the male reproductive tract (2, 6). To our knowledge, however, no study examined the effects of curcumin on sodium arsenite mediated toxicity in epididymal spermatozoa of adult mice. The present study investigated the effects of curcumin on epididymal sperm parameters in adult mice exposed to sodium arsenite.

**Materials and Methods**

**Animals and exposures**

In this experimental study, adult male Navel Medical Research Institute (NMRI) mice (8-9 weeks old, 32 ± 5 g) were purchased from Pasteur Institute, Tehran, Iran. The animals were housed in plastic cages on a 12-hour light/dark cycle, temperature of 24 ± 2°C, with water and food *ad libitum*. Adult mice (n=24) were randomly divided into four groups: control, sodium arsenite (5 mg/kg, Sigma, USA) (2, 6), curcumin (100 mg/kg, Sigma, USA) (22, 23) and curcumin+sodium arsenite. Exposures were performed by intraperitoneal injections for a period of five weeks (one spermatogenic cycle for mice) (24). The local Ethical Committee at Arak University approved the experiments. Sodium arsenite and curcumin were dissolved in distilled water and dimethyl sulfoxide (DMSO, Merck, Germany), respectively. Based on the solvents, we chose two control groups, distilled water and DMSO. Since there were no significant differences between the results of the control groups, we considered data from the distilled water group as the control group. At the end of the exposures, the animals were weighed, anesthetized and dissected. Their left testes and cauda epididymides were removed. We recorded the testes weights.

**Sperm count**

The dissected epididymis from each animal was transferred into 10 ml Ham’s F10 medium and cut into small slices in order to release the spermatozoa into the medium. After 10 minutes, one ml of the sperm suspension was diluted with 9 ml of formaldehyde. The diluted spermatozoa were transferred into a Neubauer hemocytometer chamber and the sperm heads were counted with a microscope. The sperm count was performed according to the World Health Organization (WHO) guidelines (25) and data were expressed as the number of sperm per ml.

**Sperm motility**

Assessment of sperm motility was performed according to a WHO protocol (25). Briefly, 10 μl of the sperm suspension was placed on a prewarmed Mackler chamber. A minimum of 5 microscopic fields were assessed to evaluate sperm motility of at least 200 sperm for each of the animals. The percentage of sperm motility was analyzed for the following motion patterns: progressively motile sperm (PMS), nonprogressively motile sperm (NPMS), and nonmotile sperm (NMS).

**Sperm viability**

Sperm viability was evaluated by eosin-nigrosin staining according to a WHO protocol (25). In brief, 40 μL of eosin stain (1% in distilled water, Merck, Germany) was mixed to 20 μL sperm suspension. After 30 seconds, we added 60 μL of nigrosin stain (10% in distilled water, Merck, Germany). One drop of the mixture was placed on a microscope slide to
generate a thin smear and examined under a light microscope at ×1000 magnification. In this method, viable spermatozoa remained colorless while non-viable spermatozoa stained red.

**Sperm morphology**

The Diff Quick staining kit (Faradid Pardaz Pars Inc., Iran) was used to evaluate sperm morphology (26). The sperm suspension was smeared onto a slide and air-dried. These smears were subsequently fixed in Diff Quick fixative for 25 seconds. The smears were then stained with Diff Quick staining solutions I and II for 25 seconds, then washed in distilled water. We observed 100 spermatozoa in order to detect the presence of sperm abnormalities in each sample. In the Diff Quick smear, acrosome stained pink (light purple) whereas the nucleus, midpiece and tail of the sperm stained dark purple.

**Acrosome integrity**

The ability of spermatozoa to undergo acrosome reaction was evaluated by the Coomassie brilliant blue staining method (27). The sperm suspension was smeared and air-dried. The air-dried smears were fixed in 5% paraformaldehyde/phosphate-buffered saline (PBS) for 15 minutes, then washed with PBS. The smears were subsequently stained with Coomassie brilliant blue solution (0.25% in 10% glacial acetic acid and 25% methanol) for 5 minutes after which they were washed with PBS. We counted 100 spermatozoa in each sample under a light microscope. In this staining, reactive acrosomes remained colorless, whereas intact acrosomes stained blue.

**Sperm chromatin structure**

In order to investigate sperm chromatin structure in epididymal sperm, acridine orange (AO) staining was used to detect DNA integrity (double strand DNA versus single strand DNA) in the sperm. Aniline blue (AB) staining was performed to detect histone-protamine replacement during the sperm maturation process. AO staining was performed according to the Tejada et al. (28) method. In brief, the smears were fixed with methanol/acetic acid (3:1) for 14 hours at 4°C and stained with the AO solution (0.19% in phosphate citrate buffer, pH=2.5) for 10 minutes. The slides were washed in distilled water, air dried, and then observed under fluorescence microscope (Olympus, Japan, excitation: 450-490 nm) at ×1000 magnification. At least 100 spermatozoa per slide were counted to evaluate double-strand DNA (green fluorescent) and single-strand DNA (yellow/red fluorescent). As a positive control, sperm DNA was denatured by heating at 96°C for 30 minutes in a thermocycler prior to staining.

The AB staining was carried out based on the Wong et al. (29) method. The smears were immersed in 4% formalin (Merck, Germany) solution for 5 minutes. Fixed smears were then washed with distilled water and dipped in 5% AB stain in 4% acetic acid (pH=3.5) solution for 5 minutes. The slides were then washed in distilled water and stained with 0.5% eosin for 1 minute. Finally, we evaluated 100 spermatozoa per slide under a light microscope at ×1000 magnification. Spermatozoa were classified as dark blue (immature sperm with histone) and red-pink (mature sperm with protamine). As a positive control, sperm samples obtained from testis of immature mice were stained as previously mentioned.

**Statistical analysis**

Results were expressed as mean ± SD for six animals per group. One-way analysis of variance (ANOVA) followed by the Tukey’s test was used to assess the statistical significance of data using SPSS (SPSS for Windows, Version 16.0., Chicago, SPSS Inc.) P<0.05 was considered significant.

**Results**

We observed no significant differences in body and testis weights among the four groups (P>0.05, Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Curcumin</th>
<th>Sodium arsenite</th>
<th>Curcumin+sodium arsenite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body (g)</td>
<td>34.57 ± 2.17*</td>
<td>34.08 ± 3.34*</td>
<td>33.75 ± 4.32*</td>
<td>33.87 ± 1.32*</td>
</tr>
<tr>
<td>Testis (g)</td>
<td>0.115 ± 0.01*</td>
<td>0.111 ± 0.03*</td>
<td>0.107 ± 0.01*</td>
<td>0.108 ± 0.01*</td>
</tr>
</tbody>
</table>

Mean ± SD, n=6 per group. P>0.05. Means with the same superscripts do not differ significantly. P>0.05. ANOVA, Tukey’s test.
There was significantly lower sperm count in the sodium arsenite group compared to the control group (P<0.01). In the curcumin+sodium arsenite group, curcumin significantly compensated the sperm count compared to the sodium arsenite group (P<0.01). Animals exposed with curcumin alone showed no significant difference in this parameter compared to the control and curcumin+sodium arsenite groups (Table 2).

Sodium arsenite significantly decreased the percentage of PMS (P<0.001) and increased the percentage of NPMS (P<0.01) as well as NMS (P<0.001) compared to the control group. In the group of animals exposed to curcumin+sodium arsenite, curcuminine significantly (P<0.001) ameliorated the adverse effect of sodium arsenite on these motility patterns compared to the sodium arsenite group. There were no significant differences in sperm motility in the curcumin group compared to the control and curcumin+sodium arsenite groups (Table 2).

Sperm viability significantly (P<0.001) decreased in the sodium arsenite group compared to the control group. Administration of curcumin significantly reversed sperm viability in the curcumin+sodium arsenite group when compared to the sodium arsenite group (P<0.001). However, the application of curcumin alone had no significant effect on sperm viability compared to the control and curcumin+sodium arsenite groups (Table 2).

This table shows that the sodium arsenite induced a significant (P<0.01) increase in abnormal sperm. In the curcumin+sodium arsenite group, curcumin significantly reversed the percentage of sperm morphological abnormalities compared to the sodium arsenite group (P<0.01). Figure 1 shows a number of sperm abnormalities induced in sodium arsenite mice. Curcumin showed no significant effect on this parameter compared to the control and curcumin+sodium arsenite groups (Table 2).

Table 2: Epididymal sperm number, sperm motility, sperm viability, sperm morphological abnormalities and acrosome integrity of male study mice

<table>
<thead>
<tr>
<th>Sperm parameter</th>
<th>Control</th>
<th>Curcumin</th>
<th>Sodium arsenite</th>
<th>Curcumin+sodium arsenite</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (10⁶)</td>
<td>9.53 ± 0.93ᵇ</td>
<td>9.69 ± 0.68ᵇ</td>
<td>5.55 ± 0.68ᵇ</td>
<td>9.02 ± 0.57ᵇ</td>
</tr>
<tr>
<td>PMS (%)</td>
<td>81.89 ± 1.29ᵇ</td>
<td>83.69 ± 1.90ᵇ</td>
<td>57.35 ± 1.52ᵇ</td>
<td>81.82 ± 0.91ᵇ</td>
</tr>
<tr>
<td>NPMS (%)</td>
<td>5.06 ± 0.71ᵇ</td>
<td>3.54 ± 0.90ᵇ</td>
<td>14.43 ± 1.42ᵇ</td>
<td>5.06 ± 0.46ᵇ</td>
</tr>
<tr>
<td>NMS (%)</td>
<td>13.05 ± 2.01ᵇ</td>
<td>12.77 ± 1.57ᵇ</td>
<td>28.22 ± 0.70ᵇ</td>
<td>13.12 ± 0.73ᵇ</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>77.39 ± 5.68ᵇ</td>
<td>85.70 ± 3.74ᵇ</td>
<td>62.07 ± 7.59ᵇ</td>
<td>83.22 ± 2.70ᵇ</td>
</tr>
<tr>
<td>Acrosome integrity (%)</td>
<td>74.96 ± 3.53ᵇ</td>
<td>78.86 ± 2.26ᵇ</td>
<td>60.56 ± 8.39ᵇ</td>
<td>75.99 ± 2.83ᵇ</td>
</tr>
</tbody>
</table>

PMS; Progressively motile sperm, NPMS; Non-PMS, and NMS; Non-motile sperm. Mean ± SD, n=6 per group. Means with the same superscripts do not differ significantly. P<0.05. ANOVA, Tukey’s test.

Fig.1: Sperm morphological abnormalities in sodium arsenite-exposed mice. A. Club-shaped head, B. Amorphous head, C. Bent head and D. Lack of the usual hook. Diff Quick staining (magnification: ×1000).
In the sodium arsenite group there was a significantly lower percentage of spermatozoa with intact acrosome (P<0.001) compared to the control. Curcumin significantly (P<0.001) compensated the adverse effects of sodium arsenite on acrosomal reaction in the curcumin+sodium arsenite group compared to the sodium arsenite group. In addition, we observed no significant difference in acrosome integrity between the curcumin and control as well as curcumin+sodium arsenite groups (Table 2).

Interestingly, in all sperm parameters where curcumin in the curcumin+sodium arsenite group significantly reversed the toxic effect of sodium arsenite, the data mean was similar to that of the control group (Table 2).

Both AO and AB staining showed that neither sodium arsenite nor curcumin had any obvious effects on sperm DNA integrity and histone-protamine replacement (Fig.2, Table 3).

### Table 3: DNA integrity and histone-protamine replacement in mouse epididymal sperm

<table>
<thead>
<tr>
<th>Sperm parameter</th>
<th>Control</th>
<th>Curcumin</th>
<th>Sodium arsenite</th>
<th>Curcumin+sodium arsenite</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA integrity (%)</td>
<td>99.67 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.83 ± 0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.33 ± 0.82&lt;sup&gt;a&lt;/sup&gt;</td>
<td>99.50 ± 0.84&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Histone-protamine replacement (%)</td>
<td>98.67 ± 1.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.83 ± 0.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.17 ± 1.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.50 ± 1.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Mean ± SD, n = 6 per group. Means with the same superscripts do not differ significantly. P>0.05. ANOVA, Tukey’s test.

![DNA integrity and histone-protamine replacement](image)

Fig.2: DNA integrity [acridine orange (AO) staining] and histone-protamine replacement [aniline blue (AB) staining] in mouse epididymal spermatozoa. AO staining: A. Control, B. Sodium arsenite (5 mg/kg), C. Positive control. AB staining: D. Control, E. Sodium arsenite (5 mg/kg) and F. Positive control (magnification: ×1000).
Discussion

This study evaluated the spermatotoxic effects of sodium arsenite in adult mice and showed that curcumin had the capability to reverse sodium arsenite toxicity.

In the present study, sodium arsenite had no significant effects on body and testis weights. Our results supported those of previous studies (7, 30), however, other reports showed that arsenic exposure induced reductions in body and testis weights (31). These different results might be attributed to the dependency of the toxic effects of sodium arsenite on the dose and exposure period (32).

In accordance with previous findings (2, 6), sodium arsenite caused a significant reduction in the number of epididymal spermatozoa. The effect of sodium arsenite might have been attributed to the endocrine disrupting potential of arsenic (33). Therefore, the decreased sperm count might be the straight effect the reduction of luteinizing hormone (LH), follicle stimulating hormone (FSH), or testosterone production (6, 34) which would reduce sperm counts in sodium arsenite-exposed mice. According to research, sodium arsenite induced apoptosis in the testis (35). Therefore, the reductions in sperm count might have been the result of sodium arsenite-mediated apoptosis in spermatogonia. In addition, arsenic is involved in the generation of free radicals (9). These indicators of oxidative stress can react with polyunsaturated fatty acids (PUFA), resulting in lipid peroxidation in the sperm membrane (36). Since curcumin is a potent antioxidant (10), it can reverse the adverse effects of sodium arsenite on sperm count. Therefore we have hypothesized that sodium arsenite-induced oxidative stress might be responsible for reductions in sperm count.

The results of the present study showed a significant decrease in sperm motility and viability in the sodium arsenite group compared to the control group. Arsenic with its electrophilic nature has been shown to readily interact with thiol and sulfhydryl groups on proteins (37). The decline in the sperm motility might be due to the high concentration of arsenic in the epididymis where the sperm undergo the process of maturation and acquire motility. Oxidative stress mediated by sodium arsenite possibly damages cellular organelles such as mitochondria and in turn lead to disruption of mitochondrial membrane potential (38) and cellular ATP depletion (39). Therefore, we assumed that induction of oxidative stress by arsenic led to toxic effects on sperm motion kinetics and sperm viability. To support this idea we demonstrated that curcumin, with its antioxidant property, ameliorated the adverse effects of sodium arsenite on sperm motility and sperm viability parameters.

This study has explored the toxic effect of sodium arsenite on sperm morphology. Arsenic can cause changes in pituitary-gonadal axis hormones (34). Therefore, it may be speculated that the appearance of sperm abnormalities are due to reductions in LH and FSH, with subsequent reduction in testosterone production. These hormonal alterations induced by arsenic may in turn affect spermatogenesis and reduce normal sperm morphology in the sodium arsenite-exposed mice.

We showed that sodium arsenite significantly increased abnormal acrosome reaction in spermatozoa. Oxidative stress, by inducing lipid peroxidation, affected both fluidity and flexibility of spermatozoa membrane (40). The increase in the ability to undergo an abnormal acrosome reaction observed in animals exposed with sodium arsenite might have resulted from oxidative damage to the plasma membrane of spermatozoa. Acrosome reaction is a membrane fusion phenomenon which requires a high intracellular calcium concentration (41). Arsenic, by disrupting the endoplasmic reticulum, (38) can perturb calcium homeostasis to increase the cytoplasmic calcium concentration (42). Previous studies have shown that arsenic exposure caused a significant increase in calcium influx (43). Therefore, in the present study, sodium arsenite possibly increased cytosolic calcium in the spermatozoa, which led to a premature acrosome reaction. Compensation of this effect in the curcumin+sodium arsenite group might explain this possibility.

Previous studies reported DNA damage induced by ROS (44). Antioxidant deprivation could cause DNA damage in spermatozoa (45). Furthermore, exposure of cells to arsenic induced DNA fragmentation (35). However, in this study sodium arsenite had no significant effect on epididymal sperm chromatin structure as evaluated by AO and AB staining. Several studies have shown that...
sodium arsenite exerts its effects in a dose- and duration-dependent manner (32). It is reasonable to assume that this is the fact for these sperm parameters in the present study. In addition, mammalian sperm DNA has tightly compacted DNA compared to the other cells in the body (46). Tight packaging by protamines might protect sperm DNA against damaging agents.

Conclusion
Curcumin, as a major component of turmeric and a natural antioxidant, is not toxic. Its adverse effect on the fetus has not been reported. Possibly, curcumin can be used in human diets as a therapeutic agent against different pathological conditions induced by oxidative stress.

Acknowledgments
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References


37. Wang TC, Jan KY, Wang AS, Gurr JR. Trivalent arsenicals induce lipid peroxidation, protein carbonylation, and oxidative DNA damage in human urothelial cells. Mutat Res. 2007; 615(1-2): 75-86.


Zeta Sperm Selection Improves Pregnancy Rate and Alters Sex Ratio in Male Factor Infertility Patients: A Double-Blind, Randomized Clinical Trial

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Abstract

Background: Selection of sperm for intra-cytoplasmic sperm injection (ICSI) is usually considered as the ultimate technique to alleviate male-factor infertility. In routine ICSI, selection is based on morphology and viability which does not necessarily preclude the chance injection of DNA-damaged or apoptotic sperm into the oocyte. Sperm with high negative surface electrical charge, named “Zeta potential”, are mature and more likely to have intact chromatin. In addition, X-bearing spermatozoa carry more negative charge. Therefore, we aimed to compare the clinical outcomes of Zeta procedure with routine sperm selection in infertile men candidate for ICSI.

Materials and Methods: From a total of 203 ICSI cycles studied, 101 cycles were allocated to density gradient centrifugation (DGC)/Zeta group and the remaining 102 were included in the DGC group in this prospective study. Clinical outcomes were compared between the two groups. The ratios of X- and Y bearing sperm were assessed by fluorescence in situ hybridization (FISH) and quantitative polymerase chain reaction (qPCR) methods in 17 independent semen samples.

Results: In the present double-blind randomized clinical trial, a significant increase in top quality embryos and pregnancy rate were observed in DGC/Zeta group compared to DGC group. Moreover, sex ratio (XY/XX) at birth significantly was lower in the DGC/Zeta group compared to DGC group despite similar ratio of X/Y bearings spermatozoa following Zeta selection.

Conclusion: Zeta method not only improves the percentage of top embryo quality and pregnancy outcome but also alters the sex ratio compared to the conventional DGC method, despite no significant change in the ratio of X- and Y- bearing sperm population (Registration number: IRCT201108047223N1).

Keywords: Zeta Potential, Density Gradient Centrifugation, Sex Ratio, Embryo Quality, Pregnancy

Introduction

Intra-cytoplasmic sperm injection (ICSI) is usually considered as the ultimate technique to alleviate male-factor infertility when other assisted reproductive technologies (ART) fail to help a couple conceive. During ICSI, a single sperm is directly deposited into the cytoplasm of a mature oocyte, thereby bypassing all natural selection barriers to fertilization (1).

Accordingly, studies demonstrate that selection of sperm based on viability and morphology does not necessarily preclude the chance of oocyte injection with a DNA-damaged or apoptotic sperm when there are no other criteria for selection of sperm in conventional ICSI procedure (2, 3).

To address this obstacle, a series of advanced strategies for non-invasive selection of intact sperm based on cellular and molecular principles have been implemented (for more detail see review by Nasr-Esfahani et al. (4), and Simon L et al. (5). In this regard, Chan et al. (6) and our group (7) proposed that sperm population selected based on the membrane Zeta potential represent lower degrees of DNA fragmentation. Zeta potential is a negative electro-kinetic potential of around -16 to -20 mV which is acquired by sperm-membrane during spermatogenesis and epididymal maturation as a result of sperm surface coating with sialic acids (8).

Our recent study has provided preliminary data on the capacity of Zeta potential to improve the ICSI outcomes on small population (9). Therefore, we aimed to compare clinical outcomes of ICSI using sperm selected by using of Zeta potential or routine density gradient centrifugation (DGC) methods. Moreover, considering differential Zeta-potential of X- and Y-bearing sperm (10), we designed to understand whether Zeta method of sperm selection has any bearing influence on the sex ratio of developed pregnancies developed by ICSI. In the present double-blind randomized clinical trial, we showed that Zeta procedure not only improves the pregnancy outcome but also alters the sex ratio of developed pregnancies, despite no significant change in the ratio of X- and Y-bearing sperm.

Materials and Methods

Patients

This prospective study was approved by the Research Ethics Committee involving human subjects at Royan Institute and Isfahan Fertility and Infertility Center. A total of independent 228 ICSI cycles were included in a parallel double-blind randomized clinical trial spanning the period between September 2010 and March 2014. The power of sample size was calculated to be around 200 based on a previous study (9). Furthermore, we assessed the ratio of X- and Y-bearing sperm by fluorescence in situ hybridization (FISH) and quantitative polymerase chain reaction (qPCR) methods in 17 independent samples of all 228 semen samples subjected to DGC/Zeta and DGC procedures.

Inclusion criteria

A trained nurse was asked to assess the last ultrasound and semen analysis of ICSI candidates on the day of human chorionic gonadotropin (hCG) injection. Accordingly, women below 40 years who had adequate number of follicle in their last ultrasound scan (at least 4 dominate follicle greater 16 mm) and at least one semen parameter (volume, total motility, progressive motility, concentration and morphology) of their partner was below normal threshold based on World Health Organization (WHO 2010) (11). The verified couples were randomly allocated using block designed between the control (DGC) or treatment (DGC/Zeta) trial groups by one of the staff who was unaware of the experimental study. On the day of ICSI, semen samples from men were assessed according to WHO (2010) (11) and only this data for semen samples are provided in this study.

Exclusion criteria

Women with poor quality oocyte (abnormal zona pellucida, large perivitelline space, refractile bodies, increased cytoplasmic granularity, smooth endoplasmic reticulum clusters, and abnormal, fragmented, or degenerated polar bodies) and endometrial thickness greater than 7 mm (type C) were excluded from this study.

Semen processing by density gradient centrifugation

All procedures were conducted under sterile conditions. Semen processing was carried out using Ham’s F-10 supplemented with 10% human serum albumin (HAS, Octalbin, Switzerland). Liquefied semen samples were placed on PureSperm column...
(80% lower, 40% upper) and centrifuged at 300 g for 20 minutes. Sperm pellets were suspended in Ham’s-F10 plus albumin and washed twice in the same medium. The pellet was finally resuspended in 1 ml of the Ham’s-F10 plus albumin for ICSI.

Sperm selection based on combined density gradient and Zeta

The Zeta method was carried out according to modified protocol of Chan et al. (6). For DGC/Zeta, Ham’s-F10 was used without serum supplementation, unless otherwise stated. Immediately after DGC, sperm pellets were washed with Ham’s-F10, re-suspended and diluted in 4 ml Ham’s-F10 in 5 ml Falcon plastic tubes. The prepared sperm suspension was subsequently exposed to the positive charge which was induced by placing the tube inside a latex glove up to the cap. For induction of the charge, the glove was rotated or twisted two or three turns around the tube which was grasped by its cap. Finally, the tube was rapidly removed from the glove and kept at room temperature for 1 minute to allow adherence of the "intact" sperm to the charged tube wall. The medium then was dispensed from the tube to eliminate any non-adhering sperm and the tube wall was washed with 4 ml Ham’s-F10 plus albumin to neutralize the charge on the tube wall and to detach adhering sperm. The tube was centrifuged and the pellet was re-suspended in 1 ml of Ham’s-F10 plus albumin to be used for ICSI. The entire centrifugation step was carried out at 300 g for 5 minutes. For verification of Zeta procedure, an electrostatic voltmeter (Alpha lab, Salt Lake City, USA) was used (7). To minimize variation, a trained individual carried out all procedures and the tubes were labeled by codes. In addition, the embryologist who performed the ICSI procedure was unaware of the individual allocation to the groups (DGC or DGC/Zeta) or the type of sperm preparation implemented.

Intra-cytoplasmic sperm injection

A single standard stimulation and ovulation induction protocol, and ICSI procedure were performed for all the cases (9).

Fertilization rate was calculated from the ratio of fertilized oocytes (2PN) by the total number of injected metaphase II oocytes, multiplied by 100.

Embryo quality was assessed by a certain staff who was not involved and aware of trail on day 3 post-oocyte retrieval and a top quality embryo was defined as an embryo between 6-8 cells with equal blastomere size and less than 25% fragmentation (12, 13). Percentage of top quality embryos was assessed by dividing number of top quality embryo by the total number of embryos, multiplied by 100. Chemical pregnancy was defined when β-hCG level was higher than 10 IU and clinical pregnancy rate was defined by ultrasonography findings showing at least one embryo with a fetal heart beat, 5 weeks after transfer. Implantation rate was defined by the number of observed gestational sacs per number of transferred embryos.

Assessing X- and Y- bearing sperm ratio

Ratio of X- and Y- bearing sperm was determined by FISH technique according to Alahmad et al. (14). Quantitative PCR was also conducted according to Ainsworth et al. (15) 2011 for determining the ratio of X and Y bearing sperm.

Quantitative polymerase chain reaction method

Genomic DNA was extracted using the DNeasy® Blood & Tissue Kit (Qiagen™, Germany), according to the manufacturer’s instructions with some modifications. In brief, semen and blood samples were centrifuged at 3000 g for 3 minutes. The sperm pellet was re-suspended in 200 μl phosphate-buffered saline (PBS) and the samples were treated with proteinase K (40 mAU/mg protein, supported by DNeasy® Blood & Tissue Kit) and incubated at 56°C for 30 minutes. Genomic DNA was harvested by Mini spin column and stored at -20°C. Sex determining region Y (SRY) and Amelogenin genes were candidate as Y and X chromosome determinations, respectively. SRY gene located on p11.3 region of the Y chromosome encodes a transcription factor that belongs to the high mobility group (HMG) box that has a DNA binding domain and was used as a dominant gene in mammalian male sex determination (16). Amelogenin gene is located on the X and Y chromosomes at X p22.1-22.3 and Y p11.2. This gene could be used as a sequence for mammalian female sex determination because it has a 177-bp fragment which inserted just in X-sequence (15). To amplify Y and X specific chromosome fragments by PCR, two pairs of primers were designed (Table 1).
SRY-forward/reverse primers were designed to amplify a 167-bp fragment from SRY gene. For amplification of an X chromosome specific fragment, Amel-reverse primer was designed to anneal to inserted 177-bp fragment in X-sequence. Amel-forward/reverse primers amplified a specific 107-bp fragment for X chromosome (Fig.1). Sex ratio was quantified by quantitative real-time PCR (RT-qPCR) using the Rotorgene 2000 Real Time Cycler (Corbett Research, Sydney, Australia). For each sample, RT-qPCR was performed in triplicate. PCR was conducted by adding 1 µL genomic DNA to the 20 µL of PCR mixture that contained 1 × SYBR® Premix Ex Taq™ (Takara Bio Inc., Otsu, Japan), 0.4 µM of each specific primer, and DNase-free water. The PCR protocol included an initial step of 94°C (4 minutes), followed by 40 cycles of 94°C (30 seconds), 60°C (30 seconds), and 72°C (30 seconds). Primer efficiency was evaluated by making a 5-fold serial dilution of each samples reaction for each primer pairs and was calculated by 10-1/slope equation. The sex ratio in each reaction was calculated by the ratio of threshold cycle (CT) of X to Y (X/Y).

Fluorescence in situ hybridization technique

Mixture of X (DXZ1) red and Y (DYZ3) green (Abbott) labeled probes were prepared for detection of X and Y chromosomes in sperm nuclei. Under a cover slip, ten microliter of probe mixture was added and sealed with rubber cement. For hybridization of the DNA probes, spermatozoa and probe DNA were concomitantly denatured for 5 minutes at 75°C. Then, slides were incubated in a moist chamber at 37°C for 4 hours. After washing with 0.4× SSC/0.3% NP40 at 73°C for 2 minutes and then in 2× SSC/0.1% NP40 at room temperature for 1 minute, slides were counterstained with 10 ml of 4(6-diamidino-2-phenylindole) (DAPI) mixed with antifade (Cytocell Technologies Ltd, UK) using a fluorescence microscope (Nikon E800, Japan) equipped with a triple-band 476 pass filter for DAPI/spectrum green/spectrum orange. At least 1000 spermatozoa with intact nuclei were counted and green or orange fluorescent spot were considered as X and Y chromosomes in sperm, respectively (14).

Statistical analysis

For statistical analysis, the Chi-square, Student’s t test, one-way analysis of variance (ANOVA) and logistic regression model were carried out using the Statistical Package for the Social Sciences software (SPSS 18, Chicago, IL, USA). All data were presented as means ± SEM, and differences were considered significant at P<0.05.

Table 1: The list of primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Size</th>
<th>Gene</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRY</td>
<td>F: CGTCGGAAAGCCGAAGATGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: TTGATGGCCGGTAAAGTGCC</td>
<td>167-bp</td>
<td>SRY</td>
<td>NW_001842360.1</td>
</tr>
<tr>
<td>Amel</td>
<td>F: GTGTCTCTTGGCTGCTCTGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: GGAGAACCTCAAACCCCGACG</td>
<td>107-bp</td>
<td>Amelogenin</td>
<td>NW_001842422.1</td>
</tr>
</tbody>
</table>

Fig.1: PCR amplicons of the Amelogenin and Sex determining region Y [SRY] gene from sperm genomic DNA. A. Schematic illustration of annealing of primers for specific amplification of X and Y chromosome in PCR reaction and B. A 167-bp and 107-bp fragment was amplified in PCR reaction from SRY and Amelogenin gene as indicators for sex determination. PCR; Polymerase chain reaction.
Results

Patient cohorts

251 couples were informed about the trial and 228 accepted to participate in the trial. Due to technical limitation, two cases were included per day (one for DGC and one for DGC/Zeta) and therefore, 20 cases which had the inclusion criteria were excluded from the study. Of the 208 remaining cases, 5 cases (3 from DGC/Zeta and 2 from control group) were excluded from the study based on the exclusion criteria. Of the 203 ICSI cycles included in this study, 101 cases were designated to the DGC/Zeta group while 102 were allocated to DGC group.

Confounding factors

Table 2 compares possible confounding factors between DGC/Zeta and DGC groups. As shown, no significant difference in term of semen parameters, number of oocyte retrieved (Table 3), female and male ages were observed between the two groups.

Table 2: Comparison of possible confounding factors between DGC/Zeta and DGC groups

<table>
<thead>
<tr>
<th></th>
<th>DGC/Zeta group Mean (SE) n=102</th>
<th>DGC group Mean (SE) n=101</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male age (Y)</td>
<td>35.76 ± 5.91</td>
<td>36.79 ± 6.18</td>
<td>0.22</td>
</tr>
<tr>
<td>Sperm concentration (10⁶/ml)</td>
<td>44.27 ± 3.42</td>
<td>42.14 ± 3.43</td>
<td>0.41</td>
</tr>
<tr>
<td>Total sperm motility (%)</td>
<td>38.84 ± 1.20</td>
<td>39.09 ± 1.45</td>
<td>0.89</td>
</tr>
<tr>
<td>Progressive motility (%)</td>
<td>16.86 ± 1.05</td>
<td>16.21 ± 1.24</td>
<td>0.68</td>
</tr>
<tr>
<td>Sperm normal morphology (%)</td>
<td>3.78 ± 0.18</td>
<td>4.13 ± 0.15</td>
<td>0.12</td>
</tr>
<tr>
<td>Female age (Y)</td>
<td>30.73 ± 0.48</td>
<td>31.34 ± 0.53</td>
<td>0.26</td>
</tr>
</tbody>
</table>

DGC; Density gradient centrifugation.

Intra-cytoplasmic sperm injection outcomes

Table 3 shows ICSI outcome between the two groups. No difference in fertilization rates was observed between the DGC/Zeta and DGC groups (77.89 ± 1.87 vs. 76.91 ± 2.08%, respectively). Even though, the respective percentages of top quality embryos (45.83 ± 3.11 vs. 35.38 ± 4.64%), chemical pregnancy (43.13% vs. 23.7%), clinical pregnancy (39.2 vs. 21.8%) and abortion (7.5 vs. 18.2%) were significantly improved in DGC/Zeta group when compared with DGC group. The implantation rate was similar between the two groups (21.01 vs. 12.75% in DGC/Zeta and DGC group, respectively). The mean numbers of embryos transferred were 2.51 ± 0.08 vs. 2.48 ± 0.09 in DGC/Zeta and DGC group, respectively without any significant difference.

Table 3: Comparison of ICSI outcome between DGC/Zeta and DGC groups

<table>
<thead>
<tr>
<th></th>
<th>DGC/Zeta group n=102</th>
<th>DGC group n=101</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of oocyte retrieved</td>
<td>8.65 ± 0.40</td>
<td>8.06 ± 0.35</td>
<td>0.17</td>
</tr>
<tr>
<td>Fertilization rate (%)</td>
<td>77.89 ± 1.87</td>
<td>76.91 ± 2.08</td>
<td>0.72</td>
</tr>
<tr>
<td>Top quality embryo (%)</td>
<td>45.83 ± 3.11</td>
<td>35.38 ± 4.64</td>
<td>0.04*</td>
</tr>
<tr>
<td>Mean of transferred embryos</td>
<td>2.51 ± 0.08</td>
<td>2.48 ± 0.09</td>
<td>0.78</td>
</tr>
<tr>
<td>Mean of vitrified embryos</td>
<td>2.06 ± 0.26</td>
<td>1.78 ± 0.25</td>
<td>0.45</td>
</tr>
<tr>
<td>Chemical pregnancy rate (%)</td>
<td>44/102 (43.13%)</td>
<td>24/100 (23.7%)</td>
<td>0.004*</td>
</tr>
<tr>
<td>Clinical pregnancy rate (%)</td>
<td>40/102 (39.2%)</td>
<td>22/100 (21.8%)</td>
<td>0.009*</td>
</tr>
<tr>
<td>Abortion rate (%)</td>
<td>3/40 (7.5%)</td>
<td>4/22 (18.2%)</td>
<td>0.03*</td>
</tr>
<tr>
<td>Stillbirth rate (%)</td>
<td>0%</td>
<td>2%</td>
<td>0.00</td>
</tr>
<tr>
<td>% Implantation rate (%)</td>
<td>54/257 (21.01%)</td>
<td>32/251 (12.75%)</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Independent student’s t test and Chi-square carried out for statically analyzing. ICSI; Intra cytoplasmic sperm injection, DGC; Density gradient centrifugation, and *; Indicates significant difference (P<0.05).

Confounding factors of intra-cytoplasmic sperm injection outcomes

To compare the clinical pregnancy rate between the two groups and evaluate the possible confounding factors on ICSI outcomes, we applied binary regression model (Table 4). Results showed the odds ratio of clinical pregnancy between DGC/Zeta versus DGC group was 2.304 with P=0.01. Therefore, the chance of clinical pregnancy rate in DGC/Zeta group was 2.3 fold higher than DGC group. These data revealed that confounding factors which had significant influence on the ICSI outcome were male smoking, female age, total oocyte retrieved and injected, ovarian factor and polycystic ovarian syndrome.
Table 4: Multiple regression analysis for DGC vs. DGC/Zeta

<table>
<thead>
<tr>
<th>Parameters</th>
<th>P value</th>
<th>Odds ratio</th>
<th>95% CI for EXP(B)</th>
<th>Lower</th>
<th>Upper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male smoking</td>
<td>0.042*</td>
<td>0.383</td>
<td>0.152</td>
<td>0.965</td>
<td></td>
</tr>
<tr>
<td>Female age</td>
<td>0.012*</td>
<td>0.903</td>
<td>0.834</td>
<td>0.978</td>
<td></td>
</tr>
<tr>
<td>Total oocyte retrieved</td>
<td>0.019*</td>
<td>0.795</td>
<td>0.656</td>
<td>0.963</td>
<td></td>
</tr>
<tr>
<td>Injected oocyte</td>
<td>0.020*</td>
<td>1.290</td>
<td>1.040</td>
<td>1.601</td>
<td></td>
</tr>
<tr>
<td>Tubal factor</td>
<td>0.608</td>
<td>1.238</td>
<td>0.547</td>
<td>2.805</td>
<td></td>
</tr>
<tr>
<td>Endometriosis</td>
<td>0.248</td>
<td>2.075</td>
<td>0.601</td>
<td>7.165</td>
<td></td>
</tr>
<tr>
<td>Uterine factor</td>
<td>0.798</td>
<td>1.143</td>
<td>0.410</td>
<td>3.185</td>
<td></td>
</tr>
<tr>
<td>Polycystic ovarian Syn.</td>
<td>0.005*</td>
<td>5.618</td>
<td>1.699</td>
<td>18.577</td>
<td></td>
</tr>
<tr>
<td>Ovarian factor</td>
<td>0.049*</td>
<td>0.352</td>
<td>0.124</td>
<td>1.002</td>
<td></td>
</tr>
<tr>
<td>Duration of infertility</td>
<td>0.293</td>
<td>0.743</td>
<td>0.427</td>
<td>1.292</td>
<td></td>
</tr>
<tr>
<td>No. Previous ART</td>
<td>0.994</td>
<td>1.000</td>
<td>0.914</td>
<td>1.093</td>
<td></td>
</tr>
<tr>
<td>Clinical pregnancy</td>
<td>0.018*</td>
<td>2.304</td>
<td>1.154</td>
<td>4.601</td>
<td></td>
</tr>
</tbody>
</table>

Binary logistic regression carried out for statically analyzing. *: Indicates statistical significance (P<0.05), CI; Confidence interval, DGC; Density gradient centrifugation, and ART; Assisted reproduction technique.

Sex ratios

Figure 2 compares the percentage of girl baby delivery to total baby delivery between DGC and DGC/Zeta groups. As shown, the percentage of girls delivered after DGC/Zeta sperm selection procedure was significantly higher than DGC procedure with a P<0.001. In addition, we observed statistical significance in sex ratio between DGC and DGC/Zeta groups. Sex ratio was significantly lower in the DGC/Zeta group compared to DGC group (P=0.04). Therefore, the number of girl birth was higher in the DGC/Zeta group compared to DGC group.

Table 5: Comparison of X and Y chromosome-bearing sperm populations between washed sperm, DGC and DGC/Zeta groups by real-time PCR

<table>
<thead>
<tr>
<th>Groups</th>
<th>Sample number</th>
<th>Replicate per sample</th>
<th>Ct_{XY}/Ct_{Amel}</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male blood</td>
<td>17</td>
<td>3</td>
<td>0.99 ± 0.01</td>
<td>0.21</td>
</tr>
<tr>
<td>washed sperm</td>
<td>17</td>
<td>3</td>
<td>0.99 ± 0.01</td>
<td>0.22</td>
</tr>
<tr>
<td>DGC</td>
<td>17</td>
<td>3</td>
<td>0.99 ± 0.01</td>
<td>0.20</td>
</tr>
<tr>
<td>DGC/Zeta</td>
<td>17</td>
<td>3</td>
<td>1.00 ± 0.01</td>
<td>0.17</td>
</tr>
</tbody>
</table>

One-way analysis of variance (ANOVA) carried out for statistically analyzing. DGC; Density gradient centrifugation, Ct; Cycle threshold and PCR; Polymerase chain reaction.

Discussion

The association between sperm maturation and chromatin integrity with ICSI outcome is well established in several studies (17–19). Moreover, intensive studies on sperm morphology and chromatin status have revealed that that spermatozoa with apparently normal morphology may have fragmented DNA. Therefore, a simple selection of ICSI sperm based on viability and morphology does not necessarily preclude the chance of oocyte injection with a DNA-damaged or apoptotic sperm (3). This notion has been supported by inverse association observed between the increased propor-
tion of normal spermatozoa with damaged DNA with embryo quality and also pregnancy outcome after ICSI (20). There are evidence that sperm selected based on their Zeta capacity represent lower degree of DNA damage (6, 21). A recent study by Simon et al. (22) showed that selection of negatively-charged sperm through micro-electrophoresis decreased the degree of DNA damage. Therefore, to reduce the chance of selection of morphologically normal spermatozoa with damaged DNA during ICSI, we carried out a double-blind randomized clinical trial to investigate the efficiency of Zeta sperm selection method to distinguish between intact and damaged sperm.

The results of this study revealed that selection of sperm based on Zeta method increases embryo quality, and chemical and clinical pregnancy rates taking into account all the possible confounding factors which may affect the ICSI outcomes. The confounding factors which had significant influence on the ICSI outcome were male smoking, female age, total numbers of oocyte retrieved and injected, ovarian factors and polycystic ovarian syndrome. These findings are in agreement with the available studies (23-25). Furthermore, the improved ICSI outcomes are consistent with our previous study which suggested that selection of sperm based on sperm functional characteristics reduces the possibility of insemination of DNA damaged sperm during ICSI (4). These results are also in concordance with previous preliminary studies which have implemented SpermSep® CS-10 technique based on sperm surface negative charge (21). To our knowledge, this is the first clinical trial on a large cohort patient group that evaluates the outcome of novel sperm selection based on Zeta potential after ICSI procedure.

The Zeta potential of human Y-bearing sperm has been estimated to be around -16 mV, while the corresponding value for the X-bearing sperm is around -20 mV. The higher negative charge of X-bearing sperm has been attributed to 25% more densely charge sialated proteins residues on their plasma membrane (10). Based on these reports, we compared the sex ratio of children born in DGC and DGC/Zeta groups which was significantly in favor of higher females born in the DGC/Zeta procedure. To our knowledge, this is the first clinical trial on a large cohort patient group that evaluates the outcome of novel sperm selection based on Zeta potential after ICSI procedure.

Considering the fact that the study was a double-blind trial in which the individuals who carried out the ICSI procedure were unaware of sperm selection procedure (DGC or DGC/Zeta), the tentative difference or the skewed sex ratio of children born through Zeta procedure may be attributed to other possible unknown factors. It seems that the difference could be due to higher resistance of X-bearing sperm to stressful conditions. We had previously shown that during Zeta procedure, sperm with negative Zeta potential attached to the positive surface of the tube and the selected sperm underwent a capacitation-like process. This was confirmed by Chlortetracycline (CTC) staining for detection of capacitated sperm and also externalization of phosphatidyl serine (EPS) as an early marker of apoptosis by annexin V staining (26). EPS is attributed to early apoptosis and part of natural process of capacitation and acrosome reaction when two membranes (inner acrosome and sperm lemma) are in the process of fusion. Based on the present data, we proposed that Y-bearing sperm may be less resistant to this process and become immotile during the Zeta procedure. Therefore, following Zeta procedure, we might be selecting X-bearing sperm which may have resisted the Zeta procedure. This proposition is consistent with a previous report which showed that X-bearing sperm are more resistant to stressful conditions like thermal stress (27).

Literature background regarding changes in sex ratio from fertilization to birth in ART cycles suggest that “In-vitro-culture-induced precocious X-chromosome inactivation together with ICSI-induced decrease in number of trophectoderm cells in female blastocysts may account for preferential female mortality at early post-implantation stages and thereby variations in sex ratios at birth in ART cycles”. Whether selection of normal sperm, by procedures like Zeta or Time-lapse, may help to overcome these in vitro induced defects, remains to be explored (28, 29).
Conclusion
Selection of sperm based on Zeta potential improves ICSI outcome. Furthermore, the sex ratio is tentatively affected in favor of female sex. However, further studies are required to confirm this possibility and the mechanism by which Zeta selection may alters the sex ratio.

Acknowledgements
This study was support by Royan institute and we would like to express our gratitude to staff of Isfahan Fertility and Infertility for their full support. None of the authors has any conflicts of interest to disclose and all authors support submission to this journal.

References
An Introduction to The Royan Human Ovarian Tissue Bank

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Abstract
From December 2000 until 2010, the researchers at Royan Institute conducted a wide range of investigations on ovarian tissue cryopreservation with the intent to provide fertility preservation to cancer patients that were considered to be candidates for these services. In 2010, Royan Institute established the Royan Human Ovarian Tissue Bank as a subgroup of the Embryology Department. Since its inception, approximately 180 patients between the ages of 7-47 years have undergone consultations. Ovarian samples were cryopreserved from 47 patients (age: 7-35 years) diagnosed with cervical adenocarcinoma (n=9); breast carcinoma (n=7), Ewing’s sarcoma (n=7), opposite side ovarian tumor (n=7), endometrial adenocarcinoma (n=4), malignant colon tumors (n=3), as well as Hodgkin’s lymphoma, major thalassemia and acute lymphoblastic leukemia (n=1-2 patients for each disease). Additionally, two patients requested ovarian tissue transplantation after completion of their treatments.

Keywords: Fertility Preservation, Human, Cancer, Ovarian Tissue Cryopreservation


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There is a concerning increase in cancer diagnoses according to the Iranian Cancer Society. Today, due to medical advances, many cancers are treatable with timely diagnosis and follow up. The patient can return to a normal life after radiotherapy, chemotherapy, or surgical tumor excision. Therefore, many cancers are no longer considered incurable. Although, in many cases, chemotherapy and radiotherapy aim to save lives, premature ovarian failure and reduction of follicular reserve is undeniable. By taking into consideration the probable infertility of cancer patients, preservation of their reproductive ability prior to onset of cancer treatment is crucial (1, 2). Different methods of assisted reproductive techniques that include oocyte, embryo and ovarian tissue cryopreservation have helped these patients. The use of these techniques in single or married, as well as young and older women differ. Hence, the most appropriate technique is selected according to the patients’ circumstances (1-7). In cases where adequate time exists for ovarian stimulation, embryo cryopreservation is considered the gold standard and an acceptable clinical technique. However, if embryo cryopreservation is not an option due to the absence of a sexual partner, unwillingness to use donor sperm, or for any other reason, the oocytes can be frozen (6). Ovarian tissue cryopreservation is another technique that has a long history of use, but with a new purpose. Limitations of oocyte cryopreservation exist, such as the impossibility of stimulating ovaries in patients with hyperstimulation syndrome. Under these circumstances, ovarian tissue cryopreservation is more accepted and approved (2, 5). In this technique numerous follicles at different stages of maturity are preserved without delays to cancer treatment. In addition, for single or young girls this is the best choice to preserve their reproductive ability (3, 4).

The Royan Human Ovarian Tissue Bank was established in 2010 with the intent to provide fertility preservation services to cancer patients eligible for preservation of reproductive ability. We have established the maximum age for inclusion in the Tissue Bank as 35 years. Cases of malignancy where tumors have metastasized to the ovarian tissue are not accepted for cryopreservation. In other cases there is
no exclusion for acceptance. Patients undergo an initial consultation that determines individual factors of age, marital status, physical and mental conditions, cancer type, its progression stage and grade, level of previous treatments, earlier infertility treatment and prognosis after treatment. After the initial consultation, the best fertility preservation technique is selected. A contract is signed between the Ovarian Tissue Bank and the patient after the consultation. This contract includes patients’ rights and sample maintenance insurance, as well as informing patients about the use of her own sample after treatment, which is approved by the Royan Ethical Committee.

The procedure for ovarian tissue cryopreservation at the Royan Human Ovarian Tissue Bank is as follows. An ovarian tissue sample is removed from the patient by laparoscopy, laparotomy, unilateral or bilateral oophorectomy according to the patient’s condition. The sample is transferred to the Ovarian Bank in the shortest possible time (approximately 1 hour) in Medium 199+Heppes (HTCM, Gibco, Paisley, UK)+20% human serum albumin (HAS, Biotest, Germany) as transfer medium at 4°C and on ice. In the laboratory, initially, the transferred tissue is washed in HTCM+20% HSA medium, after which the medullary part is removed. Next, the cortical part is thinned and 10×5×1 mm strips are obtained from the thin cortex. These steps are all performed on a cool pad. Finally, the stripes are vitrified in a two-step process, equilibration and vitrification. In the first step (equilibration), each strip is washed in equilibrium medium composed of HTCM, ethylene glycol (EG, Sigma, St. Louis, MO, USA), Dimethyl sulphoxide (DMSO, Sigma, USA, each 7.5%) and 20% HSA for 15 minutes at 4°C. In the second step (vitrification), each strip is washed in 15% HTCM, 15% DSMO and 15% EG, 0.25 M sucrose, and 20% HSA for 10 minutes at 4°C. The extra medium is completely removed from the strips, after which they are directly transferred into liquid nitrogen. Of note, we randomly fix one strip before cryopreservation for histological evaluation (H&E staining and Semi thin). For tissue evaluation, one vitrified strip is warmed and assessed histologically. Warming is performed in 4 steps in descending concentrations (1, 0.5, 0.25, and 0.125) of sucrose. The base medium is comprised of HTCM+20% HSA. The histological assessment markers considered for tissue evaluation include total integrity, follicular population, oocyte degeneration, vacuolization and granulation of the nucleus, oolemma and ooplasm conditions, zona pellucida situation (in secondary or preantral follicle), coherence and connectivity of granulosa cells (Fig.1).

Fig.1: Primordial (short arrows), primary (long arrows) and preantral (black arrow) follicles in A., B., C. Control, D., E. and F. Vitrified human ovarian tissues. Hematoxylin and eosin (H&E) staining (magnification: ×20, ×50 μm).
Human Ovarian Tissue Cryopreservation

Functionality of the entire ovarian tissue is considered by the presence or absence of the corpus luteum or corpus albicans in tissue. Finally, the information is kept and filed in a histology description form.

Acknowledgments

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References

Commentary

Minimal Stimulation In Vitro Fertilization: A Better Outcome

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In in vitro fertilization (IVF) programme, the advantages of mild-stimulation have long been appreciated, while there was a called for more patient-friendly approach in ovarian stimulation around 20 years ago (1). However, the concept is yet to get wide-spread acceptance in the IVF community. The main impediment has been a lack of robust outcome data that can assure the success of mild-IVF at least as good as those of conventional IVF. The randomized controlled trials (RCTs) that compared sequential clomiphene citrate (CC) and low-dose gonadotropins (as mild/ minimal stimulation) with conventional long protocol were either small in sample size or heterogeneous in character (2). Nevertheless, recent meta-analyses and systematic reviews found no difference in pregnancy rates or live birth rates (LBRs) between sequential CC-gonadotropin protocol and conventional IVF (3, 4). More recently, a prospective cohort of 163 good prognosis patients undergoing IVF with sequential CC and low-dose gonadotropin regimen reported a cumulative-LBR (C-LBR) of 70% from a fresh and subsequent frozen embryo transfer (ET) up to 3 cycles (5). A large retrospective cohort study of 20, 244 cycles from Japan using a protocol comprising of extended CC (up to the trigger day)+gonadotropin and subsequent vitrified-thawed ET found the treatment outcomes of in all age-groups were comparable with those in the Registry of the Society for Assisted Reproduction (SART) in the USA (6).

The article by Zhang et al. (7) intended to improve the treatment outcomes of minimal stimulation IVF by introducing certain modifications. They recommended the following protocol that was almost identical to the aforementioned Japanese study: extended course of CC up to the day of trigger, the final maturation of oocytes (trigger) by gonadotropin-releasing hormone (GnRH)-agonist and subsequent vitrified-thawed ET. In this protocol, human chorionic gonadotropins (hCG) as trigger was considered only if the couples insisted on fresh ET. The authors reminded us of the proven advantages of mild/ minimal stimulation protocol, especially with regards to its safety and patients’ tolerance. Each of the suggested modifications in the course of minimal-IVF cycles was backed up by recent evidence supporting improved clinical outcomes: GnRH-agonist trigger has been shown to increase oocytes maturation, while better LBR and perinatal outcome have been linked with frozen-thawed ET. A distinct advantage of this regimen which the authors’ group had shown in a previous publication, was its better outcome in treating over-weight women (7). Not the least, continuation of CC throughout the follicular phase, by preventing the luteinizing hormone (LH) surge, effectively circumvented the need for expensive GnRH-antagonists. Due to its simplicity and savings on the cost of medications, the suggested minimal stimulation protocol could potentially be considered in low-resourced communities worldwide. As a whole, this strategy seems to be a step forward in establishing a successful patient-friendly IVF programme.

Even though the protocol sounded attractive theoretically, the crude data-evidence on the treatment success has still been limited to few retrospective studies and a yet unpublished RCT (n=564) by the authors’ own team. By randomly allocating good prognosis patients between mini-IVF with single ET and conventional IVF with double ET, the au-
The authors found lower cumulative C-LBRs (6 months) with the mini-IVF protocol [49 vs. 63%; relative risk (RR): 0.76; 95% confidence interval (CI) 0.64-0.89], albeit no incidence of ovarian hyperstimulation syndrome. The proposed strategies, therefore, need further evaluation through RCTs, by directly comparison of its LBRs per single ET (fresh or frozen), as well as C-LBRs with those of conventional IVF.

The publication by Zhang et al. (7) was not a review article in true sense. It was an effort to disseminate a certain minimal stimulation IVF-ET protocol with specific modifications on the ovulation trigger and ET strategies. In some places, the proposed treatment plan appeared rather too inflexible and specific. For example, it was not convincing why buserelin as a trigger had to be administered by intranasal route only (other than protecting patients from another needle-prick), or why frozen ET was recommended on a medicated cycle only, overlooking potential cost-savings on a natural cycle. Also, routine pre-treatment with combined contraceptive pill remained questionable. General acceptability of the recommended strategy might be restricted by the fact that not all embryology laboratories run an effective vitrification programme, and that the tariff of additional interventions e.g. freezing-thawing and storage of embryos for all patients may be considered as a limiting factor for many clinics. There was evidence from a number of RCTs that mild-IVF cycles, where fresh ETs were performed, resulted in a significant financial benefit, as compared to conventional IVF (4, 8, 9). However, comparative data on the cost-effectiveness of obligatory frozen-thawed ET versus fresh ET in the setting of mild/minimal-IVF are lacking.

The bulk of evidence of better LBRs and superior perinatal outcomes in frozen ET are largely derived from studies with conventional IVF (10). A compromised endometrial receptivity secondary to supra-physiological estrogen and progesterone levels following conventional ovarian stimulation has been implicated (11). Pre-trigger serum estrogen and progesterone levels that were lower than those of conventional IVF caused better endometrial receptivity following milder stimulation IVF and fresh ET (12). In fact, a meta-analysis found better implantation rates in mild-stimulation IVF (2). Adverse perinatal outcomes including low-birth weight and preterm birth have also been linked with the higher number of retrieved oocytes and high late follicular estrogen levels in conventional IVF, not with mild-IVF (10, 13). The mean birth-weight has been found to be higher following natural modified protocol than of conventional IVF (14). Until more evidence in support of using vitrified-thawed embryos in mild-IVF programme is available, the practice of fresh ET seems to continue. The compulsion of frozen ET in the protocol proposed by Zhang et al. (7) actually originated from the deleterious effects of both GnRH-agonist and CC (without gonadotropin) on endometrial receptivity. The former agent is known to be responsible for a luteal phase insufficiency, while the latter tends to cause endometrial thinning. Future studies may explore the possibility of fresh ET in this situation that is possible by replacing CC with tamoxifen (which does not affect endometrial thickness and has successfully been used in patients with estrogen-sensitive cancer) and by applying the emerging methods of enhancing luteal phase support following agonist trigger (15, 16). There was some evidence that sequential addition of CC in an antagonist cycle might improve the corpus luteal function by maintaining a good LH level in both follicular and luteal phase (17). Extrapolating this benefit of CC in GnRH-agonist-triggered cycles, a study found no rectification of the luteal defect induced by agonist trigger (18). Although the peak luteal LH and progesterone levels were elevated, the duration of luteal activity was no different from that of GnRH agonist-induced LH surge in this study. It would be interesting to examine if extended course of anti-estrogens up to the day of trigger, as proposed by Zhang et al. (7), could uphold the LH levels long enough to adequately support the luteal phase.

Acknowledgments

The authors declare no conflict of interest.

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