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Editorial Office Address: P.O.Box: 16635-148, Royan Institute, Tehran, Iran
(Mohammad Hossein Nasr Esfahani, Ph.D.)
Tel & Fax: +9821-22510895
Web: www.ijfs.ir
Emails: ijfs@royaninstitute.org & info@ijfs.ir

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Original Article

Recurrent Vulvovaginal Candidiasis: Could It Be Related to Cell-Mediated Immunity Defect in Response to *Candida* Antigen?

Zahra Talaei, M.D.[1], Saba Sheikhbahaei, M.D.[1], Vajihe Ostadi, Ph.D.[1], Mazdak Ganjalikhani Hakemi, Ph.D.[2], Mohsen Meidani, M.D.[3], Elham Naghshineh, M.D.[4], Majid Yaran, Ph.D.[1], Alireza Emami Naeini, M.D.[1], Roya Sherkat, M.D.[1*]

1. Acquired Immunodeficiency Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
2. Cellular and Molecular Immunology Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
3. Infectious Diseases and Tropical Medicine Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
4. Department of Obstetrics Gynecology, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: Recurrent vulvovaginal candidiasis (RVVC) is a common cause of morbidity affecting millions of women worldwide. Patients with RVVC are thought to have an underlying immunologic defect. This study has been established to evaluate cell-mediated immunity defect in response to *candida* antigen in RVVC cases.

Materials and Methods: Our cross-sectional study was performed in 3 groups of RVVC patients (cases), healthy individuals (control I) and known cases of chronic mucocutaneous candidiasis (CMC) (control II). Patients who met the inclusion criteria of RVVC were selected consecutively and were allocated in the case group. Peripheral blood mononuclear cells were isolated and labeled with CFSE and proliferation rate was measured in exposure to candida antigen via flow cytometry.

Results: T lymphocyte proliferation in response to *candida* was significantly lower in RVVC cases (n=24) and CMC patients (n=7) compared to healthy individuals (n=20, P<0.001), but no statistically significant difference was seen between cases and control II group (P>0.05). Family history of primary immunodeficiency diseases (PID) differed significantly among groups (P=0.01), RVVC patients has family history of PID more than control I (29.2 vs. 0%, P=0.008) but not statistically different from CMC patients (29.2 vs. 42.9%, P>0.05). Prevalence of atopy was greater in RVVC cases compared to healthy individuals (41.3 vs. 15%, P=0.054). Lymphoproliferative activity and vaginal symptoms were significantly different among RVVC cases with and without allergy (P=0.01, P=0.02).

Conclusion: Our findings revealed that T cells do not actively proliferate in response to Candida antigen in some RVVC cases. So it is concluded that patients with cell-mediated immunity defect are more susceptible to recurrent fungal infections of vulva and vagina. Nonetheless, some other cases of RVVC showed normal function of T cells. Further evaluations showed that these patients suffer from atopy. It is hypothesized that higher frequency of VVC in patients with history of atopy might be due to allergic response in mucocutaneous membranes rather than a functional impairment in immune system components.

Keywords: Allergy, *Candida albicans*, Cell Mediated Immunity, Vulvovaginal Candidiasis, Atopy

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***Corresponding Address: P.O.Box: 81876-98191, Acquired Immunodeficiency Research Center, Isfahan University of Medical Sciences, Isfahan, Iran**
Email: sherkat@med.mui.ac.ir

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Introduction

Vulvovaginal candidiasis (VVC) is the second most common cause of genital tract infections (1). It has been shown that VVC affects 75% of female population at least once during their lives and 5-10% at higher frequencies (2). In more than 85% of the cases, VVC is primarily caused by *candida albicans*, followed by *candida glabrata* with an incidence of 4-5%, and to a lesser extent by *candida tropicalis* and *candida parapsilosis* (3, 4). Recurrent VVC (RVVC) is defined as occurrence of four or more episodes of VVC during 12 months (5). Several risk factors have been proposed for RVVC including pregnancy, diabetes mellitus, corticosteroid therapy, antibiotic therapy, and some hygiene habits. Primary impaired immune response can also be considered as a predisposing factor in RVVC patients with none of the risk factors above (6-9). Our knowledge about immune response against fungal pathogens has advanced considerably in recent years. A low rate of immune cell proliferation following antigenic or mitogenic stimulation is assumed to be an indicator for immunodeficiency diseases in clinical and research projects (10). Previous studies showed reduction in *candida*-related lymphocyte proliferation in RVVC patients, focusing on cellular immunity involvement in the process of VVC (11-13). Anergy to *candida* in *in vivo* skin test of RVVC patients is in accordance with impaired T cell proliferation in these patients (14). However, paradoxical evidences indicate no difference in lymphocyte transformation, leukocyte migration inhibition and lymphokines produced by Th1 cells among RVVC patients and healthy individuals (15, 16).

There are several ways to evaluate cellular immunity against *candida* infection. Among the different laboratory methods to test T lymphocyte proliferation stimulated by *candida* antigens flow cytometry using carboxy fluorescein diacetate succinimidyl ester (CFSE), is an established method describes cell division with simple intuitive and meaningful parameters. CFSE provides clear tracking of cell division via a 50% concentration reduction of the fluorescent dye in each divided cell. In the case of T cells this fluorescence reduction may be detected after 5 days. The goal of the current study was to measure T cell proliferation stimulated by *candida* antigen in RVVC patients in comparison with healthy controls and chronic mucocutaneous candidiasis (CMC) subjects using CFSE. Moreover, our aim was to introduce a simple and cost effective diagnostic test to be used routinely in patients suffering from RVVC.

Materials and Methods

Our cross-sectional study was performed from January 2014 till May 2015. Patients with 4 or more episodes of VVC infection during the past year who were initially visited by gynecologists and referred to immunology clinics were enrolled in RVVC case group. All episodes of RVVC were confirmed with vaginal swab smear and culture. Control I subjects were healthy individuals without history of vulvovaginitis during the past year and also had less than 3 episodes of vulvovaginitis in a year during the previous years. Age and educational level did not vary among cases and individuals in control I group. Patients with chronic, persistent or recurrent non-invasive mucocutaneous candidiasis associated with organ infections, autoimmunity, vasculopathy and absence of predisposing conditions such as diabetes or HIV were enrolled in the study as control II group (CMC patients) (17).

Patients with pregnancy, history of using any antibiotic, corticosteroid, hormone therapy, antifungal within the past 30 days and medical history of diabetes mellitus were excluded. Also patients with refractory VVC were excluded because RVVC means episodes of candida infection, with complete response to treatment each time. Required information including age, education status, family history of primary immunodeficiency diseases (PID) (in 1st, 2nd or 3rd degree relatives), history of allergy (confirmed by the clinical immunology and allergy specialist), history of hypothyroidism, history of using antifungal and frequency of VVC within last year was collected using a questionnaire. PID is defined as a heterogeneous group of diseases with higher susceptibility to infections as a result of immunity defect. International Union of Immunological Societies classifies PIDs in 8 large categories according to the impaired components of immune system (18). Severity of vaginitis was measured with a semiquantitative basis scoring from 0-3: 0 (absent), 1 (mild), 2 (moderate), 3 (severe). Sign and symptoms like pruritus, erythema, burning, edema and excoriation/fissure have been scored according to the patient's statement. The sum-score of <4 is considered as asymptomatic/mild vulvovaginitis

and excluded from our study and total score of >7 is defined as severe vulvovaginitis (19). Written and signed informed consents were obtained from all participants. The study was approved by Ethical Committee of Isfahan University of Medical Sciences (reference number: 283457). All enrolled patients were assisted by only one clinician.

At first, prior to blood sampling, phytohemagglutinin (PHA)-induced skin test was done in patients and control II group as an index of cell-mediated immunocompetence. By this test the mitogen PHA is injected subcutaneously and the swelling is measured 24 hours later. Blood samples were taken from the subjects. White blood cell count, immunoglobulin level and basic immunological markers were measured. Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-hypaque gradient separation (Amersham Biosciences, Germany). The number of PBMCs was set at 5-10×10^6 million cells per milliliter in PBS (Cayman Kit, Canada), were labeled with CFSE (Cayman Kit, Canada), and incubated for 30 minutes at 37°C with 5% CO_2. Cells were then centrifuged and the supernatant was discarded. Cell pellet was re-suspended in RPMI-1640 culture medium containing 10% fetal calf serum (FCS) and incubated again at 37°C with 5% CO_2 (Cyman kit, Canada). Triplicate cultures of 2×10^5 cells in 200 µl medium per well were established in 96-well round-bottomed cell culture plates. The candida antigen (HollisterStier, Germany) was diluted at a ratio of 1 to 10 V/V in RPMI-1640 culture medium containing 10% FBS and added to the wells containing the cell suspension. After addition of the antigen, cell plate was incubated at 37°C and 5% CO_2. After 5 days, the cells were transferred to microtubes and washed with PBS. Finally, Proliferation was evaluated with flow cytometery (Partec, Denmark) using the FloMax software. The assays were done in totally blinded manner. Statistical analyses were done by Student's t test, ANOVA and chi square test using SPSS16 software program (SPSS Inc., Chicago, IL, USA).

Results

Twenty-eight patients with RVVC, 28 healthy individuals (control I) and 7 patients with chronic mucocutaneous candidiasis (control II) entered the study. Four RVVC cases and 8 healthy subjects were excluded; hence 24 cases, 20 controls and 7 CMC cases enrolled the study. Characteristics of individuals in each group are shown in Table 1. Age and educational level were not different among the 3 groups. Immunoglobulin level, white blood cell (WBC) counts, immunological biomarkers and PHA skin test were all normal among controls and patients. Mean proliferation of T lymphocytes in response to *candida* antigen was 1.89 ± 1.6 in cases, 3.94 ± 1.0 in healthy controls and 0.81 ± 0.42 in CMC patients (Fig.1).

Table 1: Characteristic of individuals divided in 3 groups of recurrent vulvovaginal candidiasis (RVVC) cases, control and chronic mucocutaneous candidiasis (CMC)

	RVVC case n=24	Control I n=20	Control II (CMC) n=7	P value
Age (Y)	33.3 ± 8.6	32.8 ± 7.9	29.1 ± 8.4	0.5
Educational status				0.3
Lower than high school	15 (62.5%)	8 (40%)	4 (57.1)	
Higher than high school	9 (37.5%)	12 (60%)	3 (42.9%)	
Family history of PID				0.013
Yes	7 (29.2%)	0	3 (42.9%)	0.008 (post hoc case-control I)
No	17 (70.8%)	20 (100%)	4 (57.1%)	0.49 (post hoc case-control II)
History of atopy				NS
Yes	10 (41.7%)	3 (15%)	1(14.3%)	0.054 (post hoc case-control II)
No	15 (58.3%)	17 (85%)	6 (86.6%)	
Drug history (antifungal)				
Yes	15 (60%)	0	2 (28.6%)	
No	10 (40%)	20 (100%)	5 (71.4%)	
Clinical symptom severity (mean ± SD)	5.8 ± 1.5	-	6.8 ± 1.3	0.1

PID; Primary immunodeficiency diseases.

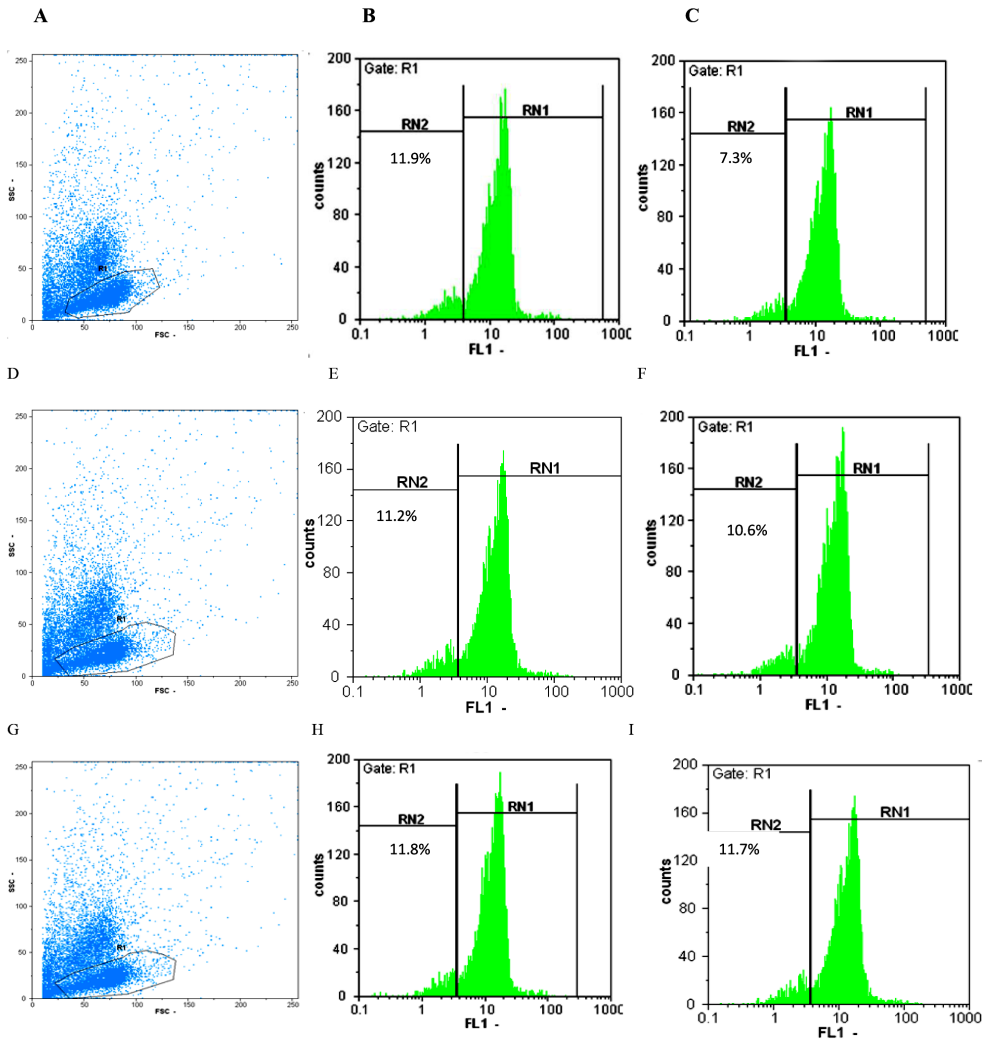

Fig.1: Plots of flow cytometry in recurrent vulvovaginal candidiasis (RVVC), healthy individuals (control I) and chronic mucocutaneous candidiasis (CMC) patients (control II). **A.** CFSE- labeled lymphocytes in a healthy control after 5 days, **B.** Proliferation of lymphocytes after 5 days with antigen in a healthy control, **C.** Proliferation of lymphocytes after 5 days without antigen in a healthy control, **D.** CFSE-labeled lymphocytes in a RVVC case, **E.** Proliferation of lymphocytes after 5 days with antigen in a RVVC case, **F.** Proliferation of lymphocytes after 5 days without antigen in a RVVC case, **G.** CFSE- labeled lymphocytes in a CMC case, **H.** Proliferation of lymphocytes after 5 days with antigen in a CMC case, and **I.** Proliferation of lymphocytes after 5 days without antigen in a CMC case.

Although T cell response was significantly different among the 3 groups (P<0.001), it did not differ statistically between cases and CMC patients (P>0.05). Family history of PID was seen in 29.2% of cases, 42.9% of CMC patients and none of the healthy individuals. History of PID in family members was significantly different among the groups (P=0.01). The prevalence of allergy in RVVC cases was higher than control II group (P=0.054, 41.3 vs. 15%). The median of recurrence in patients during last year was 5.5 times with 4 times as minimum and 8 times as maximum episodes of recurrences. Vaginal symptoms in RVVC cases were not different from CMC group (P>0.05). T cell proliferation was negatively correlated with frequency of RVVC and clinical symptom severity respectively (r=-0.7, P<0.001, r=-0.4, P=0.013). T cell activation was greater in RVVC cases who had allergy compared to the ones without allergy (P=0.01) and also in patients who had used antifungals in comparison with patients who did not have the history of using antifungals (P=0.057). Vaginal clinical symptoms were different in cases with or without allergy and cases with or without history of using antifungal agents (Table 2).

Table 2: T cell proliferation and vaginal symptom severity in patients with/without atopy and in patients with/without history of antifungal consumption

	n	T cell proliferation (mean ± SD)	P value	Clinical symptom severity (mean ± SD	P value
Atopy in RVVC cases			0.01		0.02
Yes	10	2.90 ± 1.5		5.0 ± 1.1	
No	14	1.27 ± 1.31		6.4 ± 1.5	
Antifungal			0.57		0.005
Yes	16	1.53 ± 0.89		5.3 ± 1.2	
No	15	0.93 ± 0.78		6.8 ± 1.5	

RVVC; Recurrent vulvovaginal candidiasis.

Discussion

VVC is a fungal infection predominantly caused by *candida albicans*. Despite large number of surveys on mechanisms involving localized vaginal yeast infections, the pathophysiology is not determined yet. Clinical studies demonstrated the role of both innate and adaptive immunity in VVC (20). Both arms of adaptive immunity (cell mediated immunity and humoral immunity) are thought to be protective against *candida* infection (21). A study found infiltration of T cells predominantly in vaginal fluid of fungal infected rats; however, another study showed proliferation of vaginal B-lymphocytes isolated from *candida*-infected rats in response to fungal antigen (22).

There is considerable conflict about susceptibility to RVVC in the literature, whether it is mainly due to impairment in T cell function. A study done by Corrigan et al. (23) revealed that subjects with RVVC have decreased T cell proliferation and IFN-γ secretion in stimulation with *candida*. Alternative clinical studies detected significant decrease in lymphoproliferative activity of helper T cells and pro-inflammatory cytokines (24, 25). It is necessary to notice that response to subcutaneous injection of PHA in RVVC patients and healthy individuals did not differ. The test is an approval document indicating that T cells are responsive and have normal function in exposure to other antigens. Our results showed that T cells did not proliferate normally in 58% of RVVC cases compared to control I group. However there were RVVC cases (42%) who had normal LTT but were suffering from allergy at the same time or had the history of allergic reactions. So RVVC patients with allergies showed higher T cell proliferation than RVVC patients without allergies. Our findings suggest two hypotheses for host defense against recurrent *candida* infection. One emphasizes on the importance of T cell mediated defect in response to *candida* predisposing vaginal tissue to yeast infection, and the other one proposes an underlying immune hypersensitivity reaction in vaginal mucosa rendering the signs and symptoms of vulvovaginitis in allergic patients who had normal T cell function. We indicated that sever form of VVC is related to lower proliferation of T cells, as higher clinical score was seen in patients with cell-mediated immunity (CMI) defects in response to *candida* than patients with normal T cells.

Early clinical studies have shown that defects in CMI by Th1 cells lead to recurrent fungal infections (26-28). It has been revealed that mutations affecting Th17/IL17 increase susceptibility to CMC and was confirmed with results of the study assessing vaginal yeast problems in response to inhibition of Th17 (29). These results are consistent with our findings while they are in contrast with some other studies, which reported normal cellular immunity in evaluation of RVVC patients (15, 30, 31). Controversial results have been published about contribution of Th2 cells in protection against *candida* infection. Some studies propose no immunologic role of Th2 and secretory cytokines (32, 33), while other studies report higher levels of Th2-related chemokines in vaginal fluid (34, 35).

Some of the studies conducted on the relationship between patient's immunity and the incidence of VVC have focused on the evaluation of local safety and allergic reactions in the vaginal environment. Several evidences exist suggesting that RVVC has strong correlation with atopy (36-39). Treatment with zafirlukast, cetirizine or other allergy immunotherapy medicines induce remission and are sometimes considered as mainte-

nance therapy in patients who failed to get resolution of symptoms by variant antifungal treatments (40, 41). Weissenbacher et. al. (42) have studied immunological factors including IL-4, IL-5, IL-13 and PGE2 in vaginal discharge in women with RVVC proposing that infected cases had a specific local immune deficiency in that area. Another study evaluating patients with hypersensitivity to their spouse's seminal plasma proteins suggests that IgE-mediated immune responses may be involved in this process (43). We concluded that the etiology of RVVC in allergic patients is an inflammatory response to allergens in different mucosal membranes (oropharynx, sinus and vagina), which provide vaginal environment susceptible to fungal growth.

Other studies have demonstrated that maintenance therapy with antimycotic drugs is effective in lowering sign and symptom severity and frequency of recurrence (44, 45). We found that patients with history of using antifungal agents did not have significantly higher lymphoproliferative activity but presented milder symptoms than the groups not having received such drugs. In our studies patients taking antifungal medicine in the past 30 days were excluded and only patients who had the history of receiving antimycotics prior to that were analyzed. Previous *in vitro* studies performed by Fidel et al. (46, 47) found that immunity against *candida* is not mediated by systemic host defense and it is mostly associated with local acquired mucosal immunity.

As opposed to this publication, a study done by Leigh et al. (48) indicated that the incidence of mucosal infection was not different between HIV+ patients and healthy persons. However, another study evaluating HIV+ patients with oral and vaginal candidiasis, suggested that immunity to *candida* is mediated by immunity from both systemic and local sources (49). Further studies demonstrated that reduced protection against *candida* is mostly due to *candida albicans*-specific adaptive immunity (23, 50). As we extracted lymphocytes from peripheral blood, it would be an evidence of systemic immunity involvement in this process. Our study did not assess local immunity to *candida* so it could not reveal the role of local immunity in developing RVVC. Other hypotheses about susceptibility to candida infection are increased polymononuclear leukocyte (PMN) response and chemotactic factors (23, 51), impaired innate immune response (52) and defect in Th17 response to *candida* due to dectin-1 mutation (53).

Conclusion

The result of this study showed that the patients with RVVC could have had a pre-existing defect in their CMI or a proven history of allergy, which could increase the susceptibility of mucosa to get *candida* infection. Since T cell mediated immunity seems to have an important role in development of RVVC, diagnosis and treatment of this infection should be performed with regard to T cell immunity. LTT is recommended to be employed routinely using CFSE staining and measured by flow cytometry in patients who complain about recurrent incidents of *candida* vulvovaginitis. By further studies confirming our results, RVVC may be classified as CMC, one of the PIDs, which should then receive treatment required for CMC patients, while others with simultaneous history of allergy could take benefit from allergy treatments.

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References

1. Mikamo H, Matsumizu M, Nakazuru Y, Okayama A, Nagashima M. Efficacy and safety of a single oral 150 mg dose of fluconazole for the treatment of vulvovaginal candidiasis in Japan. J Infect Chemother. 2015; 21(7): 520-526.
2. Mårdh PA, Rodrigues AG, Genç M, Novikova N, Martinez-de-Oliveira J, Guaschino S. Facts and myths on recurrent vulvovaginalcandidosis--a review on epidemiology, clinical manifestations, diagnosis, pathogenesis and therapy. Int J STD AIDS. 2002; 13(8): 522-539.
3. Linhares LM, Witkin SS, Miranda SD, Fonseca AM, Pinotti JA, Ledger WJ. Differentiation between women with vulvovaginal symptoms who are positive or negative for Candida species by culture. Infect Dis Obstet Gynecol. 2001; 9(4): 221-225.
4. Saporiti AM, Gómez D, Levalle S, Galeano M, Davel G, Vivot W, et al. Vaginal candidiasis: etiology and sensitivity

profile to antifungal agents in clinical use. Rev Argent Microbiol. 2001; 33(4): 217-222.
5. Mandell GL, Bennett JE, Dolin R. Mandell, Douglas, and Bennett's Principles and practice of infectious disease. 8th ed. Philadelphia: Churchill Livingstone; 2015; 1364-1365.
6. Geiger AM, Foxman B, Gillespie BW. The epidemiology of vulvovaginal candidiasis among university students. Am J Public Health. 1995; 85(8 Pt 1): 1146-1148.
7. Gunther LS, Martins HP, Gimenes F, Abreu AL, Consolaro ME, Svidzinski TI. Prevalence of Candida albicans and non-albicans isolates from vaginal secretions:comparative evaluation of colonization, vaginal candidiasis and recurrent vaginalcandidiasis in diabetic and non-diabetic women. Sao Paulo Med J. 2014; 132(2): 116-120.
8. Gonçalves B, Ferreira C, Alves CT, Henriques M, Azeredo J, Silva S. Vulvovaginal candidiasis: epidemiology, microbiology and risk factors. Crit Rev Microbiol. 2016; 42(6): 905-927.
9. Kalo-Klein A, Witkin SS. Candida albicans: cellular immune system interactions during different stages of the menstrual cycle. Am J Obstet Gynecol. 1989; 161(5): 1132-1136.
10. Hori T, Ohnishi H, Teramoto T, Tsubouchi K, Naiki T, Hirose Y, et al. Autosomal-dominant chronic mucocutaneous candidiasis with STAT1-mutation can be complicated with chronic active hepatitis and hypothyroidism. J Clin Immunol. 2012; 32(6): 1213-1220.
11. Puel A, Cypowyj S, Maródi L, Abel L, Picard C, Casanova JL. Inborn errors of human IL-17 immunity underlie chronic mucocutaneous candidiasis. Curr Opin Allergy Clin Immunol. 2012; 12(6): 616-622.
12. Witkin SS, Hirsch J, Ledger WJ. A macrophage defect in women with recurrent Candida vaginitis and its reversal in vitro by prostaglandin inhibitors. Am J Obstet Gynecol. 1986; 155(4): 790-795.
13. Witkin SS, Yu IR, Ledger WJ. Inhibition of Candida albicans-inducedlymphocyte proliferation by lymphocytes and sera from women with recurrentvaginitis. Am J Obstet Gynecol. 1983; 147(7): 809-811.
14. Syverson RE, Buckley H, Gibian J, Ryan GM Jr. Cellular and humoral immune status in women with chronic Candida vaginitis. Am J Obstet Gynecol. 1979; 134(6): 624-627.
15. Fong IW, McCleary P, Read S. Cellular immunity of patients with recurrent or refractory vulvovaginal moniliasis. Am J Obstet Gynecol. 1992; 166(3): 887-890.
16. Fidel PL Jr, Lynch ME, Redondo-Lopez V, Sobel JD, Robinson R. Systemic cell-mediated immune reactivity in women with recurrent vulvovaginal candidiasis. J Infect Dis. 1993; 168(6): 1458-1465.
17. Conley ME, Notarangelo LD, Etzioni A. Diagnostic criteria for primary immunodeficiencies. Representing PAGID (Pan-American Group for Immunodeficiency) and ESID (European Society for Immunodeficiencies). Clin Immunol. 1999; 93(3): 190-197.
18. Al-Herz W, Bousfiha A, Casanova JL, Chatila T, Conley ME, Cunningham-Rundles C, et al. Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. Front Immunol. 2014; 5: 162.
19. Sobel JD, Kapernick PS, Zervos M, Reed BD, Hooton T, Soper D, et al. Treatment of complicated Candida vaginitis: comparison of single and sequential doses of fluconazole. Am J Obstet Gynecol. 2001; 185(2): 363-369.
20. Ashman RB. Protective and pathologic immune responses against Candida albicans infection. Front Biosci. 2008; 13: 3334-3351.
21. de Bernardis F, Santoni G, Boccanera M, Spreghini E, Adriani D, Morelli L, et al. Local anticandidal immune responses in a rat model of vaginal infection by and protection against Candida albicans. Infect Immun. 2000; 68(6): 3297-3304.
22. De Bernardis F, Santoni G, Boccanera M, Lucciarini R, Arancia S, Sandini S, et al. Protection against rat vaginal candidiasis by adoptive transfer of vaginal B lymphocytes. FEMS Yeast Res. 2010; 10(4): 432-440.
23. Corrigan EM, Clancy RL, Dunkley ML, Eyers FM, Beagley KW. Cellular immunity in recurrent vulvovaginal candidiasis. Clin Exp Immunol. 1998; 111(3): 574-578.
24. Fidel PL Jr, Barousse M, Lounev V, Espinosa T, Chesson RR, Dunlap K. Local immune responsiveness following intravaginal challenge with Candida antigen in adult women at different stages of the menstrual cycle. Med Mycol. 2003; 41(2): 97-109.
25. Nawrot U, Grzybek-Hryncewicz K, Zielska U, Czarny A, Podwińska J. The study of cell-mediated immune response in recurrent vulvovaginal candidiasis. FEMS Immunol Med Microbiol. 2000; 29(2): 89-94.
26. Klein RS, Harris CA, Small CB, Moll B, Lesser M, Friedland GH. Oral candidiasis in high-risk patients as the initial manifestation of the acquired immunodeficiency syndrome. N Engl J Med. 1984; 311(6): 354-358.
27. Samaranayake LP. Oral mycoses in HIV infection. Oral Surg Oral Med Oral Pathol. 1992; 73(2): 171-180.
28. Clift RA. Candidiasis in the transplant patient. Am J Med. 1984; 77(4D): 34-38.
29. Whibley N, Gaffen SL. Brothers in arms: Th17 and Treg responses in Candida albicans immunity. PLoS Pathog. 2014; 10(12). e1004456.
30. Cu-Uvin S, Hogan JW, Warren D, Klein RS, Peipert J, Schuman P, et al. Prevalence of lower genital tract infections among human immunodeficiency virus (HIV)-seropositive and high risk HIV-seronegative women. HIV Epidemiology Research Study Group. Clin Infect Dis. 1999; 29(5): 1145-1150.
31. White MH. Is vulvovaginal candidiasis an AIDS-related illness? Clin Infect Dis. 1996; 22 Suppl 2: S124-127.
32. Mathur S, Koistinen GV, Horger EO 3rd, Mahvi TA, Fudenberg HH. Humoral immunity in vaginal candidiasis. Infect Immun. 1977; 15(1): 287-294.
33. Barousse MM, Van Der Pol BJ, Fortenberry D, Orr D, Fidel PL Jr. Vaginal yeast colonisation, prevalence of vaginitis, and associated local immunity in adolescents. Sex Transm Infect. 2004; 80(1): 48-53.
34. Babula O, Lazdāne G, Kroica J, Linhares IM, Ledger WJ, Witkin SS. Frequency of interleukin-4 (IL-4) -589 gene polymorphism and vaginal concentrations of IL-4, nitric oxide, and mannose-binding lectin in women with recurrent vulvovaginal candidiasis. Clin Infect Dis. 2005; 40(9): 1258-1262.
35. Chen S, Li S, Wu Y, Liu Z, Li J. Local expression of vaginal Th1 and Th2cytokines in murine vaginal candidiasis under different immunity conditions. J Huazhong Univ Sci Technolog Med Sci. 2008; 28(4): 476-479.
36. Witkin SS, Jeremias J, Ledger WJ. A localized vaginal allergic response inwomen with recurrent vaginitis. J Allergy Clin Immunol. 1988; 81(2): 412-416.
37. Kudelko NM. Allergy in chronic monilial vaginitis. Ann Allergy. 1971; 29(5): 266-267.
38. Rigg D, Miller MM, Metzger WJ. Recurrent allergic vulvovaginitis: treatment with Candida albicans allergen immunotherapy. Am J Obstet Gynecol. 1990; 162(2): 332-336.
39. Tomsíková A, Tomaierová V, Kotál L, Nováčková D. An

immunologic study of vaginal candidiasis. Int J Gynaecol Obstet. 1980; 18(6): 398-403.
40. Neves NA, Carvalho LP, Lopes AC, Cruz A, Carvalho EM. Successful treatment of refractory recurrent vaginal candidiasis with cetirizine plus fluconazole. J Low Genit Tract Dis. 2005; 9(3): 167-170.
41. Rigg D, Miller MM, Metzger WJ. Recurrent allergic vulvovaginitis: treatment with Candida albicans allergen immunotherapy. Am J Obstet Gynecol. 1990; 162(2): 332-336.
42. Weissenbacher TM, Witkin SS, Gingelmaier A, Scholz C, Friese K, Mylonas I. Relationship between recurrent vulvovaginal candidiasis and immune mediators in vaginal fluid. Eur J Obstet Gynecol Reprod Biol. 2009; 144(1): 59-63.
43. Bernstein JA, Herd ZA, Bernstein DI, Korbee L, Bernstein IL. Evaluation and treatment of localized vaginal immunoglobulin E-mediated hypersensitivity to human seminal plasma. Obstet Gynecol. 1993; 82(4 Pt 2 Suppl): 667-673.
44. Sobel JD. Recurrent vulvovaginal candidiasis. A prospective study of the efficacy of maintenance ketoconazole therapy. N Engl J Med. 1986; 315(23): 1455-1458.
45. Bolouri F, Moghadami Tabrizi N, Davari Tanha F, Niroomand N, Azmoodeh A, Emami S, et al. Effectiveness of fluconazole for suppressive maintenance therapy in patients with rvvc: a randomized placebo-controlled study. Iran J Pharm Res. 2009; 8(4): 307-313.
46. Fidel PL Jr, Lynch ME, Sobel JD. Circulating CD4 and CD8 T cells have little impact on host defense against experimental vaginal candidiasis. Infect Immun. 1995; 63(7): 2403-2408.
47. Fidel PL Jr, Lynch ME, Sobel JD. Effects of preinduced Candida-specific systemic cell-mediated immunity on experimental vaginal candidiasis. Infect Immun. 1994; 62(3): 1032-1038.
48. Leigh JE, Barousse M, Swoboda RK, Myers T, Hager S, Wolf NA, et al. Candida-specific systemic cell-mediated immune reactivities in human immunodeficiency virus-positive persons with mucosal candidiasis. J Infect Dis. 2001; 183(2): 277-285.
49. Fidel PL Jr. Immunity to Candida. Oral Dis. 2002; 8 Suppl 2: 69-75.
50. LeBlanc DM, Barousse MM, Fidel PL Jr. Role for dendritic cells in immunoregulation during experimental vaginal candidiasis. Infect Immun. 2006; 74(6): 3213-3221.
51. Yano J, Lilly E, Barousse M, Fidel PL Jr. Epithelial cell-derived S100 calcium-binding proteins as key mediators in the hallmark acute neutrophil response during Candida vaginitis. Infect Immun. 2010; 78(12): 5126-5137.
52. Bernstein JA, Seidu L. Chronic vulvovaginal Candida hypersensitivity: an underrecognized and undertreated disorder by allergists. Allergy Rhinol (Providence). 2015; 6(1): 44-49.
53. Ferwerda B, Ferwerda G, Plantinga TS, Willment JA, van Spriel AB, VenselaarH, et al. Human dectin-1 deficiency and mucocutaneous fungal infections. N Engl J Med. 2009; 361(18): 1760-1767.

Original Article

Association of *CYP1A1*2A* Polymorphism with Idiopathic Non-Obstructive Azoospermia in A South Indian Cohort

Shalaka S Ramgir, M.Sc., Nishu Sekar, M.Sc., Divya Jindam., B.Sc., Abilash V.G., Ph.D.*

Department of Biomedical Sciences, School of Bio Sciences and Technology (SBST), VIT University, Vellore, Tamilnadu-632014, India

Abstract

Background: Infertility is the inability of a couple to conceive after one and a half years of unprotected sex. Male infertility, which accounts for almost half of infertility cases, is considered as a major problem all over the world. The aim of this study was to investigate the association of *CYP1A1* polymorphisms with idiopathic non-obstructive azoospermia in a South Indian cohort.

Materials and Methods: An experimental study was conducted with idiopathic non-obstructive azoospermia. A total of 120 infertile and 80 fertile samples were collected, and DNA was then extracted from all samples. The *CYP1A1*2A* polymorphism genotyping was carried out by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP).

Results: The genotype distribution of *CYP1A1*2A* polymorphism showed significant difference between patients and controls. Moreover, the CC genotype was associated with decreased risk of idiopathic non-obstructive azoospermia in comparison with the TT and TC genotypes.

Conclusion: The current experimental study identified that the CT genotype of *CYP1A1*2A* polymorphism may contribute to the pathogenesis of male infertility in the South Indian population.

Keywords: CYP1A1, Restriction Fragment Length Polymorphism, Infertility, Cohort

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Introduction

Infertility is the inability of a couple to conceive after one and a half years of unprotected sex. It is one of the major medical problems where about 10-15% of couples are affected with infertility of which 50% of these cases are male-related (1). The literature suggests that about 15% of male infertility cases are due to genetics factors (2). Besides genetic and environmental factors, about 30% of cases of infertility in men remain poorly understood in terms of etiology and pathogenesis, and their condition is thus considered idiopathic (3). A decrease in sperm count and motility from 38.18 million/ml and 61.16% in 1993-1994 to 26.61 million/ml and 47.14% respectively by 2004-2005 was recorded in a study on the Indian population. Sperm morphology was 40.51% in 1993-1994 and was decreased to 19.75% by 2004-2005 (4). Ageing or environmental toxicants initiate DNA strand break in the spermatozoa of affected males, eventually leading to a mutation in the embryo (5). Genetic factors can be identified in male infertility with congenital hypogonadotropic hypogonadism, congenital absence of vas deferens and primitive testicular failure (6). Epidemiological studies have been unequivocal about the effects of lead (Pb^{2+}) and cadmium (Cd^{2+}) on hormone concentrations, male fertility and sperm parameters (7).

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***Corresponding Address: Department of Biomedical Sciences, School of Bio Sciences and Technology (SBST), VIT University, Vellore, Tamil Nadu-632014, India**
Email: abilash.vg@vit.ac.in

Royan Institute
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CYP1A1 (cytochrome P450, family 1, subfamily A, polypeptide 1) (8) encodes the CYP1A1 enzyme that catalyzes the bioactivation of polycyclic aromatic hydrocarbons (PAHs). In the natural environment, PAHs are capable of forming DNA adducts after it has being activated to generate DNA reactive metabolism. In sperm cells DNA adducts may be considered as a sign of severe DNA damage and infertility is thought to be associated with such damage, which may affect meiotic division during spermatogenesis (9). The four most important enzyme families involved in the metabolism of xenobiotics are the N-acetyltransferase (NAT), cytochrome P450 (P450), glutathione-S-transferase (GST) and microsomal epoxide hydrolase (mEH) enzymes (10). A study on the Chinese population suggested that a CYP1A1 polymorphism may contribute to the pathogenesis of male infertility (11). *CYP1A1*2A* (T→C substitution at nucleotide 3801 in the 3′-non-coding region; rs464693) is the most prevalent in the Asian population (12). Increase in smoking, alcohol consumption and high exposure to chemicals may lead to infertility. The study was therefore designed to investigate the association of the *CYP1A1*2A* polymorphism with idiopathic non-obstructive azoospermia and to assess the impact of the status of life style factors on the relationship between the polymorphism and susceptibility to idiopathic non-obstructive azoospermia.

Materials and Methods

In this experimental study, 120 idiopathic azoospermic men were included but excluding those with known cases such as Y chromosome microdeletion, obstructive azoospermia and Klinefelter syndrome all of which were tested at the Andrology Department, Stanley Medical College and Hospitals, Chennai, India. The age of azoospermic men ranged from 24-38 years and the 80 fertile healthy subjects (control group) in the same age range were included in the study. The criterion for including healthy controls was to have at least one child without assisted reproductive technologies. Couples reported with female factors were excluded from the study. With the help of an experienced urologist at Stanley Hospital, for each patient, a detailed case history was obtained and a clinical examination was carried out. The lifestyle habit and chemical exposure of the probands were recorded including smoking habits, alcohol drinking and exposure to toxic chemicals. Semen was collected from both infertile and fertile males after three days of abstinence from sex and semen volume, sperm count, and motility were recorded. Blood sample from each participant was collected by a physician with written consent. The study was approved by the University Human Ethical Committee of the VIT University.

Genotype determination

DNA was extracted from 2 ml of venous blood according to lab procedure and stored at +4°C and then subjected to agarose gel electrophoresis. Oligonucleotide sequences of the polymerase chain reaction (PCR) primers were 5′-CAGTGAAGAGGTGTAGCCGC-3′ and 5′-TAGGAGTCTTGTCTCATGCC-3′, and the product length was 340 bp. Three µl of DNA was amplified with initial temperature of 95°C for 5 minutes, 30 cycles of denaturation at 94°C for 45 seconds, annealling at 60°C for 50 seconds and extension at 72°C for 1 minute, followed by a final extension at 72°C for 10 minutes in a thermal cycling machine. The 20 µl PCR mixture contained 10 pmol of each forward and reverse primer, 6 µl of master mix, 9 µl of autoclaved miliq water and 4 µl of DNA. The PCR products were separated by gel electrophoresis on a 3% agarose gel containing ethidium bromide (50 µg/µl) and were visualized under UV illumination. The results were analyzed with a gel analysis software (MEDCARE). 2 µl of amplified PCR products were then mixed with 7 µl of nuclease free water, 2 µl of 10X buffer and 2 units of Msp1 restriction enzyme (Eurofins Genomic India pvt Ltd). The digested fragments were visualized on an agarose gel as above. When an Msp1 restriction site was present, the fragment of 340 bp was digested into two fragments of 140 and 200 bp. Homozygotes for the ancestral allele lacked the 140 and 200 bp fragments and had the PCR band of 340 bp while heterozygous individuals had all the three bands and homozygotes for the derived allele has the two smaller bands (9).

Statistical analysis

Hardy-Weinberg equilibrium deviation was assessed by using the Chi-Square goodness-of-fit test. The difference in genotypic distribution was analyzed using Fisher's exact test (two-sided). The

statistical package used to estimate the odds ratio and 95% confidence intervals was Graphpad Prism 6.1. A P<0.05 was interpreted as statistically significant.

Result

In total of 120 non-obstructive azoospermic men, higher number of men with CT genotype were observed in the 25-30 age group (>30%) followed TT genotype with (>15%) than any other groups with other genotypes (Fig.1) and this study revealed that 74% of TC, 70% of TT and 40% of CC genotype men had reduced semen volume (<1.5 ml) (Table 1).

Bands corresponding to the 340 bp PCR fragment were observed, confirming amplification of this region of *CYP1A1*. The RFLP analysis of *CYP1A1*2A* polymorphism results of the 120 patients (Fig.2, RFLP results of a few samples), showed that the genotype counts were 70 heterozygous, 40 homozygous for the ancestral allele and 10 homozygous for the derived allele. In the control group the counts were 27, 53 and 1 respectively. The observed frequency of patients with homozygous CC was 8.34% of which all were exposed to smoking, the percentage of homozygous TT was 33.34% of which 50% were exposed to smoking and 50% were exposed to chemicals, and the percentage of heterozygous CT was 58.34% of which 57.14% were not exposed to any harmful chemicals and 28.57% were exposed to smoking and 14.2% were exposed to both alcohol as well as smoking. In the control group homozygous CC was 1.25% of which 100% were exposed to smoking, the percentage of homozygous TT was 65.00% of which 61.53% were exposed to alcohol and 38.46% were not exposed to any harmful chemicals, and the percentage of heterozygous CT was 33.75% of which 25.92% were not exposed to alcohol and 74.07% were not exposed to any harmful chemicals (Fig.3).

The differences in allele frequencies of this *CYP1A1*2A* polymorphism between fertile and infertile men were found to be statistically significant (P=0.0001). Differences in genotypic was also observed between infertile and fertile men (P=0.0001). The semen analysis report showed the frequencies of TT, CT and CC genotypes in azoospermic men were found to be 23.33, 43.33 and 3.33% in patients with less than 1.5 ml of reduced semen volume and 10, 15, 5% in patients with more than 1.5 ml of reduced semen volume respectively. In fertile controls with normal semen volume, we observed 65% TT, 33.75% CT and 1.25% CC genotypes (Table 2).

Table 1: Semen volume in relation to the *CYP1A1* polymorphism in azoospermic and fertile men

	Group and genotype	Semen volume	
		Reduced (<1.5 ml)	Normal (>1.5 ml)
Infertile	TT (wild) n=40	28	12
	TC (hetero) n=70	52	18
	CC (homo) n=10	4	6
Fertile	TT (wild) n=52	0	52
	TC (hetero) n=27	0	27
	CC (homo) n=1	0	1

Table 2: Genotype frequencies of the *CYP1A1*2A* polymorphism among infertile and fertile men (controls) and their association with male infertility

CYP1A1 genotypes	Fertile men (Control) n=80	Infertile men (Patients) n=120	P value	OR (95% CI)
TT (Wild)	52	40	Reference	
TC (Hetero)	27	70	0.0001*	3.43 (1.87-6.29)
CC (Mutant)	1	10	0.0001*	13 (1.597-105.8)
TC+CC	28	80	0.0001*	3.714 (2.046-6.741)

OR; Odds ratio and CI; Confidence interval.

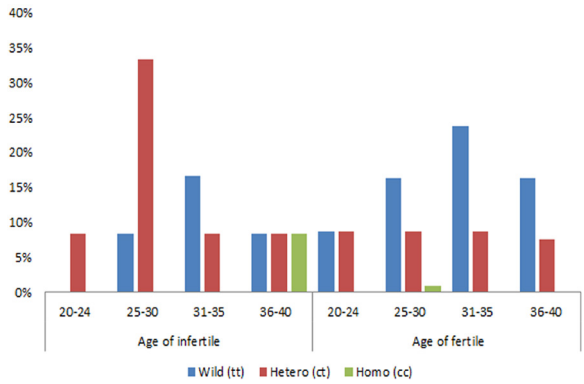

Fig.1: Age-wise distribution of *CYP1A1* polymorphism genotypes in infertile and fertile men.

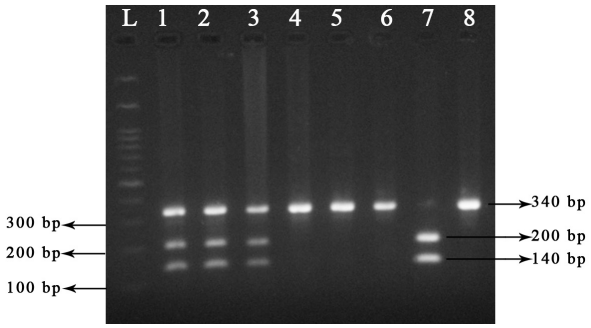

Fig.2: *CYP1A1* gene polymorphism was analyzed by polymerase chain reaction (PCR). Description: Lanes L; Marker, Lanes 1-3; Heterozygous genotype (CT), Lanes 4-6 and 8; Homozygous wild (TT), and Lane 7; Homozygous mutant (CC).

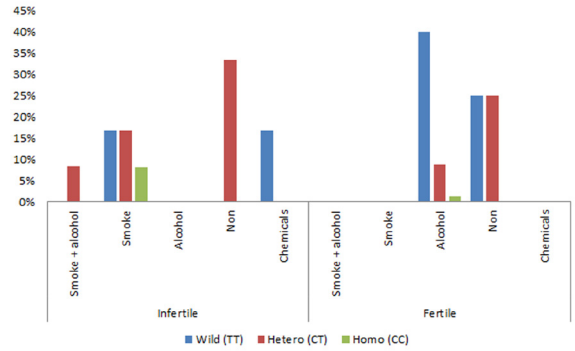

Fig.3: Association of smoke, alcohol and chemical exposure with the *CYP1A1* polymorphism in infertile and fertile men.

Discussion

CYP1A1 is an important phase I enzyme and plays a key role in the metabolism of lipophilic xenobiotics. The enzyme is vitally expressed in male reproductive organs and its polymorphisms may be a determinant of individual susceptibility to infertility. Metabolic activation or inactivation of xenobiotics is catalyzed by hemethilate enzymes like CYP1A1, which catalyzes PAHs in the first step of metabolism. For instance, the process of converting the carcinogen benzo[a]pyrene (B[a] P) to its ultimate DNA-binding form is metabolized by CYP1A1 (13). These metabolites have been shown to cause small cell lung carcinoma (14), recurrent pregnancy loss (15), coronary artery disease and diabetes (16). It is thought that CYP1A1 also plays a vital role in metabolism of endogenous substrates like steroid hormones through catalyzing the hydroxylation of 17b-estradiol at the C-2 position (17-19).

The genotypic distribution of *CYP1A1*2A* polymorphism in the infertile male group deviated from the Hardy-Weinberg equilibrium. There have not been any reports describing such an incompatibility for *CYP1A1* polymorphisms in the South Indian population. To remain in the Hardy-Weinberg equilibrium, the population must be very large and must follow random mating. Our study population is relatively small and consanguineous marriages are 5% common in this population. The observed incompatibility may thus be inherent to the studied population. In the overall analysis, we found that individuals heterozygous for this polymorphism had an increased risk. In the subsequent analysis, we found that patients exposed to smoking, alcohol or chemicals have an overrepresentation of the homozygous ancestral genotype TT, leading to male infertility. The patients with smoking, alcohol consumption and high exposure to chemicals may also have an increased risk in heterozygous type polymorphism leading to male infertility.

It is suggested that in infertility, genetic polymorphisms of xenobiotic metabolism may play an important role (20). Based on an Indian study, the pathogenesis of male infertility was associated with the CC genotype of the *CYP1A1*2A* polymorphism (9). Besides the study on the Indian population, other studies have shown that being homozygous for the *CYP1A1*2A* variant increased susceptibility to estrogen-related breast cancer in African-Americans (21). However, a case-control study on Japanese women showed a decreased risk with homozygous *CYP1A1*2A* among breast cancer patients (22). A study of *CYP1A1* in the Chinese population showed that variants in this gene may

contribute to the pathogenesis of male infertility in the Han population (10). To completely understand the etiology of idiopathic male infertility, an understanding of the complex gene-environment interactions is necessary. This is particularly relevant for genes such as CYP1A1 which is in direct contact with environmental toxins. Smoking, which was reported at a moderately high percentage in the infertile group of this study, could be an additional contributory factor in the development of male infertility by increasing levels of PAH in the body (23). The study carried out by Abilash et al. (24) estimated the frequency of Y chromosome microdeletion in infertile men to explore the effect of smoking, alcohol drinking, chemical exposure and cellular chromosomal aberration among 34 azoospermia and 55 oligospermia patients. They found that the chromosome aberrations per cell in azoospermia and oligospermia were higher than that of the control. The percentage of microdeletion observed in unexposed azoospermia had 15%, azoospermia smokers 22%, azoospermia smokers and alcoholics 25%; whereas the unexposed oligospermia had 7%, oligospermia smokers had 12%, and oligospermia smokers and alcoholics had 37%. Based on these results, they concluded that the etiology of male infertility may differ between ethnicities and smoking, alcohol drinking and chemical exposure may have deleterious effects on human fertility (24).

Conclusion

Our study indicates that the CT genotype of *CYP1A1*2A* may contribute to the pathogenesis of idiopathic non obstructive azoospermia. This result thus suggests that the relationship between this genetic variation and the vulnerability to the disease depends on personal habits such as smoking, alcohol drinking and other environmental factors such as exposure to chemicals and heavy metals. Since this study is a preliminary step in investigating this association, further studies are needed to identiyfing the underlying mechanism and to validate our results.

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References

1. Drugkar Amol Z, Gangane SD, More Rakhi M, Drugkar Swati A. Cytogenetic study in male infertility. IOSR Journal of Dental and Medical Science (IOSR-JDMS). 2013; 5(2): 5-11.
2. Foresta C, Ferlin A, Gianaroli L, Dallapiccola B. Guidelines for the appropriate use of genetic tests in infertile couples. Eur J Hum Genet. 2002; 10(5): 303-312.
3. Pasqualotto FF, Pasqualotto EB, Sobreiro BP, Hallak J, Medeiros F, Lucon AM. Clinical diagnosis in men undergoing infertility investigation in a university hospital. Urol Int. 2006; 76(2): 122-125.
4. Sk A, V J, G K, D U, P K. Declining semen quality among south Indian infertile men: A retrospective study. J Hum Reprod Sci. 2008; 1(1): 15-18.
5. Aitken RJ, Baker MA, Sawyer D. Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. Reprod Biomed Online. 2003; 7(1): 65-70.
6. Krausz C. Male infertility: pathogenesis and clinical diagnosis. Best Pract Res Clin Endocrinol Metab. 2011; 25(2): 271-285.
7. Benoff S, Jacob A, Hurley IR. Male infertility and environmental exposure to lead and cadmium. Hum Reprod Update. 2000; 6(2): 107-121.
8. Kawajiri K. Cyp1a1. IARC Sci Publ. 1999; (148): 159-172.
9. Vani GT, Mukesh N, Siva Prasad B, Rama Devi P, Hema Prasad M, Usha Rani P, et al. Association of CYP1A1* 2A polymorphism with male infertility in Indian population. Clin Chim Acta. 2009; 410(1-2): 43-47.
10. Wormhoudt LW, Commandeur JN, Vermeulen NP. Genetic polymorphisms of human N-acetyltransferase, cytochrome P450, glutathione-S-transferase, and epoxide hydrolase enzymes: relevance to xenobiotic metabolism and toxicity. Crit Rev Toxicol. 1999; 29(1): 59-124.
11. Lu N, Wu B, Xia Y, Wang W, Gu A, Liang J, et al. Polymorphisms in CYP1A1 gene are associated with male infertility in a Chinese population. Int J Androl. 2008; 31(5): 527-533.
12. Yager JD, Leihr JG. Molecular mechanisms of estrogen carcinogenesis. Annu Rev Pharmacol Toxicol. 1996; 36: 203-232.
13. McManus ME, Burgess WM, Veronese ME, Huggett A, Quattrochi LC, Tukey RH. Metabolism of 2-acetylaminofluorene and benzo (a) pyrene and activation of food-derived heterocyclic amine mutagens by human cytochromes P-450. Cancer Res. 1990; 50(11): 3367-3376.
14. Androutsopoulos VP, Tsatsakis AM, Spandidos DA. Cytochrome P450 CYP1A1: wider roles in cancer progression and prevention. BMC Cancer. 2009; 9: 187.
15. Suryanarayana V, Deenadayal M, Singh L. Association of CYP1A1 gene polymorphism with recurrent pregnancy loss in the South Indian population. Hum Reprod. 2004; 19(11): 2648-2652.
16. Wang XL, Greco M, Sim AS, Duarte N, Wang J, Wilcken DE. Effect of CYP1A1 MspI polymorphism on cigarette smoking related coronary artery disease and diabetes. Atherosclerosis. 2002; 162(2): 391-397.

17. Wang H, Napoli KL, Strobel HW. Cytochrome P450 3A9 catalyzes the metabolism of progesterone and other steroid hormones. Mol Cell Biochem. 2000; 213(1-2): 127-135.
18. Dannan GA, Porubek DJ, Nelson SD, Waxman DJ, Guengerich FP. 17 beta-estradiol 2-and 4-hydroxylation catalyzed by rat hepatic cytochrome P-450: roles of individual forms, inductive effects, developmental patterns, and alterations by gonadectomy and hormone replacement. Endocrinology. 1986; 118(5): 1952-1960.
19. Spink DC, Eugster HP, Lincoln DW 2nd, Schuetz JD, Schuetz EG, Johnson JA, et al. 17 beta-estradiol hydroxylation catalyzed by human cytochrome P450 1A1: a comparison of the activities induced by 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin in MCF-7 cells with those from heterologous expression of the cDNA. Arch Biochem Biophys. 1992; 293(2): 342-348.
20. Aydos SE, Taspinar M, Sunguroglu A, Aydos K. Association of CYP1A1 and glutathione S-transferase polymorphisms with male factor infertility. Fertil Steril. 2009; 92(2): 541-547.
21. Taioli E, Trachman J, Chen X, Toniolo P, Garte SJ. A CYP1A1 restriction fragment length polymorphism is associated with breast cancer in African-American women. Cancer Res. 1995; 55(17): 3757-3758.
22. Miyoshi Y, Takahashi Y, Egawa C, Noguchi S. Breast cancer risk associated with CYP1A1 genetic polymorphisms in Japanese women. Breast J. 2002; 8(4): 209-215.
23. Goldman R, Enewold L, Pellizzari E, Beach JB, Bowman ED, Krishnan SS, et al. Smoking increases carcinogenic polycyclic aromatic hydrocarbons in human lung tissue. Cancer Res. 2001; 61(17): 6367-6371.
24. Abilash VG, Saraswathy R, Marimuthu KM. The frequency of Y chromosome microdeletions in infertile men from Chennai, a South East Indian population and the effect of smoking, drinking alcohol and chemical exposure on their frequencies. Int J Genet Mol Biol. 2010; 2(7): 147-157.

Original Article

Comparative Expression Analysis of *HSP70*, *HSP90*, *IL-4*, *TNF*, *KITLG* and *KIT-receptor* Gene between Varicocele-Induced and Non-Varicocele Testes of Dog

Hossein Hassanpour, Ph.D.[1*], Amin Bigham Sadegh, Ph.D.[2], Iraj Karimi, Ph.D.[3], Heidar Heidari Khoei, D.V.M.[1], Azarnoush Karimi, D.V.M.[1], Parinaz Edalati Shaarbaf, D.V.M.[4], Tahereh Karimi Shayan, M.Sc.[1]

1. Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran
2. Department of Veterinary Surgery and Radiology, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran
3. Department of Pathobiology, Faculty of Veterinary Medicine, Shahrekord University, Shahrekord, Iran
4. Department of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran

Abstract

Background: This study was designed to create an experimental varicocele model by a simple surgical procedure in dog with minimum invasion and to investigate the effect of varicocele-induced infertility on the expression of six related genes (*HSP90*, *HSP70*, *IL-4*, *TNF*, *KITLG* and *KIT receptor*).

Materials and Methods: In this experimental study, the proximal part of the pampiniform plexus of dog testes was partially occluded without abdominal incision which was confirmed by venographic examination. To evaluate varicocele in its acute form, dogs were castrated after 15 days and testes were dissected. Histopathologic evaluation was undertaken and the relative expression of the six genes was assessed by quantitative real-time polymerase chain reaction (PCR).

Results: Microscopic changes showed tubule degeneration. The Johnson score was significantly decreased in the varicocele testes when compared with non-varicocele testes. Expressions of *HSP90*, *TNF*, *KITLG* and the *KIT-receptor* gene were significantly down-regulated (P=0.029, 0.047, 0.004 and 0.035 respectively) in varicocele-induced testes while *HSP70* was upregulated (P=0.018). IL-4 did not show differential expression (P=0.377).

Conclusion: We conclude that partial occlusion of the proximal part of the pampiniform plexus induces varicocele in the testis of dog. Differential expression of the mentioned genes may be responsible for the pathophysiology of varicocele and related subfertility.

Keywords: Varicocele, Dog, Gene Expression

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Introduction

Varicocele is a pathologic dilation of the venous pampiniform plexus in the spermatic cord (1) and is thought to be associated with male infertility. Diagnostic techniques such as scrotal ultrasonography and color Doppler imaging have demonstrated that varicocele may be the cause of 91% of subfertile human cases (2, 3). The pathophysiology of testicular damage in varicocele is not completely understood, however, histopathologic testicular damages due to varicocele are well documented. The effect of varicocele varies, but may often result in a generalized failure of sperm production (from oligozoospermia to complete nonobstructive azoospermia) (4). Varicocele not only affects the normal function and the fertilizing capacity of the sperm, but it also affects the reproductive potential of the haploid male gamete (5). Several studies have suggested varicocele-mediated mechanisms to explain impaired spermatogenesis (6-8). Im-

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***Corresponding Address: P.O.Box: 115, Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran**
Email: Hassanpour-h@vet.sku.ac.ir

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paired temperature regulation and reactive oxygen species (ROS) production may lead to DNA damage and progressive apoptosis of testicular cells (9-12). Research at cellular and molecular level, while still in its infancy, may provide additional insights into the varicocele puzzle (8).

The signaling of KIT is well-known for its ability to potentiate cell survival, proliferation and differentiation. The KIT receptor and its ligand, KIT ligand (KITLG), have been widely studied (13). The KIT receptor is a transmembrane protein with tyrosine kinase activity and a member of the type III receptor tyrosine kinase family. Binding of KITLG to KIT leads to the activation of multiple pathways including Src kinase, phospholipase C (PLC)-γ, Janus kinase (JAK)/signal transducers and activators of transcription (STAT), mitogen activated protein (MAP) kinase and phosphatidyl-inositol-3 (PI3)-kinase pathways (14, 15). Dysfunction of KIT signaling thus results in an array of developmental defects in melanogenesis, hematopoiesis, gametogenesis and spermatogenesis (14, 16, 17).

Cytokines are small soluble proteins with a crucial role in the regulation of inflammatory responses. Also, they transmit signals to surrounding cells for the regulation of cell growth and differentiation. They could trigger complex intracellular signaling events that regulate gene expression required for the cellular response (18). A number of studies have reported that KIT expression is regulated by various proinflammatory signals (16, 19). Differential effects are induced by some cytokines depending on the type of the cell system. Among cytokines, interleukin 1 (IL-1), tumour necrosis factor (TNF), IL-4, granulocyte-macrophage colony stimulating factor fibroblast growth factor (FGF) and IL-10 have been reported to change KIT synthesis (20, 21). However, the effects of cytokines and KIT signaling in the inflammatory process of varicocele are predicatable.

The heat shock proteins (HSPs), a family of endogenous, protective proteins, are located in the cytoplasm and nucleus (e.g. HSP70 and HSP90 respectively) to maintain normal cellular function. ROS, cytotoxic lysosomal enzymes and cytoskeletal alterations are able to activate HSP expression. HSPs, in turn, suppress pro-inflammatory cytokines, reduce oxidative bursts, repair ion channels, protect against the toxic effect of nitric oxide, modulate immune-mediated injuries and prevent apoptosis. The function of HSP and its dependant factors in inflammation provides a basis for its possible involvement in the pathophysiology of varicocele. Indeed, the presence of many HSPs in varicocele has been confirmed by previous studies (22, 23). In the present study, we therefore aimed to investigate histopathologic changes in the varicocele testis and whether same changes can be identified in non-varicocele testis. Variation in the expression of *HSP90*, *HSP70*, *IL-4*, *TNF*, *KITLG* and the *KIT-receptor* gene, and their potential contribution to varicocele-mediated infertility is discussed.

Materials and Methods

In this experimental study, six adult male cross-bred dogs (2-4 years old) with normal quality and approximately 30 kg weight were used in this experiment. They were cared for in the Faculty of Shahrekord Veterinary Medicine and housed in pens with ample run. Commercial food was provided twice a day and the dogs had free access to water. Anti-parasitic drugs were administrated to all dogs (mebendozole, 22 mg/kg, orally for 6 days and praziquantel, 5 mg/kg, orally once). All animals were maintained according to the guidelines of Animal Care and Use Committee of the Faculty of Shahrekord Veterinary Medicine.

Experimental varicocele induction in dog

To induce experimental varicocele by surgery, the inguinal canal region of dogs was prepared aseptically for operation. Dogs were sedated with 2% acepromazine (0.2 mg/kg) and anesthetized by ketamine and then maintained with 2% halothane. An incision was made in the skin of the inguinal canal region while animals were in the dorsal recumbent position. Spermatic cord was exposed and tunica vaginalis was incised to expose the pampiniform plexus. To make a partial occlusion and congestion in the pampiniform plexus, a piece of silicone tube (INWAY® Suprapubic Catheter, pfm Medical Co., Germany) of 1 cm long was longitudinally incised and opened, and then proximal part of the pampiniform plexus was cited in it. To prevent the movement of the tube, three interrupted sutures were applied by 2.0 absorbable suture material and the skin was sutured by non-absorbable suture material. Dogs were kept for 2 weeks and the diameter of the testes were examined and recorded. On the 15th postoperative day, the animals were anesthetized, their spermatic cord was

incised and 2 milliliters of iohexol contrast media (iodixanol, Visipaque 320, GE Healthcare, Canada) was injected in the testicular vein and radiographs were taken immediately from the injected area. This venography was done to confirm congestion and dilation of the venous pampiniform plexus in the spermatic cord of varicocele-induced testis. Finally, non-varicocele (left) and varicocele-induced (right) testes were dissected by castration of dogs. This was undertaken after two weeks to evaluate varicocele in its acute form (short time) as observed in many adult men (24). Half of each testis was immediately frozen in liquid nitrogen and stored at -70°C for subsequent RNA and expression analyses. Another half was fixed in formalin solution followed by embedding in glycol methacrylate for histopathologic evaluation.

Histopathologic evaluation

Histopathologic evaluation of the induced varicocele model was carried out by hematoxylin and eosin staining in the non-varicocele and varicocele-induced testes. To examine spermatogenic activity, spermatogenesis was categorized by using the Johnson score (25). A grade from 1 to 10 for each tubule cross section was provided according to the following criteria: i. No germ cells and no Sertoli cells present, ii. No germ cells but only Sertoli cells present, iii. Only spermatogonia present, iv. Only a few spermatocytes present, v. No spermatozoa or spermatids but many spermatocytes present, vi. Only a few spermatids present, vii. No spermatozoa but many spermatids present, viii. Only a few spermatozoa present, ix. Many spermatozoa present and disorganized spermatogenesis, and x. Complete spermatogenesis and perfect tubules.

RNA extraction and cDNA synthesis

Total RNA from left (non-varicocele) and right (varicocele) testes was extracted using the Rimazol reagent (Sinaclon Bioscience, Iran) and then homogenized (Sinaclon Bioscience, Iran). The quantity of extracted RNA was then measured by spectrophotometry. Only RNA samples with an absorbance ratio (A260/280) of ≥1.9 was used for synthesis of cDNA (26). Gel agarose (2%) electrophoresis (stained with ethidium bromide) was applied to analyze the quality of extracted RNA. The cDNA was produced from total RNA using M-MLV reverse transcriptase (Sinaclon Bioscience, Iran) according to the protocol of a previous study (27). To denature residual RNA in the cDNA mix, the sample was heated at 75°C for 15 minutes and subsequently stored at -20°C.

Quantitative real time polymerase chain reaction analysis

The expression levels of *HSP90*, *HSP70*, *IL-4*, *TNF*, *KITLG*, the *KIT-receptor* gene and *GAPDH* (encoding glyceraldehyde-3-phosphate dehydrogenase) transcripts were determined by reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) using the EvaGreen chemistry (Sinaclon Bioscience, Iran). To normalise the input load of cDNA between samples, *GAPDH* levels were used as an internal control which was confirmed as a strong reference gene using Normfinder v20 (Skejby Sygehus, Denmark) in this experiment. Specific primers were designed based on mRNA sequences with Primer-Blast (www.ncbi.nlm.nih.gov/tools/primer-blast/). All primer sequences are given in Table 1. PCR reactions were carried out in a real-time PCR cycler (Rotor Gene Q 6000, Qiagen, USA) in triplicate for each sample of testis. The reaction mixture contained 1 µl cDNA, 0.5 µM of each specific primer and 4 µl of Titan Hot Taq Eva-Green Ready Mix in a total volume of 20 µl. The thermal profile was 95°C for 15 minutes, 35 cycles of 94°C for 40 seconds, 60°C for 35 seconds and 72°C for 32 seconds. At the end of each stage, the level of fluorescence emission was obtained for quantification of expression levels. Data were analyzed using the LinRegPCR software version 2012.0 (Amsterdam, Netherland) to obtain the threshold cycle (Ct) and reaction efficiency (28). The transcript level of each target gene relative to *GAPDH* was estimated for each sample in two experimental testes by using efficiency (E) in the formula $E_{GAPDH}^{(Ct\ sample)}$ / E target $^{(Ct\ sample)}$. The comparison was then statistically analyzed between the two groups of testes. To determine fold change for each gene, the relative gene expression of varicocele-induced testes relative to the non-varicocele testes were calculated as following (29).

$$\text{Ratio}=\frac{E_{GAPDH}^{(Ct\ sample)}}{E_{traget}^{(Ct\ sample)}} \div \frac{E_{GAPDH}^{(Ct\ control)}}{E_{traget}^{(Ct\ sacontrol)}}$$

Statistical analysis

Data are represented as mean ± SE. Differential expression was assessed statistically by using paired

t test between the non-varicocele and varicocele-induced testis pair. When the assumptions behind a parametric test were violated, comparisons were made by the Wilcoxon test. All statistical analyses were performed with the Statistical Package for Social Sciences software version 17 (SPSS Inc., Chicago, IL, USA). When paired t test was done, differences between paired values were consistent and $P<0.05$ were considered statistically significant.

Results

Venographic assessment

As observed in the right testicular venogram (Fig.1), dilatation and toruosity of veins of the pampiniform plexus, secondary to retrograde flow, were apparent.

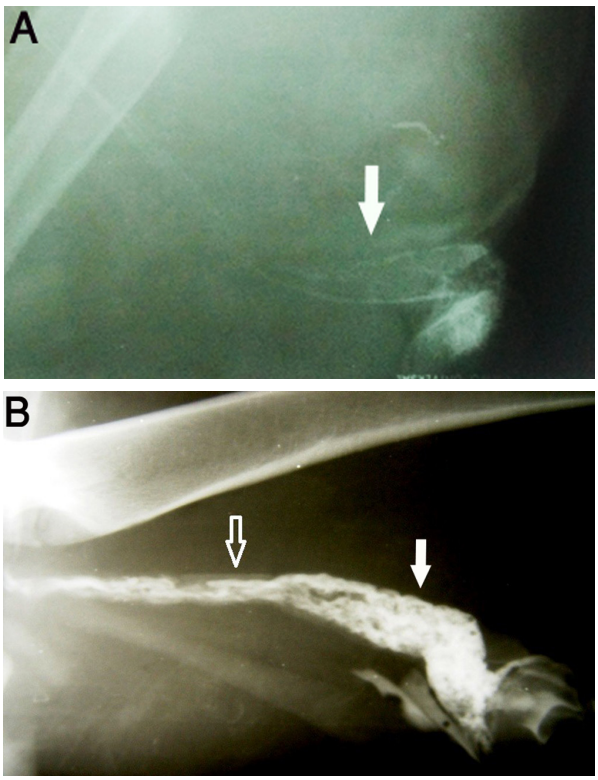

Fig.1: Venography of the right testis in a dog using iohexol contrast media. **A.** Non-varicocele testis; normal circulation in the pumpiniform plexus (white arrow) and **B.** Varicocele-induced testis on the 15th postoperative day; dilatation and congestion in the veins of the pampiniform plexus (white arrow) due to partial occlusion in its proximal part (black arrow).

Histopathologic evaluation

Gross pathologic changes of varicocele-induced testes were congestion, edema and enlargement. Microscopic changes were evaluated after hematoxylin and eosin staining of different sections of testes and were then compared between non-varicocele and varicocele-induced testes. The histopathologic changes consisted of testicular degeneration as well as spermatogenic arrest at the spermatocyte stage and formation of multinucleated spermatid due to failure in spermatid separation (Fig.2A). In addition, coagulative necrosis in the seminiferous epithelium and the presence of eosinophilic material in the seminiferous tubules along with hemorrhage in the interstitium were induced (Fig.2B). Testicular atrophy was also present in the form of complete absence of spermatogenesis (but with normla Sertoli cells) and shrinkage of some seminiferous tubules (Fig.2C). Furthermore, epididimyal atrophy as a prominent dilation of epididimyal tubules with pressure atrophy of their columnar epithelia (Fig.2D), severe congestion and dilation of the spermatic cord vessels with inter-vascular fibrosis (Fig.2E), and epididymal squamous metaplasia and intertubular fibrosis (Fig.2F) were also among the induced histopathologic changes.

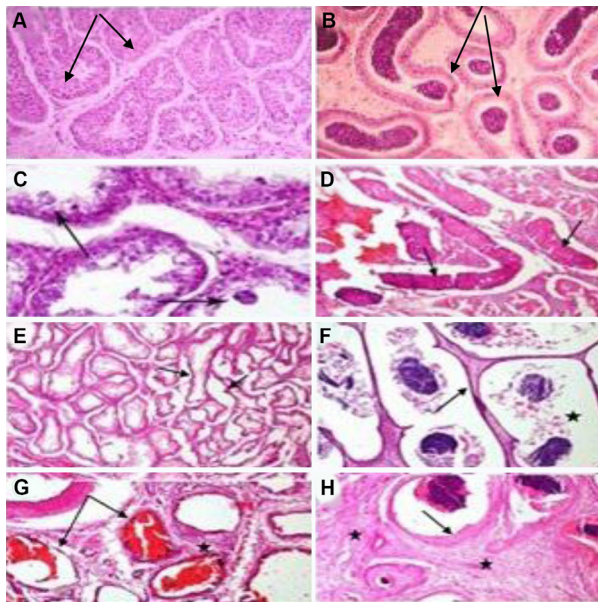

Fig.2: Histopathologic evaluation of varicocele-induced testes after hematoxylin and eosin staining. **A.** Normal testis with complete spermatogenesis (arrows) (×10), **B.** Normal epididymis containing spermatozoids and columnar epithelium lining the tubular walls (arrows) (×10), **C.** Testicular degeneration as spermatogenic arrest at the spermatocyte stage and formation of multinucleated spermatids (arrows) (×40), **D.** Coagulative necrosis in the seminiferous epithelium (arrows) (×10), **E.** Testicular atrophy as complete absence of spermatogenesis (arrows) (H&E, ×10), **F.** Epididimyal epithelial cell pressure (arrows) and dilation of the epididymal tubules (star) (×10), **G.** Severe congestion (arrows) and dilation of the spermatic cord vessels with inter-vascular fibrosis (star) (×10), and **H.** Epididymal epithelial metaplasia (arrows) and intertubular fibrosis (star) (×10).

Table 1: Primers used for reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) of canine transcripts

Target	Sequencing primer (5′-3′)	PCR product (bp)	Accession no.
GAPDH	CCCACTCTTCCACCTTCGAC CCTTGGAGGCCATGTAGACC	135	NM_001003142.1
KITLG	GGACTTGGAGACGGTGGCAT CCTAAGGGAGCTGGCTGCAA	130	NM_001012735.1
KIT-receptor	CAGAGCCCGCAGTAGATTGG CAGACGGTGACAGAGACGAG	108	AF448148.1
IL-4	TGGGTCTCACCTCCCAACTG GTCAGCTCCATGCACGAGTC	154	NM_001003159.1
TNF	GCCGTCAGATGGGTTGTACC TCTGGTAGGAGACGGCGAAG	116	NM_001003244.4
HSP90	TGTGGGAATGACCCGGGAAG TGGCCATCCTCTTGTGCCT	106	AB981782.1
HSP70	GACCGCCTTTCGGAAACCTG GTGTCGGTGAAGGCCACGTA	200	XM_005627164.1

Table 2: Relative expression of the six genes analyzed in the testis

Gene	Relative gene expression		Ratio (varicocele/non-varicocele)	Pooled SD	P value
	Non-varicocele testis	Varicocele testis			
KITLG	0.084	0.0627*	83%	0.009	0.047
KIT- receptor	0.187	0.131*	71%	0.034	0.004
IL-4	0.057	0.070	110%	0.027	0.377
TNF	0.030	0.020*	76%	0.007	0.035
HSP90	0.704	0.434*	62%	0.054	0.029
HSP70	0.010	0.029*	290%	0.009	0.018

The Johnson score in the varicocele-induced and non-varicocele testes was 4 (1-8) and 9.6 (9, 10) respectively with the difference being statistically significant (P=0.031).

Expression analysis of the six related genes

Expression level changes of all six genes were quantified using real time quantitative PCR (RT-qPCR) and are shown in Table 2. Expression level of *GAPDH* was not different in non-varicocele and varicocele-induced testes. Expression of *HSP90*, *KITLG*, the *KIT-receptor* gene and *TNF* transcripts in varicocele-induced testes was significantly lower than non-varicocele testes (P=0.029, 0.047, 0.004 and 0.035 respectively) with fold-changes of 0.62, 0.83, 0.71 and 0.76 respectively. On the contrary, *HSP70* was significantly up-regulated (P=0.018, 2.9 fold-change) in varicocele-induced testes. *IL-4* transcript levels did not show differential expression between varicocele-induced and non-varicocele testes (P=0.377).

Discussion

This study was designed to induce an experimental varicocele model by a simple surgical procedure in dog with minimum invasion and to also investigate the expression of a number of genes involved in varicocele-induced infertility. There are many limitations in the study of varicocele pathophysiology in humans with most studies being non-invasive. In addition, there are other factors such as the status of the varicocele, patient age and level of fertility in the subject population that further hinder the identification of its pathologic basis, thus limiting research on varicocele in humans. Because of these limitations, varicocele has been induced in several species as animal models (7). The induction of varicocele in most animal models involves partially occluding the left renal vein medial near to the kidney. Increased venous pressure proximal to the partial occlusion creates the increased pressure in the left internal spermatic vein, thus resulting in dilatation of the left internal

spermatic vein and the pampiniform venous plexus. In all models, a midline abdominal incision must be made from xyphoid to pubis to expose the renal and pelvic vasculature (30). In the present study, the surgical approach was only in the inguinal canal region and contrary to other studies abdominal incision was not made, rendering this method more advantageous. This route was also preferred by the Animal Care Committee and was therefore approved. The histopathologic and venographic evaluations of manipulated testes confirmed the induction of varicocele and subsequent infertility (caused by azoospermia), while the non-varicocele testis was shown to be slightly influenced as the Jonson score showed values ranging 9-10. This may be due to a transient inflammation in the non-varicocele testis.

Some studies have suggested a relationship between cytokine levels and subfertility. It has been found that concentrations of interleukins such as IL-1, IL-6 and TNF were significantly increased in semen of infertile patients (31). In varicocele, it has been also suggested that expression of IL-1α and IL-1β, as proinflammatory cytokines, were increased. These cytokines in varicocele shift the balance in favor of inflammation and immune responses and therefore result in harmful effects in testicular tissue, which may lead to male infertility. In the present study, the expression of *IL-4* and *TNF* were evaluated in varicocele. We only observed a significant down-regulation for *TNF* but not for *IL-4*. It has been shown that IL-4 and TNF play anti-inflammatory and pro-inflammatory roles respectively (32). Previous studies have indicated that the level of TNF or the TNF-related apoptosis-inducing ligand does not change in varicocele (33), however, expression of receptors of the TNF-related apoptosis-inducing ligand were different (34). These reports, nevertheless, evaluated TNF or its receptors at the protein level by ELISA, immunohistochemical and Western blotting techniques, while in our study, expression was evaluated at the transcript level by RT-qPCR. It has been shown that post-transcriptional and post-translational factors affecting activity of TNF at both gene and protein levels react to different pathways in varicocele. It must be noted that anti-inflammatory cytokines are able to suppress pro-inflammatory cytokines at both transcriptional and post-transcriptional levels. In fact, the balance between pro-inflammatory and anti-inflammatory cytokines determines the outcome and severity of this disease. Therefore, *TNF* downregulation in our study may be due to the effects of anti-inflammatory cytokines such as IL-4 (35).

Another possibility is that the levels and the subsequent effects of many cytokines alters with varicocele duration. These changes could be to some extent related to the interaction of anti-inflammatory (e.g., IL-4) and pro-inflammatory (e.g., TNF) cytokines in a time-dependent manner. It must be, however, noted that non-varicocele testes probably had a slight inflammation, resulting in an increase in pro-inflammatory cytokines such as TNF.

In the current study, the expression of *KITLG* and the KIT-receptor gene were evaluated. These results, for the first time, demonstrated the downregulation of both genes at the transcript level in varicocele-induced testis. Based on various reports, KITLG/KIT-receptor represent one of the key regulators of testicular formation, development and function since its impairment has been observed in gonadal pathologies including testicular developmental defects, infertility and testicular cancer. Downregulation of KITLG/KIT-receptor has been also observed in oligozoospermia/azoospermia, which is associated with an increase in the germ cell apoptosis process (35, 36). Overall, downregulation of KITLG/KIT-receptor, as reported in here, may be a critical factor in varicocele-mediated infertility. It has been documented that expression of KIT is influenced by various cytokines during inflammation depending on the model or type of the cell system used (18). This effect of cytokines on the KIT system may explain the downregulation of KITLG/KIT-receptor in varicocele observed in this study. Of course, this correlation between the KIT system and cytokines in varicocele needs to be demonstrated more comprehensively since the analysis of only two cytokines (i.e., TNF and IL-4) are insufficient to establish this correlation.

The HSPs are present in spermatocytes during meiosis, participating as an element of the synaptonemal complex, and during the maturation stage of spermiogenesis. We observed a significant increase in *HSP70* expression at the transcript level in the testis with varicocele. In agreement with these results, an increase in HSP proteins has been reported in sperm from oligozoospermic and varicocele individuals (23). Afiyani et al. (37) and Khosravanian et al. (38, 39) have also reported the

overexpression of HSP70-2 an HSP2A in varicocele testes. This cellular response is probably an attempt to repair spermatogenic and germ-cell damage due to heat stress. But it must be noted that the expression of all HSP members at the transcript level could not increase during damage of varicocele to protect the testicular cells as we observed here for *HSP90* expression or that observed in Lima et al. (40) who examined the expression of *HSP2A*. Probably, in a time-stage of varicocele, transcriptional apparatus for some of the HSP members would itself be prone to damage. On the other hand, this situation could exacerbate the damage of varicocele in a positive feedback.

Conclusion

Our data show that partial occlusion of the proximal part of the pampiniform plexus induces varicocele in the testis of dog. The expression of *HSP90*, *TNF*, *KITLG* and the *KIT-receptor* gene were considerably decreased in varicocele-induced testes while *HSP70* was increased. *IL-4,* however, did not show differential expression. It is likely that these expression changes may be involved in the pathophysiology of varicocele and related subfertility.

Acknowledgements

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References

1. Naughton CK, Nangia AK, Agarwal A. Pathophysiology of varicoceles in male infertility. Hum Reprod Update. 2001; 7(5): 473-481.
2. Resim S, Cek M, Fazlioğlu A, Çaşkurlu T, Gürbüz G, Sevin G. Echo-colour Doppler ultrasonography in the diagnosis of varicocele. Int Urol Nephrol. 1999; 31(3): 371-382.
3. Gat Y, Bachar GN, Zukerman Z, Belenky A, Gornish M. Varicocele: a bilateral disease. Fertil Steril. 2004; 81(2): 424-429.
4. Sandlow J. Pathogenesis and treatment of varicoceles. BMJ. 2004; 328(7446): 967-968.
5. Redmon JB, Carey P, Pryor JL. Varicocele-the most common cause of male factor infertility. Hum Reprod Update. 2002; 8(1): 53-58.
6. Jarow JP. Effects of varicoele on male infertility. Hum Reprod Update. 2001; 7(1): 59-64.
7. Salama N, Bergh A, Damber JE. The changes in testicular vascular permeability during progression of the experimental varicocele. Eur Urol. 2003; 43(1): 84-91.
8. Sheehan MM, Ramasamy R, Lamb DJ. Molecular mechanisms involved in varicocele-associated infertility. J Assist Reprod Genet. 2014; 31(5): 521-526.
9. Hamada A, Esteves SC, Agarwal A. Insight into oxidative stress in varicocele-associated male infertility: part 2. Nat Rev Urol. 2012; 10(1): 26-37.
10. Gurdal M, Kirecci S, Huri E, Karaman I, Turkeri L. Correlation between duration of varicocele and apoptosis in testicular tissue in an experimental model. Urology. 2008; 72(4): 933-936.
11. Wang YJ, Zhang RQ, Lin YJ, Zhang RG, Zhang WL. Relationship between varicocele and sperm DNA damage and the effect of varicocele repair: a meta-analysis. Reprod Biomed Online. 2012; 25(3): 307-314.
12. Saalu LC, Akunna GG, Enye LA, Ogunmodede OS, Akingbade AM. Pathophysiology of varicocele: evidence for oxidative stress as a mechanism pathway. Eur J Anat. 2013; 17(2): 82-91.
13. Rossi P, Sette C, Dolci S, Geremia R. Role of c-kit in mammalian spermatogenesis. J Endocrinol Invest. 2000; 23(9): 609-615.
14. Roskoski R Jr. Signaling by Kit protein-tyrosine kinase-the stem cell factor receptor. Biochem Biophys Res Commun. 2005; 337(1): 1-13.
15. Kazi JU, Vaapil M, Agarwal S, Bracco E, Pahlman S, Ronnstrand L. The tyrosine kinase CSK associates with FLT3 and c-Kit receptors and regulates downstream signaling. Cell Signal. 2013; 25(9): 1852-1860.
16. Reber L, Da Silva CA, Frossard N. Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. Eur J Pharmacol. 2006; 533(1): 327-340.
17. Liang J, Wu YL, Chen BJ, Zhang W, Tanaka Y, Sugiyama H. The C-kit receptor-mediated signal transduction and tumor-related diseases. Int J Biol Sci. 2013; 9(5): 435-443.
18. Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. Cytokine Growth Factor Rev. 2002; 13(4-5): 413-421.
19. Mithraprabhu S, Loveland KL. Control of KIT signalling in male germ cells: what can we learn from other systems? Reproduction. 2009; 138(5): 743-757.
20. Ronnstrand L. Signal transduction via the stem cell factor receptor/c-Kit. Cell Mol Life Sci. 2004; 61(19-20): 2535-2548.
21. Sahin Z, Celik-Ozenci C, Akkoyunlu G, Korgun ET, Acar N, Erdogru T, et al. Increased expression of interleukin-1alpha and interleukin-1beta is associated with experimental varicocele. Fertil Steril. 2006; 85 Suppl 1: 1265-1275.
22. Afiyani AA, Deemeh MR, Tavalaee M, Razi M, Bahadorani M, Shokrollahi B, et al. Evaluation of heat-shock protein A2 (HSPA2) in male rats before and after varicocele induction. Mol Reprod Dev. 2014; 81(8): 766-776.
23. Ferlin A, Speltra E, Patassini C, Pati M, Garolla A, Caretta N, et al. Heat shock protein and heat shock factor expression in sperm: relation to oligozoospermia and varicocele. J Urol. 2010; 183(3): 1248-1252.
24. Arrabal-Polo MA, Merino-Salas S. Acute left varicocele in an adult. CMAJ. 2013; 185(4): 323.
25. Dohle GR, Colpi GM, Hargreave TB, Papp GK, Jungwirth A, Weidner W. EAU guidelines on male infertility. Eur Urol. 2005; 48(5): 703-711.
26. Hassanpour H, Afzali A, Fatemi Tabatabaie R, Torabi M, Alavi Y. Cardiac renin-angiotensin system (gene expression) and plasma angiotensin II in chickens with T3-induced pulmonary hypertension. Br Poult Sci. 2016; 57(4): 444-450.
27. Hassanpour H, Yazdani A, Soreshjani KK, Asgharzadeh S. Evaluation of endothelial and inducible nitric oxide synthase genes expression in the heart of broiler chickens with experimental pulmonary hypertension. Br Poultry Sci. 2009; 50(6): 725-732.
28. Ruijter JA, Ramakers C, Hoogaars WM, Karlen Y, Bakker O, Van den Hoff MJ. Amplification efficiency: linking

baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 2009; 37(6): e45.
29. Hassanpour H, Nikoukar Z, Nasiri L, Bahadoran S. Differential gene expression of three nitric oxide synthases is consistent with increased nitric oxide in the hindbrain of broilers with cold-induced pulmonary hypertension. Br Poult Sci. 2015. 56(4): 436-442.
30. Najari BB, Li PS, Ramasamy R, Katz M, Sheth S, Robinson B, et al. Microsurgical rat varicocele model. J Urol. 2014; 191(2): 548-553.
31. Moretti E, Collodel G, Mazzi L, Campagna M, Iacoponi F, Figura N. Resistin, interleukin-6, tumor necrosis factor-alpha, and human semen parameters in the presence of leukocytospermia, smoking habit, and varicocele. Fertil Steril. 2014; 102(2): 354-360.
32. Chowdhury TT, Bader DL, Lee DA. Anti-inflammatory effects of IL-4 and dynamic compression in IL-1β stimulated chondrocytes. Biochem Biophys Res Commun. 2006; 339(1): 241-247.
33. Celik O, Kutlu O, Tekcan M, Celik-Ozenci C, Koksal IT. Role of TNF-related apoptosis-inducing ligand (TRAIL) in the pathogenesis of varicocele-induced testicular dysfunction. Asian J Androl. 2013; 15(2): 269-274.
34. Dinarello CA. Proinflammatory cytokines. Chest. 2000; 118(2): 503-508.
35. Mauduit C, Hamamah S, Benahmed M. Stem cell factor/c-kit system in spermatogenesis. Hum Reprod Update. 1999; 5(5): 535-545.
36. Zhang L, Tang J, Haines CJ, Feng HL, Lai L, Teng X, et al. c-kit and its related genes in spermatogonial differentiation. Spermatogenesis. 2011; 1(3): 186-194.
37. Afiyani AA, Deemeh MR, Tavalaee M, Razi M, Bahadorani M, Shokrollahi B, et al. Evaluation of heat-shock protein A2 (HSPA2) in male rats before and after varicocele induction. Mol Reprod Dev. 2014; 81(8): 766-776.
38. Khosravanian N, Razi M, Farokhi F, Khosravanian H. Testosterone and vitamin E administration up-regulated varicocele-reduced Hsp70-2 protein expression and ameliorated biochemical alterations. J Assist Reprod Genet. 2014; 31(3): 341-354.
39. Khosravanian H, Razi M, Farokhi F, Khosravanian N. Simultaneous administration of dexamethasone and vitamin E reversed experimental varicocele-induced impact in testicular tissue in rats; correlation with Hsp70-2 chaperone expression. Int Braz J Urol. 2015; 41(4): 773-790.
40. Lima SB, Cenedeze MA, Bertolla RP, Filho PA, Oehninger S, Cedenho AP. Expression of the HSPA2 gene in ejaculated spermatozoa from adolescents with and without varicocele. Fertil Steril. 2006; 86(6): 1659-1663.

Original Article

Physically Active Men Show Better Semen Parameters than Their Sedentary Counterparts

Paula C. Lalinde-Acevedo, B.Sc.[1], B. Jose Manuel Mayorga-Torres, B.Sc.[1], Ashok Agarwal, Ph.D.[2], Stefan S. du Plessis, Ph.D.[2, 3], Gulfam Ahmad, Ph.D.[2, 4], Ángela P. Cadavid, Ph.D.[1], Walter D. Cardona Maya, Ph.D.[1*]

1. Reproduction Group, Department of Microbiology and Parasitology, Medical School, University of Antioquia, Antioquia, Colombia
2. Center for Reproductive Medicine, Cleveland Clinic, Cleveland, Ohio, USA
3. Division of Medical Physiology, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa
4. Department of Physiology and Cell Biology, University of Health Sciences, Lahore, Pakistan

Abstract

Background: The quality of semen depends upon several factors such as environment, life style, physical activity, age, and occupation. The aim of this study was to analyze and compare the conventional and functional semen parameters in men practicing vigorous physical activity to those of sedentary men.

Materials and Methods: In this descriptive cross-sectional study, semen samples of 17 physically active men and 15 sedentary men were collected for analysis. Semen analysis was performed according to the World Health Organization (WHO) guidelines, while functional parameters were evaluated by flow cytometry.

Results: Results showed that several semen parameters (semen volume, viability, progressive motility, total motility, normal morphology, and moribund cells) were superior in the physically active group in comparison with the sedentary group. Semen parameters such as viability, progressive motility and total motility, as well as the percentage of moribund spermatozoa were significantly different between both groups. However, sperm DNA damage, lipid peroxidation and mitochondrial potential were not significantly different among the groups.

Conclusion: Nevertheless, the physical activity shows better semen parameters than sedentary group. Taken together, our results demonstrate that regular physical activity has beneficial impact in sperm fertility parameters and such a life style can enhance the fertility status of men.

Keywords: Sperm, Fertility, Physical Activity, Sedentary, Lifestyle

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Introduction

The conventional semen analysis involves the macroscopic (volume, pH, and colour) and microscopic (motility, concentration, viability, and morphology) examination (1). It reflects the secretory activity of the testes, epididymis and accessory sex glands indirectly (2). Although conventional semen analysis provides both quantitative and qualitative

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*Corresponding Address: Reproduction Group, Department of Microbiology and Parasitology, Medical School, University of Antioquia, Antioquia, Colombia
Email: wdario.cardona@udea.edu.co

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information, it does not include evaluation of the functional properties of spermatozoa (3-7). Furthermore, oxidative stress which may directly contribute to the origin of male infertility, is not measured (8). Oxidative stress occurs due to the imbalance between the reactive oxygen species (ROS), reactive nitrogen species (RNS), and seminal antioxidant reserve in the male reproductive tract (9, 10). These ROS or RNS are produced during normal cellular metabolism and can be from either endogenous (normally produced by oxidative phosphorylation in mitochondria) or exogenous origin (e.g. produced by leukocytes) (10, 11).

Physiological levels of ROS exert a critical role in spermatozoa, triggering and mediating important signaling events to acquire essential functions such as hyperactivation, capacitation, and acrosome reaction (10-12). However, an excess in ROS levels is detrimental to cellular function and spermatozoa are highly susceptible to oxidative stress due to a lack in repair mechanisms (8, 13). This may result in damage to the structural components of the axoneme which may impact on the motility patterns (14, 15). It may also induce lipid peroxidation of cellular membranes (16), thereby disrupting the fluidity of mitochondrial and plasma membranes (12, 17) and furthermore lead to oxidative damage to proteins involved in the fusing of the spermatozoon with the oolemma (15). Additionally, ROS may cause DNA damage due to impaired histone remodeling during sperm maturation (12). Oxidative damage to spermatozoa has been related with recurrent pregnancy loss (13, 18, 19) and male infertility (5, 20).

It is well known that certain environmental factors including prolonged and continued exposure of the whole body, testes or scrotum to: i. Increased temperature, even at 37°C (21-23), ii. Environmental pollutants and endocrine disruptors (21, 24), iii. Electromagnetic radiation (21, 25), as well as lifestyle factors such as smoking, recreational drug use, alcohol consumption, obesity and sedentary occupation or lifestyle (25-29), may influence sperm quality and male fertility potential (21, 23, 25) mediated by induction of oxidative stress leading to cell apoptosis. Among several life style factors, sedentarism have been found associated with several medical conditions and considered as one of the main causes of major public health issues at present (30). According to the definition of Bernstein et al. (31), individuals are considered sedentary when they spend less than 10% of their daily energy expenditure on performing moderate to vigorous-intensity activities. Also a sedentary person frequently spends much time sitting or lying down and performing activities usually associated with this low energy consumption state such as sleeping or watching television. Also it is commonly avoiding any form of exercise or sporting activities (32). Over the past five decades, changes in the occupational activities and leisure time have promoted the sedentary behavior and impacted on lifestyle (33, 34). As is true for other medical conditions (obesity and heart diseases), this phenomenon is equally deleterious for semen quality (35).

On the other hand; physical activity has beneficial effect on human health and is defined as any voluntary and repetitive body movements produced by skeletal muscle action that substantially increases energy expenditure above the basal state (34, 36). It may be included in the occupational activities or have diverse purposes like being aerobic training or training strength, flexibility and balance, therefore it encompasses exercise and sport (37, 38). Physical activity is classified according to the intensity with which it is practiced and may be quantified in terms of the energy expenditure as a multiple of the resting metabolic rate (39). Using the Metabolic Equivalent of the Task (MET, a physiological measure expressing the energy cost of physical activities), the moderate physical activity producing noticeable accelerated heart rate, ranges from 3.0 to 6.0 MET while the vigorous physical activity, demanding greater physical effort causing rapid breathing and a substantial increase in heart rate, are all the physical activities above 6.0 MET (38, 39). Some studies have reported a positive relationship (27, 33, 40-44), while others reported a negative one (45-47) between the practice of physical activity and the semen quality. Others report no impact of physical activity on sperm quality (48-51), therefore, the effect of a physically active lifestyle to improve semen quality is still controversial (52). The present study was conducted with the specific aim to evaluate and compare the semen parameters, conventional as well as functional, of men practicing vigorous physical activity to those having a sedentary life style.

Materials and Methods

In this descriptive cross-sectional study, thirty two men of reproductive age (physically active group 27.5 ± 6.0 and sedentary group 26.6 ± 5.3

years) from Medellin, Colombia were included. The inclusion criteria were: healthy men, without testicular disease, with a body mass index (BMI)<26 kg/m^2, and those who followed the same lifestyle pattern for the 12 months preceding the study, it is, either be physically active group (PAG, practice vigorous physical activities with a >6 MET for more than 2 hours per occasion at least 3 times per week; activities included are cycling, stationary cycling, calisthenetics, weightlifting, dancing, running, marital arts, football and swimming) or be sedentary group (SG, minimal physical activity ≤3 MET, do not practice sportive activities) (39, 53) (Table 1). Recreational drug and anabolic steroid users, smokers or medicated men were excluded from the study. Ethical approval was obtained from the Research Ethics Committee of the University of Antioquia and all patients gave informed consent. Semen samples were collected from volunteers between January and July 2014 by masturbation after a recommended ejaculation abstinence of 3-6 days. In addition to sample collection, certain anthropometric measurements (height and weight) necessary to calculate BMI were also measured (Table 1).

Also Participants had to complete a self-administered questionnaire by providing information regarding their reproductive history and whether or not they routinely practice any physical activity. If they did, they were asked to fill the description, type, frequency, intensity and duration of the physical activities practiced. This information was used to calculate the physical activities MET using the "Compendium of physical activities" as proposed by Ainswort et al. (39) which provided a measurement of their intensity level.

Conventional semen analysis

After complete liquefaction of the semen samples (30-60 minutes, at 37°C), a basic semen analysis was performed according to the World Health Organization (WHO) guidelines (1) while the sperm concentration was determined by using a Makler chamber (Sefi-Medical Instruments, Haifa, Israel) (54). Finally, sperm morphology was analyzed following the Tygerberg strict criteria (55), and semen samples with leukocytospermia (>1×10^6 white blood cells/mL) were excluded.

Functional analysis

All flow cytometry analysis reported in this study were conducted on an Epics XL flow cytometer (Becton Dickinson, CA, USA) with a 488 nm excitation wavelength supplied by an argon laser. Forward scatter and side scatter measurements were used to gate spermatozoa' and exclude debris' and aggregates limiting undesired effects in the overall fluorescence. All data were acquired and analyzed using WinMDI 2.9 Software (Scripps Research Institute, La Jolla, CA) and a total of 10000 events were collected per sample.

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) was measured by using 3, 3′-dihexyloxacarbocyanine iodide stain ($DIOC_6$, Molecular Probes Inc., The Netherlands) (3) a cationic lipophilic dye selective for the mitochondria of living cells. Propidium iodide (PI, Molecular Probes Inc., The Netherlands) was used as counter stain to discriminate necrotic/dead cells. Briefly, 2×10^6 spermatozoa were incubated in 300 µL of phosphate buffer saline (PBS, pH=7.4) containing $DIOC_6$ (final concentration of 10 nM) and PI (final concentration of 12 µM) in the dark (30 minutes, at 25°C). Then, samples were washed in PBS (180 x g, 5 minutes), the pellet re-suspended in PBS and subjected to flow cytometry. Data were acquired as the percentage of living spermatozoa showing high ($\Delta\Psi_{m\ high}$) or low ($\Delta\Psi_{m\ low}$) green fluorescence and dead spermatozoa-red fluorescence.

Intracellular reactive oxygen species production

The intracellular ROS and RNS (specifically H_2O_2, HO^-, ROO^- and $ONOO^-$) levels were evaluated using 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St Louis, MO, USA). Upon cleavage of the acetate groups by intracellular esterases, DCFH is selectively oxidized by the above mentioned ROS and RNS to the green fluorescent DCF. PI was used to exclude the necrotic/dead cells (5). DCFH-DA was diluted to a final concentration of 1 µM in 300 µL of PBS containing 2×10^6 spermatozoa and PI (final concentration 12 µM). The cell suspensions were incubated in the dark for 5 minutes, at 25°C, washed three times with PBS (180 x g, 5 minutes) and the pellet re-suspended in PBS before being analyzed by flow cytometry. Results are expressed as the percentage of live spermatozoa exhibiting the green DCF fluorescent response (DCF positive spermatozoa), as well as the green media fluorescence intensity (MFI).

Plasma membrane integrity evaluation

The LIVE/DEAD® Sperm Viability Kit (Molecular Probes Inc., The Netherlands) which distinguishes three populations of sperm based on their staining patterns, was used to assess the integrity of the plasma membrane according to the manufacturer's instructions. Briefly, 2×10^6 spermatozoa were incubated in 300 µL of PBS with Sybr-14 and PI (green and red fluorescence emission, final concentration of 1 µM and 12 µM, respectively) in the dark (30 minutes, 25°C), washed once and re-suspended in PBS prior to flow cytometry analysis. Data are expressed as the percentage of viable spermatozoa-intact plasma membrane cells (positive to SYBR-14 and negative to PI), necrotic/dead cells (positive for PI only) or moribund sperm (positive for both dyes).

Lipid peroxidation assay

Oxidative degradation of lipids was measured using the BODIPY (581/591) C11 (Molecular Probes Inc., The Netherlands) according to the method proposed by Aitken et al. (16). BODIPY (581/591) C11 once incorporated into sperm membranes, undergoes a fluorescent emission shift from orange to green upon peroxidation by ROS. Briefly, 2×10^6 spermatozoa suspended in 300 µL of PBS were incubated in the dark (30 minutes, at 25°C) with BODIPY C11 (final concentration 6.6 µM), washed and re-suspended in PBS before flow cytometry analysis. Results are expressed as the percentage of spermatozoa exhibiting the green fluorescence response.

Sperm Chromatin Structure Assay

The Sperm Chromatin Structure Assay (SCSA) was used to determine the sperm DNA fragmentation index by Evenson (56) as previously described and modified in our laboratory (5, 13, 18, 19). Briefly, 400 µl of acid detergent solution (HCl, NaCl, Tritón X-100, water, pH=1.2) were added to 10×10^6 spermatozoa suspended in 200 µL of TNE buffer (TRIS-HCL, NaCl and EDTA, pH=7.4). After 30 seconds, spermatozoa were stained with 600 µl of acridine orange (Sigma-Aldrich, St Louis, MO, USA) staining solution (final concentration of 6 µg/mL). The ratio of single stranded DNA (red) to single plus double stranded DNA (green) MFI was expressed as the DNA fragmentation index (DFI).

Statistical analysis

The distribution of the data was evaluated with the normality test of residuals. The t test was used to compare groups of data that assumed Gaussian distribution, while the Mann-Whitney test used to compare the variables that did not assume Gaussian distribution. Correlations between sperm variables were determined with the Pearson correlation coefficient. Data were analyzed by using Prism 5.0 (GraphPad Software, San Diego, CA) statistical software and a $P<0.05$ considered to be significant. Data following Gaussian distribution are expressed as the mean ± SD and those not assuming Gaussian distribution are expressed as median and range.

Results

According to the MET scores, men were stratified into a physically active group (PAG, 8-48 MET, n=17) and a sedentary group (SG, <3 MET, n=15). Both PAG and SG present similar characteristics with regards to abstinence (4.1 ± 0.69 vs. 3.7 ± 0.75 days), height (1.74 ± 0.06 vs. 1.72 ± 0.05 m) and BMI (23.7 ± 1.5 vs. 22.7 ± 1.8 kg/m^2). The average weight was slightly higher in the PAG in comparison with SG (71.6 ± 7.3 vs. 67 ± 5.3 kg), because these men had increased body mass in the form of muscle not of fat (Table 1).

Table 1: Characteristics of the participants

Characteristic	Physically active group n=17	Sedentary group n=15	P value
Age (Y)	27.5 ± 6.0	26.6 ± 5.3	0.66$^+$
Sexual abstinence (days)	4.1 ± 0.69	3.7 ± 0.75	0.56$^+$
Weight (Kg)	71.6 ± 7.3	67 ± 5.3	0.07$^+$
Height (m)	1.74 ± 0.06	1.72 ± 0.05	0.3$^+$
BMI (Kg/m^2)	23.7 ± 1.5	22.7 ± 1.8	0.11$^+$
Metabolic Equivalent of the Task (MET)	19.7 ± 10.6	1.8 ± 0.6	<0.0001$^\wedge$

Results are expressed as mean ± SD. BMI; Body mass index, $^+$; Student t test (Gaussian distribution), and $^\wedge$; Mann-Whitney test (non-gaussian distribution).

All semen samples from the PAG appeared normal with regards to viscosity and showed no agglutination, however various samples from SG showed moderate to high viscosity (33%) as well as isolated agglutination (47%) and moderate to abundant agglutination (13%) respectively. Among the conventional sperm parameters, total sperm motility, progressive motility and the percentage of viable sperm were significantly higher ($P<0.05$) in the PAG compared to the SG (Table 2). The only functional parameter that showed significant difference ($P<0.05$) between the PAG and SG was the percentage of moribund spermatozoa (Table 3).

Table 2: Conventional semen parameters

Variable	Physically active group n=17	Sedentary group n=15	P value
Semen volume (mL)	4.3 ± 1.2	3.5 ± 1.5	0.14^{+}
Sperm concentration ($\times10^6$ sperm/mL)	95.2 ± 47	114.4 ± 63.9	0.37^{+}
Total sperm count ($\times10^6$)	353.6 (55.72-1080)	361.9 (100-997.4)	0.82$^{\wedge}$
Viability (%)	80.2 ± 7.2	71.9 ± 10.7	0.01^{+}
Progressive motility (%)	63.0 (55.7-87.7)	56.8 (35.2-82.7)	0.03$^{\wedge}$
Non-progressive motility (%)	3.7 (1.6-22.0)	5.0 (2.7-16.6)	0.13$^{\wedge}$
Total motility (%)	66.5 (70.0-89.3)	62.3 (42.5-45.6)	0.03$^{\wedge}$
Normal morphology (%)	7.3 (2.3-12.0)	4.8 (2.7-13.4)	0.52$^{\wedge}$
Abnormal head (%)	90.2 ± 5.0	89.1 ± 4.7	0.54^{+}
Abnormal neck/middle piece (%)	44.9 ± 16.0	53.9 ± 18	0.14^{+}
Abnormal tail (%)	5.1 (3.3-7.1)	6.9 (2.5-8.7)	0.52$^{\wedge}$
Abnormal cytoplasmic droplets (%)	6.6 ± 4.8	5.7 ± 2.9	0.56^{+}

Values are expressed as mean ± SD in data with normal distribution, and median (range) in non-normal distribution. $^{+}$; Student t test (Gaussian distribution) and $^{\wedge}$: Mann-Whitney test (non-gaussian distribution).

Table 3: Functional seminal parameters

Variable	Physically active group n=17	Sedentary group n=15	P value
$\Delta\Psi_m$ high spermatozoa (%)	63.5 (51.5-80.6)	63.8 (18.9-77.1)	0.45$^{\wedge}$
$\Delta\Psi_m$ low spermatozoa (%)	4.0 (1.9-14.0)	4.4 (2.3-19.1)	0.54$^{\wedge}$
Sperm with intact plasma membrane (%)	68.1 (43.7-83.1)	65.2 (26.2-72.6)	0.10$^{\wedge}$
Moribund sperm (%)	4.3 (2.0-17.0)	9.0 (4.6-14.4)	0.02$^{\wedge}$
Necrotic/dead sperm (%)	23.5 ± 6.7	28.6 ± 11.4	0.13^{+}
DCF positive spermatozoa (%)	59.3 (6.83-78.2)	49.3 (14.3-68.1)	0.09$^{\wedge}$
DCF positive spermatozoa (MFI)	50.6 (24.4-148.8)	57.7 (14.6-92.2)	0.74$^{\wedge}$
Sperm with lipid peroxidation (%)	3.3 (0.5-18.8)	6.3 (0.15-33.6)	0.20$^{\wedge}$
DNA fragmentation index (%)	19.6 ± 8.6	17.1 ± 8.3	0.40^{+}

Values are expressed as mean ± SD in data with normal distribution and median (range) in non-normal distribution. DCF; 2', 7'-dichlorofluorescein, MFI; Mean fluorescence intensity, $^{+}$; Student t test (Gaussian distribution), and $^{\wedge}$; Mann-Whitney test (non-gaussian distribution).

When comparing the combined data sets from both groups, significant correlations were found between total abnormal sperm forms and spermatozoa with head defects (correlation coefficient-r-=0.67, P<0.01), sperm with neck/middle piece defects and progressive motility (r=-0.56, P<0.01), ejaculation abstinence time and sperm with excess residual cytoplasm (r=0.58, P<0.01), ejaculation abstinence time and non-progressive motility (r=-0.57, P<0.01), viable sperm and intracellular ROS production (r=0.79, P<0.01), viable sperm and sperm with high mitochondrial membrane potential (ΔΨm, r=0.83, P<0.01), and sperm with high ΔΨm and intracellular ROS production (r=0.65, P<0.01). In addition, when the PAG's data were analyzed separately, all of the above mentioned significant correlations were found, together with a few significant correlations exclusive to the PAG. These include: ejaculation abstinence time and immotile sperm (r=-0.57, P<0.01), sperm concentration and normal morphology (r=0.72, P<0.01), and sperm concentration and sperm with head defects (r=-0.63, P<0.01).

Discussion

We found differences in conventional and functional seminal parameters between physically active group and sedentary group of men. The semen parameters were better in PAG, which is in favor to adopt such a life style. The average values of the conventional parameters analyzed for each group, remained above the lower limit reference values proposed by the WHO (1). The total and progressive sperm motility, sperm viability, as well as the percentage of moribund cells were significantly higher in the PAG compared to SG. This is the first study in addition to conventional semen parameters, certain sperm functional parameters i.e. $\Delta\Psi_m$, plasma membrane integrity, intracellular ROS and lipid peroxidation, were analyzed in relation to the practice of vigorous physical activity or following a sedentary lifestyle. Our results are in accordance with previous study demonstrating increased sperm motility in physical active men (41) and comparable results in a group of assisted reproduction patients classified according to their physical status (43). However, significant differences in sperm viability due to physical activity levels had not been previously reported.

Some studies reported that sperm concentration and morphology are the main parameters improved in men having moderate to vigorous physical active lifestyle (47), against being sedentary (43). Our results are not in agreement with these findings, as we did not observe any significant differences in either sperm concentration or morphology between PAG and SG. Nonetheless, a positive correlation was found between sperm concentration and normal morphology in PAG. Similar results have been reported by Munuce et al. (57) in semen samples obtained from men attending a reproductive clinic without regarding their physical status. This finding is interesting because it may be related to an increase in hormones, specially follicle-stimulating hormone (FSH), luteinizing hormone (LH) and testosterone, responsible to stimulate proper spermatogonia nutrition and division during the process of spermatogenesis (58, 59). This speculation is supported by the findings from previous studies where increased total and free blood plasma and serum testosterone, as well as higher FSH and LH levels have been demonstrated after continuous moderate physical activity (41).

The plasma membrane integrity is a key determinant for proper sperm interactions with other cells and their environment, therefore it is a prerequisite for successful fertilization (60). The percentage of dual stained (moribund) spermatozoa was statistically higher in SG in comparison with PAG. This sperm population have been described as slightly damaged sperm with compromised plasma membrane that have lost their ability to exclude PI, indicating a transitional phase in which the cell ultimately die (60-62). Although the biological importance of moribund sperm has not been well established, in works on bulls the percentage of moribund spermatozoa was positively correlated with the low fertility status of males, possibly compromising the availability of live sperm in the female reproductive tract (62). Furthermore, Garner and Johnson (61) have microscopically observed that the change from green to red fluorescence of some sperm, began at the posterior portion of the sperm head, proceed anteriorly and is accompanied by the progressive loss of motility until they are dead. This increased percentage of moribund spermatozoa and negative correlation between sperm with neck/middle piece defects and progressive motil-

ity in SG can be the possible explanations of significantly lower progressive and total motility in these men.

The evaluation of functional parameters has also been used to determine the levels of oxidative stress in spermatozoa. Common sedentary activities such as sitting for long, and some physical activities including running or bicycling may disrupt the intrascrotal temperature regulation (63, 64) and increase the pressure force to the testicles (46, 47, 49), leading to oxidative stress (58).

Although the percentage of DCF positive spermatozoa in the PAG group was higher in SG, it was not significantly different. We found higher values of DCF positive sperm in comparison with previous studies (65-67) intended to evaluate the ROS/RNS production on spermatozoa using the same method. However; the oxidative stress level as depicted by lipid peroxidation measurement was discernibly lower in PAG than SG, which is in accordance with previous findings by others (16, 68). Sperm DNA integrity did not differ significantly in our study between PAG, SG and DFI remained in the range considered normal (16-24%) (69). This is in accordance with a previous report where no relation of sperm DFI was drawn in men with sedentary lifestyle in relation to their BMI and their waist circumference (70).

In addition, no mitochondrial dysfunction was detected either in the PAG or SG group despite the total and progressive motility is significantly increased in PAG. In fact, most of the spermatozoa in the semen samples from both groups had high $\Delta\Psi_m$, which is indicative in proper mitochondrial functioning (17, 71). It may support the assumption that the rapid transition from viable to moribund sperm was influencing the loss of motility in the SG sperm rather than the viable sperm that have diminished the $\Delta\Psi_m$ as it may be commonly related. As the higher ROS detection in the PAG was not correlated with oxidative stress generation in spermatozoa (higher lipoperoxidation-LPO- and/or altered DFI), we speculate that there must have been a balance between pro-oxidants and antioxidants molecules in the PAG volunteers' semen samples. Possibly the practice of vigorous physical activity of volunteers, have contributed to attenuate the oxidative stress events in consequence of the higher ROS/RNS production, since it has been previously demonstrated that physical training promotes blood total antioxidant capacity (72), and also in semen, moderate to vigorous physical activity practitioners had superior levels of antioxidant enzymes in comparison with high performance-elite athletes or sedentary men (73).

Furthermore; it is known that sperm cells have a deficient ROS-scavenging system, in consequence of its limited cytosolic space. So they are very dependent on the antioxidant protection provided by the male reproductive tract (74). This is directly influenced by the men's nutritional status and the dietary intake of antioxidant molecules since they form an essential part of the human antioxidant defense system (75).

As we did not control the diet in our volunteers, the effect of the diet cannot be ruled out, considering that, a physically active lifestyle is commonly accompanied by a healthy diet. In the light of these results, we consider convenient to include some other informational aspects, certainly related to the physically active or sedentary lifestyle and the semen quality. For instance, nutritional aspects related with dietary antioxidants intake, the determination of blood hormonal levels (mainly LH, FSH and testosterone) and the semen total antioxidant capacity evaluation, directly involved in the developmental environment of spermatozoa and the oxidative stress dynamics. On the other hand, it has also been established that if the physical training is at least moderate but regular, it may turn into an adaptation to diminish the increased amounts of ROS producing during high oxygen consumption derived from further vigorous physical activities (41, 58, 76, 77). This physical activity linked-adaptation constitutes an advantage over the possible adverse conditions associated with the practice of some previously mentioned physical activities that may negatively affect the seminal quality.

Infertility affects approximately 15% of couples of reproductive age, with significant impact on their quality of life (11, 70). As it is estimated that men contribute equally (50%) to the causes of fertility problems (29, 59), the identification and modification of some potential risk factors such as the relationship between physical activity or inactivity and semen quality, may help some couples to achieve their reproductive goals (70). The practice of vigor-

ous physical activity is clearly not the unique solution. Most of the literature regarding the relatioship between physical activity, sedentarism and semen quality, have focused on elite athletes or men attending fertility clinics. However; various investigations have demonstrated the positive influence of moderate, constant exercise on the hormonal profile (41, 58, 76), libido (78), the psychological wellbeing (59) and on the body condition (30, 38, 76), which may also impact positively the male reproductive outcome. Our volunteers may be clasified as recreational but vigorous physical activity practitioners, since none of them were endurance sport competitors and the activities performed included strenght and aerobic training or vigorous occupational physical activities. This is important to clarify because the type of physical training, specially the higher intensity or constantly anaerobic training have been related to diminishseminal parameters (45-47, 49) and also may influence the hormonal effect on the sperm quality, specially on the testosterone metabolism (41, 59).

Conclusion

Despite the fact that some indicators of cellular oxidative stress were higher in the PAG in comparison with the SG, no signs of developing a state of oxidative stress was observed. On the contrary, the practice of vigorous physical activity in the conditions set in our study (8 to 48 MET, in sessions of two hours minimum, with a frequency of at least 3 days a week), was significantly related to better semen parameters (increased viability, progressive and total motility and lower percentage of moribund cells), when compared to individuals following a complete sedentary lifestyle at least for a year. It can therefore be concluded that the levels of physical activity reported in this study, exert a positive effect on the semen parameters of these men or at least prevent its deterioration as a result of environmental stressors. Our findings are encouraging since they contribute to elucidate the proper intensity and frequency of physical activity which may excerpt a positive effect on semen quality or at least prevent its decline related to the practice of higher intensity-endurance physical activities. Future studies are required in defining the intensity and threshold to be considered as beneficial for semen quality.

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References

1. World Health Organization. WHO laboratory manual for the examination and processing of human semen. Geneva, Switzeland: WHO Press; 2010. http://apps.who.int/iris/bitstream/10665/44261/1/9789241547789_eng.pdf. (20 May 2016).
2. Eliasson R. Semen analysis with regard to sperm number, sperm morphology and functional aspects. Asian J Androl. 2010; 12(1): 26-32.
3. Bungum M, Bungum L, Giwercman A. Sperm chromatin structure assay (SCSA): a tool in diagnosis and treatment of infertility. Asian J Androl. 2011; 13(1): 69-75.
4. Cardona Maya WD, Berdugo Gutierrez JA, de los Rios J, Cadavid Jaramillo AP. Functional evaluation of sperm in Colombian fertile men. Arch Esp Urol. 2007; 60(7): 827-831.
5. Mayorga-Torres BJ, Cardona-Maya W, Cadavid A, Camargo M. Evaluation of sperm functional parameters in normozoospermic infertile individuals. Actas Urol Esp. 2013; 37(4): 221-227.
6. Mayorga-Torres BJ, Camargo M, Agarwal A, du Plessis SS, Cadavid AP, Cardona Maya WD. Influence of ejaculation frequency on seminal parameters. Reprod Biol Endocrinol. 2015; 13: 47.
7. Mayorga-Torres JM, Agarwal A, Roychoudhury S, Cadavid A, Cardona-Maya WD. Can a short term of repeated ejaculations affect seminal parameters? J Reprod Infertil. 2016; 17(3): 177-183.
8. Tremellen K. Oxidative stress and male infertility: a clinical perspective. In: Agarwal A, Aitken RJ, Alvarez JG, editors. Studies on men's health and fertility. Oxidative stress in applied basic research and clinical practice. New York: Humana Press, Springer; 2012; 325-354.
9. Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. Oxid Med Cell Longev. 2008; 1(1): 15-24.
10. Kothari S, Thompson A, Agarwal A, du Plessis SS. Free radicals: their beneficial and detrimental effects on sperm function. Indian J Exp Biol. 2010; 48(5): 425-435.
11. Henkel R. ROS and semen quality. In: Agarwal A, Aitken RJ, Alvarez JG, editors. Studies on men's health and fertility. Oxidative stress in applied basic research and clinical practice. New York: Humana Press, Springer; 2012; 301-323.
12. Aitken RJ, Baker MA, De Iuliis GN, Nixon B. New insights into sperm physiology and pathology. Handb Exp Pharmacol. 2010; (198): 99-115.
13. Gil-Villa AM, Cardona-Maya W, Agarwal A, Sharma R, Cadavid A. Role of male factor in early recurrent embryo loss: do antioxidants have any effect? Fertil Steril. 2009; 92(2): 565-571.
14. El-Taieb MA, Herwig R, Nada EA, Greilberger J, Marberger M. Oxidative stress and epididymal sperm transport, motility and morphological defects. Eur J Obstet Gynecol Reprod Biol. 2009; 144 Suppl 1: S199-203.
15. Aitken RJ, Smith TB, Jobling MS, Baker MA, De Iuliis GN.

Oxidative stress and male reproductive health. Asian J Androl. 2014; 16(1): 31-38.
16. Aitken RJ, Wingate JK, De Iuliis GN, McLaughlin EA. Analysis of lipid peroxidation in human spermatozoa using BODIPY C11. Mol Hum Reprod. 2007; 13(4): 203-211.
17. Koppers AJ. Mitochondria as a source of ROS in mammalian spermatozoa. In: Agarwal A, Aitken RJ, Alvarez JG, editors. Studies on men's health and fertility. Oxidative stress in applied basic research and clinical practice. New York: Humana Press, Springer; 2012; 21-40.
18. Gil-Villa AM, Cardona-Maya W, Agarwal A, Sharma R, Cadavid A. Assessment of sperm factors possibly involved in early recurrent pregnancy loss. Fertil Steril. 2010; 94(4): 1465-1472.
19. Rodriguez E, Gil-Villa AM, Aguirre-Acevedo DC, Cardona-Maya W, Cadavid AP. Evaluation of atypical semen parameters in individuals whose couples had a history of early recurrent embryo death: in search for a reference value. Biomedica. 2011; 31(1): 100-107.
20. Bungum M. Sperm DNA integrity assessment: a new tool in diagnosis and treatment of fertility. Obstet Gynecol Int. 2012; 2012: 531042.
21. Agarwal A, Desai NR, Ruffoli R, Carpi A. Lifestyle and testicular dysfunction: a brief update. Biomed Pharmacother. 2008; 62(8): 550-553.
22. Ivell R. Lifestyle impact and the biology of the human scrotum. Reprod Biol Endocrinol. 2007; 5: 15.
23. Ahmad G, Moinard N, Esquerre-Lamare C, Mieusset R, Bujan L. Mild induced testicular and epididymal hyperthermia alters sperm chromatin integrity in men. Fertil Steril. 2012; 97(3): 546-553.
24. Sharpe RM. Environment, lifestyle and male infertility. Baillieres Best Pract Res Clin Endocrinol Metab. 2000; 14(3): 489-503.
25. Mendiola J, Torres-Cantero AM, Agarwal A. Lifestyle factors and male infertility: an evidence-based review. Arch Med Sci. 2009; 5(1A): S3-S12.
26. Kumar S, Kumari A, Murarka S. Lifestyle factors in deteriorating male reproductive health. Indian J Exp Biol. 2009; 47(8): 615-624.
27. Jurewicz J, Radwan M, Sobala W, Ligocka D, Radwan P, Bochenek M, et al. Lifestyle and semen quality: role of modifiable risk factors. Syst Biol Reprod Med. 2014; 60(1): 43-51.
28. Bujan L, Daudin M, Charlet JP, Thonneau P, Mieusset R. Increase in scrotal temperature in car drivers. Hum Reprod. 2000; 15(6): 1355-1357.
29. Du Plessis SS, Cabler S, McAlister DA, Sabanegh E, Agarwal A. The effect of obesity on sperm disorders and male infertility. Nat Rev Urol. 2010; 7(3): 153-161.
30. Blair SN. Physical inactivity: the biggest public health problem of the 21st century. Br J Sports Med. 2009; 43(1): 1-2.
31. Bernstein MS, Morabia A, Sloutskis D. Definition and prevalence of sedentarism in an urban population. Am J Public Health. 1999; 89(6): 862-867.
32. Fox M. What is sedentarism? J Acad Nutr Diet. 2012; 112(8): 1124-1128.
33. Magnusdottir EV, Thorsteinsson T, Thorsteinsdottir S, Heimisdottir M, Olafsdottir K. Persistent organochlorines, sedentary occupation, obesity and human male subfertility. Hum Reprod. 2005; 20(1): 208-215.
34. Brownson RC, Boehmer TK, Luke DA. Declining rates of physical activity in the United States: what are the contributors? Annu Rev Public Health. 2005; 26: 421-443.
35. Janevic T, Kahn LG, Landsbergis P, Cirillo PM, Cohn BA, Liu X, et al. Effects of work and life stress on semen quality. Fertil Steril. 2014; 102(2): 530-538.
36. Bayol S, Brownson C, Loughna PT. Electrical stimulation modulates IGF binding protein transcript levels in C2C12 myotubes. Cell Biochem Funct. 2005; 23(5): 361-365.
37. Blair SN, LaMonte MJ, Nichaman MZ. The evolution of physical activity recommendations: how much is enough? Am J Clin Nutr. 2004; 79(5): 913S-920S.
38. World Health Organization. Global recommendations on physical activity for health. Geneva, Switzerland: WHO Press; 2010. Available from: http://whqlibdoc.who.int/publications/2010/9789241599979_eng.pdf. (20 May 2016).
39. Ainsworth BE, Haskell WL, Whitt MC, Irwin ML, Swartz AM, Strath SJ, et al. Compendium of physical activities: an update of activity codes and MET intensities. Med Sci Sports Exerc. 2000; 32(9 Suppl): S498-504.
40. Wise LA, Cramer DW, Hornstein MD, Ashby RK, Missmer SA. Physical activity and semen quality among men attending an infertility clinic. Fertil Steril. 2011; 95(3): 1025-1030.
41. Vaamonde D, Da Silva-Grigoletto ME, Garcia-Manso JM, Barrera N, Vaamonde-Lemos R. Physically active men show better semen parameters and hormone values than sedentary men. Eur J Appl Physiol. 2012; 112(9): 3267-3273.
42. Gaskins AJ, Mendiola J, Afeiche M, Jorgensen N, Swan SH, Chavarro JE. Physical activity and television watching in relation to semen quality in young men. Br J Sports Med. 2015; 49(4): 265-270.
43. Guarnizo MC, Niebla ED, García SM, González MR, Gordillo J, Fernández de Velasco JDJ, et al. Influence of physical activity and body mass index on sperm quality: analysis in assisted reproduction patients. Rev Asoc Est Biol Rep. 2011; 16(1): 25-34.
44. Parn T, Grau Ruiz R, Kunovac Kallak T, Ruiz JR, Davey E, Hreinsson J, et al. Physical activity, fatness, educational level and snuff consumption as determinants of semen quality: findings of the ActiART study. Reprod Biomed Online. 2015; 31(1): 108-119.
45. Arce JC, De Souza MJ, Pescatello LS, Luciano AA. Subclinical alterations in hormone and semen profile in athletes. Fertil Steril.1993; 59(2): 398-404.
46. De Souza MJ, Arce JC, Pescatello LS, Scherzer HS, Luciano AA. Gonadal hormones and semen quality in male runners. A volume threshold effect of endurance training. Int J Sports Med. 1994; 15(7): 383-391.
47. Vaamonde D, Da Silva-Grigoletto ME, Garcia-Manso JM, Vaamonde-Lemos R, Swanson RJ, Oehninger SC. Response of semen parameters to three training modalities. Fertil Steril. 2009; 92(6): 1941-1946.
48. Bagatell CJ, Bremner WJ. Sperm counts and reproductive hormones in male marathoners and lean controls. Fertil Steril. 1990; 53(4): 688-692.
49. Lucia A, Chicharro JL, Perez M, Serratosa L, Bandres F, Legido JC. Reproductive function in male endurance athletes: sperm analysis and hormonal profile. J Appl Physiol (1985). 1996; 81(6): 2627-2636.
50. Mínguez-Alarcón L, Chavarro JE, Mendiola J, Gaskins AJ, Torres-Cantero AM. Physical activity is not related to semen quality in young healthy men. Fertil Steril. 2014; 102(4): 1103-1109.
51. Stoy J, Hjollund NH, Mortensen JT, Burr H, Bonde JP. Semen quality and sedentary work position. Int J Androl. 2004; 27(1): 5-11.
52. Lalinde Acevedo PC, Mayorga Torres JM, Cardona Maya WD. Relation between physical activity, sedentarism and semen quality (Relación entre la actividad física, el sedentarismo y la calidad seminal). Rev Chil Obstet Ginecol. 2014; 79(4): 323-329.
53. Craig CL, Marshall AL, Sjostrom M, Bauman AE, Booth ML, Ainsworth BE, et al. International physical activity

questionnaire: 12-country reliability and validity. Med Sci Sports Exerc. 2003; 35(8): 1381-1395.
54. Cardona-Maya W, Berdugo J, Cadavid A. Comparing the sperm concentration determined by the makler and the neubauer chambers. Actas Urol Esp. 2008; 32(4): 443-445.
55. Kruger TF, Menkveld R, Stander FS, Lombard CJ, Van der Merwe JP, van Zyl JA, et al. Sperm morphologic features as a prognostic factor in in vitro fertilization. Fertil Steril. 1986; 46(6): 1118-1123.
56. Evenson DP. Sperm chromatin structure assay (SCSA(R)). Methods Mol Biol. 2013; 927: 147-164.
57. Munuce MJ, Cardona-Maya W, Berta CL. Is there an association between sperm normal morphology and their kinetic displacement? Actas Urol Esp. 2006; 30(6): 591-597.
58. Lanfranco F, Minetto MA. Endocrinology of physical activity and sport. In: Constantini N, Hackney AC, editors. The male reproductive system, exercise, and training: endocrine adaptations. 2nd ed. New York: Springer; 2013; 121-132.
59. du Plessis SS, Kashou A, Vaamonde D, Agarwal A. Is there a link between exercise and male factor infertility. Open Reprod Sci J. 2011; 3: 105-113.
60. Kim S, Lee YJ, Kim YJ. Changes in sperm membrane and ROS following cryopreservation of liquid boar semen stored at 15 degrees C. Anim Reprod Sci. 2011; 124(1-2): 118-124.
61. Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. Biol Reprod. 1995; 53(2): 276-284.
62. Shojaei H, Kroetsch T, Wilde R, Blondin P, Kastelic JP, Thundathil JC. Moribund sperm in frozen-thawed semen, and sperm motion end points post-thaw and post-swim-up, are related to fertility in Holstein AI bulls. Theriogenology. 2012; 77(5): 940-951.
63. Jung A, Schuppe HC. Influence of genital heat stress on semen quality in humans. Andrologia. 2007; 39(6): 203-215.
64. Koskelo R, Zaproudina N, Vuorikari K. High scrotal temperatures and chairs in the pathophysiology of poor semen quality. Pathophysiology. 2005; 11(4): 221-224.
65. Mahfouz R, Sharma R, Lackner J, Aziz N, Agarwal A. Evaluation of chemiluminescence and flow cytometry as tools in assessing production of hydrogen peroxide and superoxide anion in human spermatozoa. Fertil Steril. 2009; 92(2): 819-827.
66. Mahfouz RZ, du Plessis SS, Aziz N, Sharma R, Sabanegh E, Agarwal A. Sperm viability, apoptosis, and intracellular reactive oxygen species levels in human spermatozoa before and after induction of oxidative stress. Fertil Steril. 2010; 93(3): 814-821.
67. Sullivan MJ, Beltz TG, Johnson AK. Amastatin potentiation of drinking induced by blood-borne angiotensin: evidence for mediation by endogenous brain angiotensin. Brain Res. 1990; 510(2): 237-241.
68. Barbonetti A, Vassallo MR, Cinque B, Filipponi S, Mastromarino P, Cifone MG, et al. Soluble products of Escherichia coli induce mitochondrial dysfunction-related sperm membrane lipid peroxidation which is prevented by lactobacilli. PLoS One. 2013; 8(12): e83136.
69. Evenson DP, Larson KL, Jost LK. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. J Androl. 2002; 23(1): 25-43.
70. Eisenberg ML, Kim S, Chen Z, Sundaram R, Schisterman EF, Louis GMB. The relationship between male BMI and waist circumference on semen quality: data from the LIFE study. Hum Reprod. 2014; 29(2): 193-200.
71. Wang X, Sharma RK, Gupta A, George V, Thomas AJ, Falcone T, et al. Alterations in mitochondria membrane potential and oxidative stress in infertile men: a prospective observational study. Fertil Steril. 2003; 80 Suppl 2: 844-850.
72. Child RB, Wilkinson DM, Fallowfield JL, Donnelly AE. Elevated serum antioxidant capacity and plasma malondialdehyde concentration in response to a simulated half-marathon run. Med Sci Sports Exerc. 1998; 30(11): 1603-1607.
73. Hajizadeh Maleki B, Tartibian B, Eghbali M, Asri-Rezaei S. Comparison of seminal oxidants and antioxidants in subjects with different levels of physical fitness. Andrology. 2013; 1(4): 607-614.
74. Aitken RJ, De Iuliis GN. On the possible origins of DNA damage in human spermatozoa. Mol Hum Reprod. 2010; 16(1): 3-13.
75. Agarwal A, Prabakaran SA, Said TM. Prevention of oxidative stress injury to sperm. J Androl. 2005; 26(6): 654-660.
76. Redman LM. Physical activity and its effects on reproduction. Reprod Biomed Online. 2006; 12(5): 579-586.
77. Kraemer WJ, Ratamess NA. Hormonal responses and adaptations to resistance exercise and training. Sports Med. 2005; 35(4): 339-361.
78. Wogatzky J, Wirleitner B, Stecher A, Vanderzwalmen P, Neyer A, Spitzer D, et al. The combination matters--distinct impact of lifestyle factors on sperm quality: a study on semen analysis of 1683 patients according to MSOME criteria. Reprod Biol Endocrinol. 2012; 10: 115.

Original Article

Aluminium-Induced Oxidative Stress, Apoptosis and Alterations in Testicular Tissue and Sperm Quality in Wistar Rats: Ameliorative Effects of Curcumin

Ebrahim Cheraghi, Ph.D.[1*], Alireza Golkar, M.Sc.[2], Kambiz Roshanaei, Ph.D.[3], Behrang Alani, Ph.D.[4]

1. Department of Biology, Faculty of Sciences, University of Qom, Qom, Iran
2. Sciences Research Laboratory, Department of Biology, Qom Branch, Islamic Azad University, Qom, Iran
3. Department of Biology, Qom Branch, Islamic Azad University, Qom, Iran
4. Department of Applied Cell Sciences, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran

Abstract

Background: Reproductive toxicity is a major challenge associated with aluminum (Al) exposure. No studies have evaluated the possible effects of curcumin (CUR) on Al-induced reproductive dysfunction. Therefore, this study investigated the effects of CUR treatment on Al-induced reproductive damage.

Materials and Methods: In this experimental study, 40 male Wistar rats were allocated to the five groups (n=8) based on the treatment they received: no treatment (control), solvent [dimethyl sulfoxide (DMSO) or distilled water], CUR 10 mg/kg body weight (BW), Al chloride 10 mg/kg BW, and CUR+Al chloride (10 mg/kg BW/each alone). Treatments were performed by intraperitoneal (IP) injections for 28 days. The left testis was assessed for histopathological analysis as well as the incidence of germ cell apoptosis. One-way analysis of variance (ANOVA) followed by the Tukey's test was used. $P<0.05$ was considered significant.

Results: Significant reductions in body and testis weight; plasma testosterone and luteinizing hormone levels; sperm count, motility, morphology, and viability; germinal epithelium thickness; seminiferous tubules diameter; as well as, superoxide dismutase activity were observed in rats treated with Al. Moreover, Al exposure caused significant increments in the lumen diameter of tubules, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells and malondialdehyde (MDA) levels compared to the control group. However, in rats receiving CUR+Al, CUR significantly reversed the adverse effects of Al on testis and sperm quality. No significant differences in follicle-stimulating hormone (FSH) levels and nuclear diameter of spermatogonia were detected among all groups.

Conclusion: It can be concluded that Al causes reproductive dysfunction by creating oxidative damage. CUR, on the other hand, reduces the toxic effects of Al and improves the antioxidant status and sperm quality in male rats.

Keywords: Aluminum, Toxicity, Curcumin, Male Reproductive System, Oxidative Stress

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Introduction

Aluminum (Al) is the most common metallic element and the third most common element in the Earth's crust (1). The ionic form of this metal is detectable not only in all natural waters, but also most types of animal and plant tissues. Due to its

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*Corresponding Address: P.O.Box: 3716146611, Department of Biology, Faculty of Sciences, University of Qom, Ghadir Bolvar, Qom, Iran
Email: e.cheraghi@qom.ac.ir

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reactivity, Al is naturally found in combination with other elements to form compounds such as Al sulfate and chloride (2). Al compounds are extensively used in a wide range of products from household cookware and storage utensils to water purification agents, pharmaceuticals (such as antacids, vaccines, anti-diarrhea drugs, phosphate binders, and allergy immunotherapy injections), food additives, and even toothpastes (3).

The great abundance of Al increases the risk of exposure and related health issues in humans (4). High consumption of Al-containing products will increase the concentration of this metallic element in the consumers' organs and damage their various tissues (including the testicular tissues of humans and animals). Moreover, high levels of Al in spermatozoa and seminal plasma of humans have been reported to reduce sperm viability and motility (5, 6). Krasovskiĭ et al. (7) have confirmed the gonadal toxicity of lead and Al chloride in guinea pigs and rats. Guo et al. (8) have attributed the oxidative damage and testicular toxicity caused by Al to the reduction in testis acetyl cholinesterase (AChE) activity. Chinoy et al. (9) have also found the 30-day consumption of sodium fluoride and Al chloride to cause structural changes in the testis, such as formation of giant cells. Testicular Al accumulation, necrosis of spermatocytes/spermatids, and a significant reduction in fertility were also observed in both male rats and mice (10, 11). Al may cause male reproductive toxicity through various mechanisms such as inducing oxidative stress, interfering with spermatogenesis and steroidogenesis, impairing cell signaling, disrupting the blood-testis barrier, and affecting the endocrine system (12).

In recent years, increasing attention has been paid to the application of nutritional antioxidants (such as herbal products) in diseases related to oxidative stress. The protective effects of herbal products have been attributed to their role as free radical scavengers and antioxidant defense regulators (13). Curcumin (Curcuma longa Lin, CUR), such as the active component of turmeric, can serve as an antioxidant and therapeutic agent without any side effects (14). As a free radical scavenger, CUR can largely inhibit the production of reactive oxygen species (ROS) both *in vitro* and *in vivo*. It also exhibits anti-carcinogenic, anti-inflammatory, and antibacterial properties (15), as well as acts a potent cancer chemopreventive agent (16) and tumor cell proliferation inhibitor (17).

Despite the reported antioxidant properties of CUR (18-21), its effects on apoptosis, oxidative stress and sperm quality in Al-treated rats have not been investigated. Therefore, the present study analyzed the protective effects of CUR on Al-induced damage to the reproductive system of male rats.

Materials and Methods

CUR powder ($C_{21}H_{20}O_6$, Merck & Co. Inc., Germany) was dissolved in dimethyl sulfoxide (DMSO). Al chloride (Merck & Co. Inc., Germany) was diluted with distilled water before administration.

Experimental protocol

In this experimental study, a total of 40 male Wistar rats (240-260 g) were obtained from the animal house of Razi Institute (Iran). Rats were housed in individually ventilated cages on a 12-hour light/dark cycle, temperature of 24 ± 2ºC, with water and food ad libitum. The experimental protocol was approved by the Animal Ethics Committee in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Qom University of Medical Sciences (Qom, Iran).

Over a two-week adaptation period, all rats were fed by a standard pellet diet and closely monitored to ensure normal growth and behavior. The rats were then weighed and randomly allocated to five groups of eight animals (two control groups and three experimental groups) to receive the following treatments for 28 days (22, 23): group I (control group): no injections. Group II (control group): intraperitoneal (IP) injections of only the solvent (distilled water or DMSO). Based on the solvents, we chose two control groups, distilled water and DMSO. Since there were no significant solvents, between the results of the control groups, we considered data from the distilled water group as the control group. Group III (experimental group): IP injections of CUR 10 mg/kg body weight (BW) (22) in 0.2 ml DMSO. Group IV (experimental group): IP injections of Al chloride 10 mg/kg BW (23) in 0.2 ml distilled water. Group V (experimental group): IP injections of CUR+Al chloride at the above-mentioned doses alone.

All groups were fed by a normal diet. After the treatment period, the rats were reweighed that was followed by being euthanized and dissected. Blood samples were collected into heparinized capillary tubes through cardiac puncture. In order to separate the plasma, the samples were poured into clean tubes and centrifuged at 1500 g for 20 minutes at 4°C. Testis and epididymis were detached from the adhering connective tissues, washed in cold physiological saline, and weighed accurately.

Plasma hormone assay

Plasma was obtained and maintained at -20°C until enzyme-linked immunosorbent assay (ELISA) was performed. The concentrations of luteinizing hormone (LH), follicle-stimulating hormone (FSH) and testosterone were determined using ELISA kits (Elabscience Biotechnology Co., Ltd., Germany) according to the manufacturer's instructions. All measurements were carried out in duplicate. The intra- and inter- assay coefficients of variation were less than 10%.

Assessment of lipid peroxidation

Thiobarbituric acid reactive substance (TBARS) levels were determined as a measure of plasma concentrations of malondialdehyde (MDA), the end product of lipid peroxidation (LPO) (24). MDA levels were reported as nmol/ml.

Assessment of superoxide dismutase levels

Superoxide dismutase (SOD) activity in plasma was measured using a commercial assay kit (Cayman Chemical, USA) according to the manufacturer's instructions. This kit utilized a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The SOD assay measured all three types of SOD (Cu/Zn, Mn, and Fe SOD) in U/ml (25).

Sperm parameters

Assessment of sperm motility and count

The right cauda epididymis was incised and semen was pressed on a pre-warmed slide. Two drops of warm 2.9% sodium citrate were added to semen and mixed with a coverslip. The percentage of sperm motility was evaluated visually (magnification: ×40). Motility estimates were performed from three different fields in each sample. The mean of the three successive estimations was used as the final motility score. For sperm count, the left cauda epididymis was incised and the dripped semen was quickly sucked into a red blood pipette to the 0.5 mark. The collected semen was diluted with warm normal saline up to the 101 mark. Approximately 10 µL of the semen mixture was placed on a Neubauer chamber and viewed (magnification: ×40). The total numbers of sperm cells were counted and expressed as 10^6/ml (26).

Assessment of sperm viability and morphology

Eosin/nigrosin staining was used to determine sperm viability (percentage of live spermatozoa). A drop of semen (50 µL) with two drops of the stain (100 µL) was placed on a microscope slide. Thin smears were then prepared and observed under a light microscope (magnification: ×100). While viable sperms remained colorless, nonviable sperms appeared red. The stained and unstained sperm cells were counted. The mean values for each group were then recorded and used in percentage viability calculation. In order to determine the percentage of morphologically abnormal spermatozoa, eosin-nigrosin staining was performed, and the slides were viewed under a light microscope (magnification: ×100). A total of 200 sperm cells were examined on each slide, and the head, tail and total abnormality rates of spermatozoa were expressed as a percentage (27).

Histological analysis

In brief, an abdominal incision was made, while the testes were carefully dissected and fixed in 10% formal-saline. After paraffin embedding, the sections of 5 µm thickness were obtained using a rotary microtome, stained with Heidenhain's Azan, and observed under a light microscope (magnification: ×200) (28).

TUNEL method for analysis of apoptosis

The in-situ DNA fragmentation was visualized by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method. Briefly, dewaxed testis sections were predigested with 20 mg/ml proteinase K for 20 minutes and incubated in phosphate buffered saline (PBS) solution containing 3%

H_2O_2 for 10 minutes to block the endogenous peroxidase activity. The sections were incubated with the TUNEL reaction mixture, fluorescein-d UTP (Roche Applied Science, Germany), for 60 minutes at 37°C, according to the manufacturer's instructions. The slides were then rinsed three times with PBS and incubated with secondary anti-fluorescein-POD-conjugate for 30 minutes. After washing three times in PBS, Hoechst stain (Sigma-Aldrich, USA) was added for chromogenic reaction. As a control for method specificity, the step using the TUNEL reaction mixture was omitted in negative control serial sections, so nucleotide mixture in reaction buffer was used instead. The apoptotic index was determined at 10-random locations within each seminiferous tubule. In all groups, 100 seminiferous tubules for each animal were recorded (29).

Statistical analysis

The normality of continuous variables was confirmed using the Kolmogorov-Smirnov test. Data were reported as mean ± SE and analyzed with one-way analysis of variance (ANOVA) and Tukey's test for post-hoc analysis. $P<0.05$ were considered significant. All analyses were performed with the Statistical Package for the Social (SPSS) for Windows 16.0 (SPSS Inc., USA).

Results

Effects of curcumin and aluminum on sperm characteristics, the testis and body weight

Significant reductions in sperm count (P=0.0001), motility (P=0.0001), viability (P=0.001), and morphology (P=0.001) were detected in rats treated with Al chloride compared to control group. Moreover, sperm parameters were significantly higher in rats treated with CUR alone (P=0.0001) compared to those treated with Al chloride, but this value was the same in control group (P>0.05). Rats treated with CUR+Al chloride had significantly higher sperm count (P=0.001), motility (P=0.006), viability (P=0.001), and morphology (P=0.001) compared to Al-treated rats (Table 1, Figs.1, 2). As Table 1 shows, while the testis and body weights were significantly reduced in Al-treated rats (P=0.001) compared to control group, the values were similar in other groups (P>0.05).

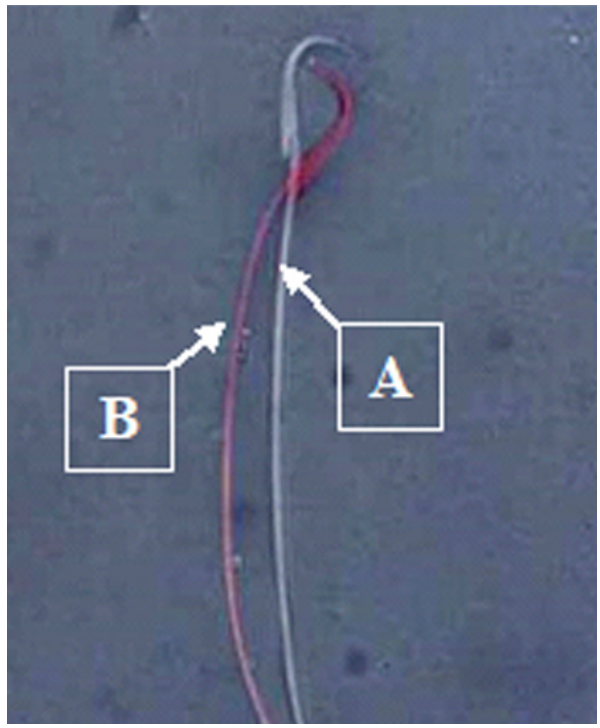

Fig.1: Assessment of sperm viability in rats treated by eosin-nigrosin stain (magnification: ×1000).
A. Alive sperm and **B.** Dead sperm.

Table 1: Changes in body weight, testis weight, sperm count, sperm motility, sperm viability and sperm abnormalities in experimental groups

Parameter	Experimental group				
	Control	Solvent	CUR	Al	CUR+Al
Body weight (g)	276.1 ± 3.1[a]	279.6 ± 3.8[a]	284.4 ± 5.1[a]	245.2 ± 4.2[b]	280.6 ± 5.4[a]
Testis weight (g)	1.36 ± 0.04[a]	1.35 ± 0.05[a]	1.37 ± 0.03[a]	1.09 ± 0.02[b]	1.34 ± 0.04[a]
Sperm count (10^6/ml)	36.22 ± 2.8[ac]	36.02 ± 3.5a[c]	45.4 ± 3.5[a]	17.5 ± 1.1[b]	32.6 ± 1.9[c]
Sperm motility (%)	84.8 ± 1.7[ac]	82.3 ± 3.1a[c]	87.1 ± 1.7[a]	43.9 ± 3.8[b]	68.3 ± 6.3[c]
Sperm abnormalities (%)	12.34 ± 1.5[ac]	12.5 ± 1.4a[c]	9.5 ± 0.7[a]	40.1 ± 3.2[b]	20.4 ± 2.8[c]
Sperm viability (%)	79.8 ± 2.4[a]	78.5 ± 2.7[a]	88.9 ± 1.9[a]	36.3 ± 3.2[b]	60.7 ± 2.7[c]

Data are shown as mean ± SE. Means within the same row with different letters are significantly differed (P<0.05) using ANOVA, Tukey's test. CUR; Curcumin and Al; Aluminum.

Effect of curcumin and aluminum on reproductive hormones

We observed that testosterone and LH levels were significantly lower in Al-treated rats compared to other groups (P=0.01). Testosterone and LH levels were higher in CUR-treated group than all other groups. LH and testosterone levels in CUR+Al treated group were significantly (P=0.04) different from Al-treated group, but this value was not significantly different compared to control and CUR-treated rats (P>0.05, Table 2). There was also no significant differences in FSH levels among the five groups (P>0.05).

Effects of curcumin and aluminum on lipid peroxidation status

Rats treated with Al chloride in comparison with other groups showed significantly increased MDA levels (P=0.01) and significantly decreased SOD activity (P=0.0001). Treatment with CUR+Al chloride resulted in significant improvement of LPO status when compared with Al-treated rats (P=0.001), but this value was not significantly different compared to control and CUR-treated rats (P>0.05, Table 2).

Effects of curcumin and aluminum on the structure of the testes

Al chloride treatment led to degeneration and necrosis with a significant reduction in the diameter of seminiferous tubules and germinal epithelium thickness compared to the control group (P=0.0001). Moreover, the lumen diameter of tubules was significantly higher in Al-treated rats than in the control group (P=0.0001). This effect was milder in rats treated with CUR+Al chloride, while the values in this group were close to those in the control group. The diameter of seminiferous tubules, germinal epithelium thickness and the lumen diameter of tubules were similar in CUR-treated rats and control group (P>0.05, Table 3, Fig.3). In addition, we observed no significant difference in the nuclear diameter of spermatogonia among the groups (P>0.05, Table 3).

Table 2: The changes of FSH, LH, Testosterone, MDA and SOD levels in experimental groups

Parameter	Experimental group				
	Control	Solvent	CUR	Al	CUR+Al
FSH (IU/L)	2.33 ± 0.4[a]	2.35 ± 0.3[a]	2.3 ± 0.4[a]	2.7 ± 0.6[a]	2.13 ± 0.2[a]
LH (IU/L)	2.3 ± 0.42[a]	2.6 ± 0.4[a]	2.8 ± 0.45[a]	0.73 ± 0.07[b]	2.2 ± 0.3[a]
T (ng/ml)	3.7 ± 0.6[a]	3.6 ± 0.7[a]	4.1 ± 0.5[a]	1.3 ± 0.3[b]	3.6 ± 0.7[a]
MDA (nmol/ml)	4.8 ± 0.44[a]	5.01 ± 0.43[a]	4.1 ± 0.53[a]	7.4 ± 0.65[b]	5.16 ± 0.54[a]
SOD (U/ml)	8.26 ± 0.4[a]	8.27 ± 0.33[a]	8.93 ± 0.43[a]	3.26 ± 0.42[b]	7.5 ± 0.43[a]

Data are shown as mean ± SE. Means within the same row with different letters are significantly differed (P<0.05) using ANOVA, Tukey's test. CUR; Curcumin, Al; Aluminum, FSH; Follicle stimulating hormone, LH; Luteinizing hormone, T; Testosterone, MDA; Malondialdehyde, and SOD; Superoxide dismutase.

Table 3: The changes of histopathology on rat testis in the experimental groups

Parameter	Experimental group				
	Control	Solvent	CUR	Al	CUR+Al
The diameter of seminiferous tubules (μ)	181.27 ± 0.8[a]	181.77 ± 0.6[a]	184.32 ± 1.5[a]	157.19 ± 1.2[b]	180.66 ± 1.3[a]
The lumen diameter of tubules (μ)	77.02 ± 1.5[a]	77.03 ± 1.4[a]	74.48 ± 1.45[a]	98.93 ± 0.73[b]	78.74 ± 0.82[a]
The nuclear diameter of spermatogonia (μ)	4.71 ± 0.01[a]	4.70 ± 0.01[a]	4.75 ± 0.02[a]	4.69 ± 0.01[a]	4.70 ± 0.01[a]
Germinal epithelium thickness (μ)	56.29 ± 0.4[a]	55.16 ± 0.8[a]	57.39 ± 0.6[a]	36.46 ± 0.6[b]	51.56 ± 0.9[a]

Data are shown as mean ± SE. Means within the same row with different letters are significantly differed (P<0.05) using ANOVA, Tukey's test. CUR; Curcumin and Al; Aluminum.

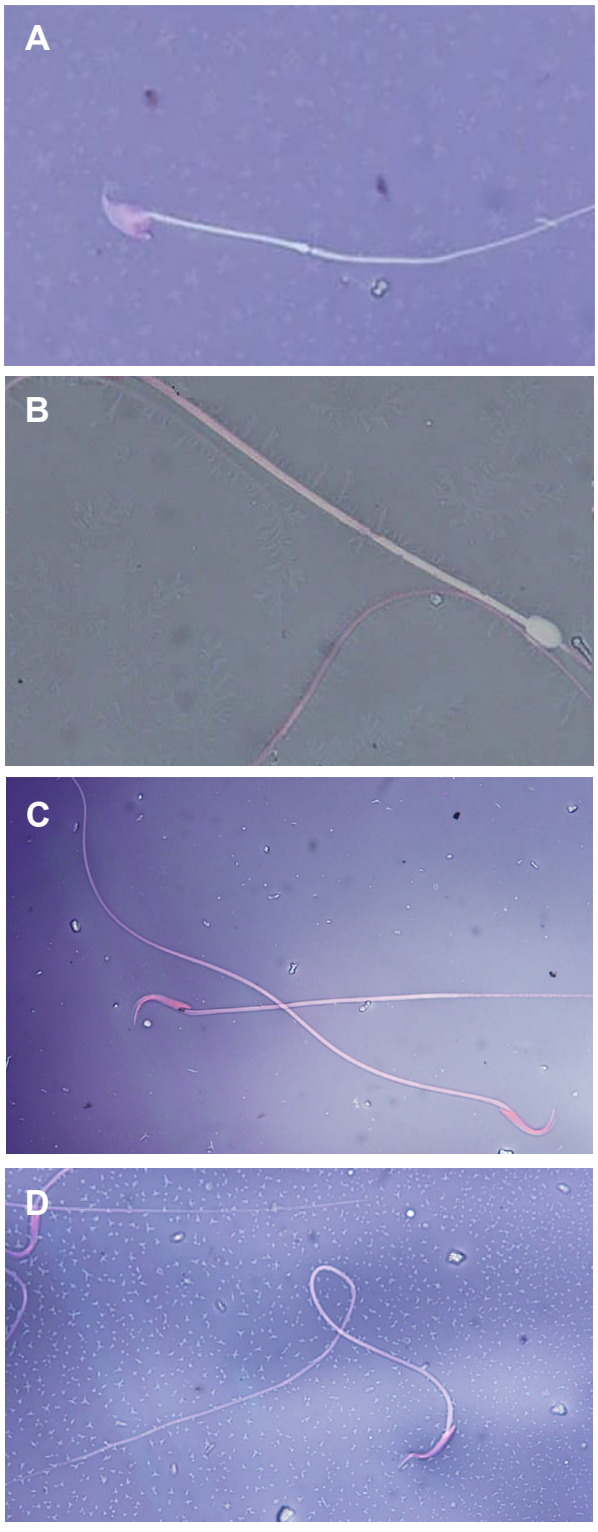

Fig.2: Some of abnormal sperm tail and head morphology after aluminum exposure by eosin-nigrosin stain (magnification: ×1000). **A.** Amorphous head, **B.** Cytoplasmic droplet, **C.** Amorphous mid-piece and tail, and **D.** Coiled or curled tail.

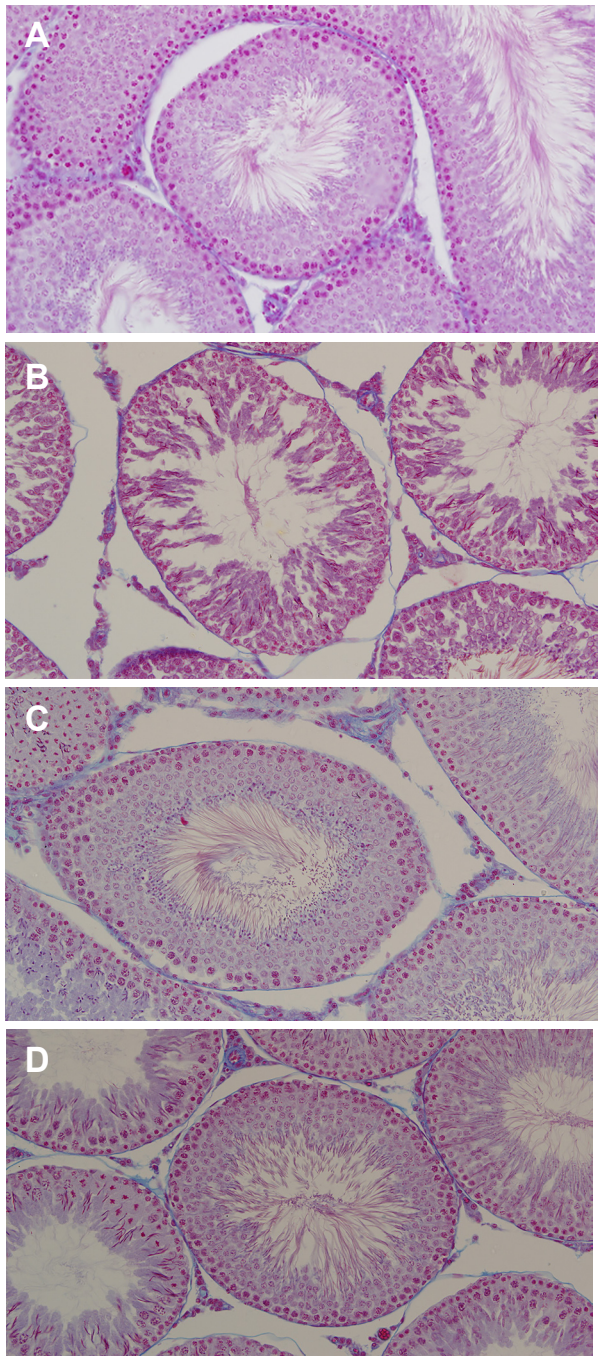

Fig.3: Photomicrographs of transverse sections in the testis (Heiden hain's azan stain, 5 µm sections, magnification: ×100). **A.** Testis of control group showing normal histological structure of seminiferous tubules, **B.** Testis of aluminum group showing an increase in the lumen diameter of tubules, a decrease in the diameter of seminiferous tubules as well as distorted seminiferous tubules with loss of normal distribution of epithelial lining and vacuolar cytoplasm (black arrow), **C.** Testis of curcumin group showing no histological changes, and **D.** Testis of CUR+Al group revealed no histopathological changes.

Effects of curcumin and aluminum on apoptosis

Rats treated with Al chloride showed significantly increased TUNEL-positive cells (P=0.001) in comparison with other groups. Treatment with CUR+Al chloride resulted in significant increase the number of TUNEL-positive cells when compared with Al-treated rats(P=0.01, Figs.4, 5). Moreover, the number of apoptotic cells were significantly decreased in rats treated with CUR alone (P=0.0001) as compared to those treated with Al alone and CUR+Al groups, but this value was not significantly different compared to control group (P>0.05).

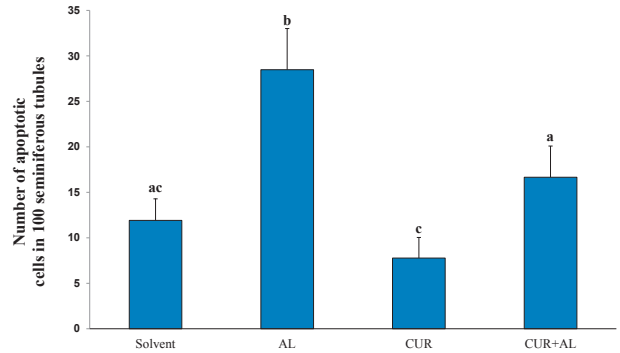

Fig.4: Photomicrograph of the different groups showing the number of apoptotic cells in 100 seminiferous tubules following TUNEL staining. Values are expressed as mean ± SE (n=8). Values bearing different superscript on the bar diagram vary significantly (P<0.05) using one way ANOVA and Tukey's test. Al; Aluminum and CUR; Curcumin.

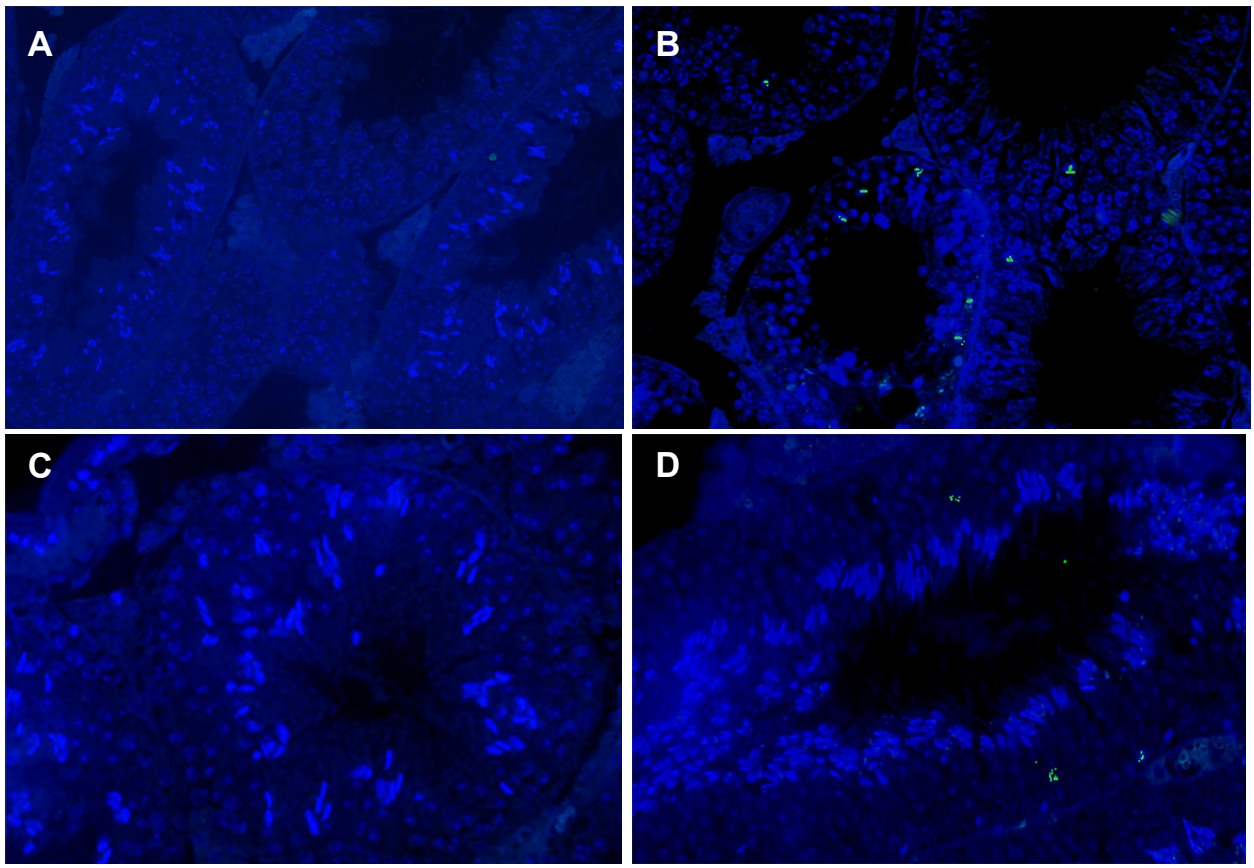

Fig.5: Effects of CUR and Al on the number of apoptotic cells by fluorescence microscope (magnification: ×400). **A.** Solvent (control), **B.** Treated with Al, **C.** Treated with CUR, and **D.** Treated with CUR+Al. Compared with the control group, the number of apoptotic cells were significantly increased in Al group. The apoptotic of cells (green) can be recognized. Al; Aluminum and CUR; Curcumin.

Discussion

This study evaluated the toxic effects of Al chloride in male rat and showed that CUR had the capability to contrary Al toxicity. The present study confirmed reductions in body and testis weight following Al exposure. This is similar to previous research (6). However, the administration of CUR either alone or in combination with Al chloride could maintain testis weight at values close to the control group. This is also in accordance with previous studies (18, 21, 22). In accordance with previous findings (30), we observed significantly lower LH and testosterone levels in the Al-treated group (compared to the control group). On the other hand, since LH and testosterone levels were significantly higher in rats treated with CUR+Al than in those exposed to Al chloride alone, CUR could effectively improve sex hormone levels and decreases the harmful effects of Al. Comparable findings were also reported by previous research (31, 32). However, in present study, the FSH values remained unchanged in all groups. In contrary, in a study, by Al-Nahari and Al Eisa (33) they have showed that Al injection was significantly decreased the rate of FSH. This different is probably due to the differences in dose and duration of administration.

Al might induce such a reduction in testosterone levels by blocking calcium channels and hence down- regulating gonadotrophins secretion in the hypophysis (34). Al exposure can also suppress steroidogenesis by increasing testicular nitric oxide concentrations and decreasing cyclic adenosine monophosphate (cAMP) (10). Previous studies showed that Al injection in rat hypocamp was significantly decreased the rate of glutamate (35). Probably blocked voltage sensitive calcium cannels (VSCCs) in cells are responsible for gonadotropin-releasing hormone (GnRH) synthesis, affecting calcium influx in those cells, and decreased the GnRH secretion. Since FSH and LH secretion is promoted by FSH-releasing hormone (FSHRH) and LH-releasing hormone (LHRH) factors which are produced in separated zones in hypothalamus nucleoli, it is probable that Al inhibited LHRH production in hypothalamus, but did not effect on FSHRH production (35). In our study, rate of FSH was not affected, which may be due to the FSH synthesis mechanism that is different from LH and not affected by calcium ion.

Based on our findings, Al exposure decreased sperm quality. Likewise, previous studies documented reduced sperm count, motility, and viability following Al treatment (36, 37). Furthermore, the alteration in antioxidant system, a decrease in cAMP and an increases in nitric oxide production caused by Al treatment might have been responsible for the observed reductions in sperm motility and viability and increased morphological abnormality (12). LH stimulates the interstitial cells of the Leydig to secrete testosterone (34). Therefore, a reduction in LH and testosterone levels in the present study, which are critical to spermatogenesis, following Al exposure can justify the reduced sperm count in Al-treated rats. In our study, CUR treatment significantly improved morphological normality and sperm count, motility, and viability in rats receiving Al chloride. In other words, CUR could counteract the negative effects of Al in the mentioned- reproductive parameters. Comparable results were reported by Salama and El-Bahr (19), Sharma and Singh (21), Jalili et al. (22), Al-Nahari and Al Eisa (33).

In agreement with previous research (31), the results of this study showed that Al increased MDA level (well-known LPO indicator) and reduced SOD activity. SOD protects spermatozoa against spontaneous O_2 toxicity and LPO. Several reports have suggested that $AlCl_3$ may inhibit the activity of SOD. Since ROS have been indicated to have a role in steroidogenesis and gametogenesis (12), the mentioned effects might have been responsible for the reduced reproductive hormones and poor sperm quality seen in Al-treated rats. The reduction in sperm counts and sex organ weights following Al exposure in the present study can confirm the role of Al toxicity in increased oxidative stress and reinforce the role of ROS. Meanwhile, CUR has been shown to affect several targets in cells for its biological activity, while it reduced LPO and enhanced antioxidant levels in rats (13). CUR exhibits protective effects against oxidative damage by decreasing the levels of free radicals, through its free radical scavenging activity, particularly against oxygen radicals, which inhibit sulfhydryl (SH)-group oxidation. It inhibits nuclear factor kappa B (NF-kB) activity, cyclooxygenase-2 (COX-2), and mitogen-activated protein kinase (MAPK) expression, while it modulates release of several cytokines and testicular enzyme activities,

mRNA expression of 17 β-hydroxysteroid dehydrogenase (17β-HSD) and cytochrome P450 side-chain cleavage (CYP450scc) enzyme in steroidogenesis (13). It seems that CUR raises testosterone and LH levels and increases the count and motility of normal sperms in treated groups through enhancing the anti-oxidant defense by increasing the expression of anti-oxidant genes in comparison with Al-treated rats.

Al cytotoxicity may be mediated by free radicals derived from this element and its capability to induce apoptosis through a wide variety of mechanisms including production of ROS, LPO, cell membrane damage, down regulation of Bax gene expression, diminished activity of alkaline phosphatase and cAMP reduction in various tissues (12, 38). In agreement with previous research (38), the results of this study showed that Al increased the amount of apoptotic cells compared with control. However, a number of studies have suggest that CUR, due to inhibition of NF-κB activation and cell scattering, can be considered as a potential therapeutic agent effective against apoptotic genes to promote cell death and proliferative processes (39). Assessment of apoptotic cells in the seminiferous tubules in the testes of rats treated with CUR+Al showed a significant reduction in the amount of apoptotic cells compared with Al group. Comparable results were reported by Aktas et al. (40). Therefore, we reported that Al induces oxidative stress and apoptosis in testicular cells, and that CUR as antioxidant prevents apoptosis induced by Al.

Histopathological analysis in the current study indicated testicular structures to be different in Al-treated rats with other groups. In fact, the Al-treated group had thinner germinal epithelium and very low spermatid and sperm counts in the lumen. Similar findings have also been reported by Guo et al. (10) and Kutlubay et al. (41). This observation could be attributed to the ability of Al to cause oxidative stress, cross the blood-testis barrier, promote lipid peroxidation, and ultimately damage the biological membrane of the testis. The low sperm count, motility, and viability, as well as the high morphological abnormality, seen in Al-treated rats confirm the mentioned mechanism. On the other hand, the protective effect of CUR on the testis may be demonstrated that it inhibits cellular damage and apoptosis occurring as a result of oxidative stress in the spermatogenic cells of seminiferous tubules and Leydig cells (13). Chandra et al. (42) have reported CUR to maintain normal serum testosterone levels and prevent the reduction in sex organ weights following chromium exposure. In a study on male Wistar rats, Sharma and Singh have highlighted the beneficial effects of CUR on decreasing the reproductive toxicity caused by lindane (organochlorine pesticide) (21). The protective effects of CUR have been attributed to its role in regulating LPO and boosting the antioxidant defense system. More specifically, CUR significantly decreased the levels of free radicals (through its free radical scavenging activity), induced the production of detoxification enzymes, and provided protection against degenerative diseases (43). The findings of the present study suggested that CUR treatment protected the cellular structure of the testes by increasing the formation of antioxidant products and decreasing LPO.

Conclusion

The results of this study highlighted the protective effects of CUR on male reproductive toxicity of Al in an experimental rat model. CUR, as powerful antioxidant, was able to reduce Al-induced damage and improve sperm quality by decreasing oxidative stress. We believe that further research on the utility of CUR may indicate its usefulness as a potential treatment for spermatogenesis after testicular injury caused by Al treatment in rats.

Acknowledgements

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References

1. Camargo MM, Fernandes MN, Martinez CB. How aluminium exposure promotes osmoregulatory disturbances in the neotropical freshwater fish Prochilus lineatus. Aquat Toxicol. 2009; 94(1): 40-46.
2. Verstraeten SV, Aimo L, Oteiza PI. Aluminium and lead: molecular mechanisms of brain toxicity. Arch Toxicol. 2008; 82(11): 789-802.
3. Karimpour Malekshah A, Torabizadeh Zh, Naghshwar F. Developmental toxicity of aluminum from high doses of AlCl3 in mice. J Appl Res. 2005; 5(4): 575-579.
4. Zhang K, Zhou Q. Toxic effects of Albased coagulants on Brassica chinensis and Raphanus sativus growing in acid and neutral conditions. Environ Toxicol. 2005; 20(2): 179-187.

5. Yousef MI, Kamel KI, El-Guendi MI, El-Demerdash FM. An in vitro study on reproductive toxicity of aluminium chloride on rabbit sperm: the protective role of some antioxidants. Toxicology. 2007; 239(3): 213-223.
6. Guo CH, Lu YF, Hsu GS. The influence of aluminum exposure on male reproduction and offspring in mice. Environ Toxicol Pharmacol. 2005; 20(1): 135-141.
7. Krasovskiĭ G, Vasukovich LY, Chariev OG. Experimental study of biological effects of leads and aluminum following oral administration. Environ Health Perspect.1979; 30: 47-51.
8. Guo CH, Huang CJ, Chiou YL, Hsu GS. Alteration of trace element distribution and testis ACE activity in mice with high peritoneal aluminum. Biol Trace Elem Res. 2002; 86(2): 145-157.
9. Chinoy N, Momin R, Jhala DD. Fluoride and aluminium induced toxicity in mice epididymis and its mitigation by vitamin C. Fluoride. 2005; 38(2): 115-121.
10. Guo CH, Lin CY, Yeh MS, Hsu GS. Aluminum-induced suppression of testosterone through nitric oxide production in male mice. Environ Toxicol Pharmacol. 2005; 19(1): 33-40.
11. Sharma S, Sharma RK, Sharma R, Sharma A, Rai A, Gupta R, et al. Synthesis and characterisation of some new aluminium derivatives of Schiff bases containing N, O and S donor atoms and the anti fertility activity of the derivative Al [SC6H4N:C(CH3)CH2COCH3]3. Bioinorg Chem Appl. 2003: 215-225.
12. Pandey G, Jain GC. A Review on toxic effects of aluminium exposure on male reproductive system and probable mechanisms of toxicity. International Journal of Toxicology and Applied Pharmacology. 2013; 3(3): 48-57.
13. Gupta SC, Patchva S, Koh W, Aggarwal BB. Discovery of curcumin, a component of golden spice, and its miraculous biological activities. Clin Exp Pharmacol Physiol. 2012; 39(3): 283-299.
14. Duvoix A, Blasius R, Delhalle S, Schnekenburger M, Morceau F, Henry E, et al. Chemopreventive and therapeutic effects of curcumin. Cancer Lett. 2005; 223(2): 181-190.
15. Ak T, Gülçin İ. Antioxidant and radical scavenging properties of curcumin. Chem Biol Interact. 2008; 174(1): 27-37.
16. Aggarwal BB, Kumar A, Aggarwal MS, Shishodia Sh. Curcumin derived from turmeric (Curcuma longa): a spice for all seasons. Bagchi D, Preuss HG, editors. Phytopharmaceuticals in cancer chemoprevention. 1st ed. CRC Press; 2005; 351-387.
17. Joe B, Vijaykumar M, Lokesh BR. Biological properties of curcumin-cellular and molecular mechanisms of action. Crit Rev Food Sci Nutr. 2004; 44(2): 97-111.
18. El-Wakf AM, Elhabiby EM, El-kholy WM, El-Ghany EA. Use of tumeric and curcumin to alleviate adverse reproductive outcomes of water: Nitrate pollution in male rats. Nature and Science. 2011; 9(7): 229-239.
19. Salama AF, El-Bahr SM. Effect of curcumin on cadmium-induced oxidative testicular damage in rats. JMRI. 2007; 28(2): 167-173.
20. Gouda SG, Khalil MS, Naim MM. Curcumin protects against testicular damage and genotoxicity induced by acrylamide in male albino mice. The Egyptian Journal of Histology. 2011; 34(2): 333-345.
21. Sharma P, Singh R. Protective role of curcumin on lindane induced reproductive toxicity in male Wistar rats. Bull Environ Contam Toxicol. 2010; 84(4): 378-384.
22. Jalili C, Khani F, Salahshoor MR, Roshankhah S. Protective effect of curcumin against nicotine-induced damage on reproductive parameters in male mice. Int J Morphol. 2014; 32(3): 844-849.
23. Abubakar MG, Taylor A, Ferns GA. Aluminium administration is associated with enhanced hepatic oxidant stress that may be offset by dietary vitamin E in the rat. Int J Exp Pathol. 2003; 84(1): 49-54.
24. Esteribauer H, Cheeseman KH. Determination of aldehdic lipid peroxidation products: malonaldehyde and 4-hydroxyonenal. Methods Enzymol. 1990; 186: 407-421.
25. Giannopolitis CN, Ries SK. Superoxide dismutases: I. Occurrence in higher plants. Plant physiol. 1977; 59(2): 309-314.
26. Bearden HJ, Fuquay JW. Semen evaluation. In: Bearden HJ, Fuquay JW, editors. Applied animal reproduction. Prentice Hall, Upper Saddle River: New Jersey; 2000; 168-182.
27. Kvist U, Bjorndahl L. Manual on basic semen analysis. ESHRE Monographs. Oxford: Oxford University Press; 2002.
28. Soleimani Mehranjani M, Noorafshan A, Momeni HR, Abnosi MH, Mahmoodi M, Anvari M, et al. Stereological study of the effects of vitamin E on testis structure in rats treated with para-nonylphenol. Asian J Androl. 2009; 11(4): 508-516.
29. Negoescu A, Lorimier P, Labat-Moleur F, Drouet C, Robert C, Guillermet C, et al. In situ apoptotic cell labeling by the TUNEL method: improvement and evaluation on cell preparations. J Histochem Cytochem. 1996; 44(9): 959-568.
30. Shahraki MR, Palan MJ. Effect of aluminium on testosterone hormones in male rat. J Med Sci. 2006; 6(2): 296-299.
31. Yousef MI, Salama AF. Propolis protection from reproductive toxicity caused by aluminium chloride in male rats. Food Chem Toxicol. 2009; 47(6): 1168-1175.
32. Abd El-Rahman NS, Al-Ahmary SH. Effect of sapogenin extract on anti-infertility induced by aluminium chloride in male rats. Adv Med Plant Res. 2013; 1(4): 77-85.
33. Al-Nahari H, Al Eisa R. Effect of turmeric (Curcuma longa) on some pituitary, thyroid and testosterone hormone against aluminum chloride (Al [Cl. sub. 3]) induced toxicity in rat. Adv Environ Biol. 2016; 10(4): 250-261.
34. Platt B, Büsselberg D. Actions of aluminum on voltage-activated calcium channel currents. Cell Mol Neurobiol. 1994; 14(6): 819-829.
35. Marubayashi U, Wen HY, McCann SM. Median eminence lesions reveal separate hypothalamic control of pulsatile follicle-stimulating hormone and luteinizing hormone release. Proc Soc Exp Biol Med. 1999; 220(3): 139-146.
36. Yousef MI, El-Morsy AM, Hassan MS. Aluminium-induced deterioration in reproductive performance and seminal plasma biochemistry of male rabbits: protective role of ascorbic acid. Toxicology. 2005; 215(1-2): 97-107.
37. Buraimoh AA, Ojo SA, Hambolu JO, Adebisi SS. Histological study of the effects of aluminium chloride exposure on the testis of wistar rats. American International Journal of Contemporary Research. 2012; 2(5): 114-122.
38. Savory J, Herman MM, Ghribi O. Intracellular mechanisms underlying aluminum-induced apoptosis in rabbit brain. J Inorg Biochem. 2003; 97(1): 151-154.
39. Akram M, Uddin Sh, Ahmed A, Usmanghani K, Hannan A, Mohiuddin E, et al. Curcuma longa and curcumin: a review article. Rom J Biol-Plant Biol. 2010; 55(2): 65-70.
40. Aktas C, Kanter M, Erboga M, Ozturk S. Anti-apoptotic effects of curcumin on cadmium-induced apoptosis in rat testes. Toxicol Ind Health. 2012; 28(2): 122-130.
41. Kutlubay R, Oğuz EO, Can B, Güven MC, Sinik Z, Tuncay OL. Vitamin E protection from testicular damage caused by intraperitoneal aluminium. Int J Toxicol. 2007; 26(4): 297-306.
42. Chandra AK, Chatterjee A, Ghosh R, Sarkar M. Effect of curcumin on chromium-induced oxidative damage in male reproductive system. Environ Toxicol Pharmacol. 2007; 24(2): 160-166.
43. Kalpana C, Menon VP. Modulatory effects of curcumin on lipid peroxidation and antioxidant status during nicotine-induced toxicity. Pol J Pharmacol. 2004; 56(5): 581-586.

Original Article

Effect of Grape Seed Extract on Lipid Profile and Expression of Interleukin-6 in Polycystic Ovarian Syndrome Wistar Rat Model

Zohreh Salmabadi, M.Sc.[1], Homa Mohseni Kouchesfahani, Ph.D.[1*], Kazem Parivar, Ph.D.[1], Latifeh Karimzadeh, M.Sc.[2]

1. Department of Animal Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran
2. Animal Center Laboratories and Cellular and Molecular Research Laboratory, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran

Abstract

Background: Polycystic ovary syndrome (PCOS) is a common but complex endocrine disorder and is the major cause of anovulation and consequent subfertility. In this study the effect of grape seed extract (GSE) on triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholestrol (HDL-C), low-density lipoprotein-cholestrol (LDL-C) and interleukin-6 (IL-6) in PCOS Wistar rats were assessed.

Materials and Methods: In this experimental study, 84 adult female Wistar rats were divided into 7 groups (n=12) including control (intact), Sham (estradiol valerate solvent injection), control PCOS and 4 experimental PCOS groups. To induce the syndrome, a single subcutaneous injection of 2 mg estradiol valerate was applied. In experimental groups, PCOS rats were treated with different doses of 50, 75, 100 and 200 mg/kg body weight (BW) GSE by intraperitoneal injection for 10 consecutive days. After harvesting blood serum, TG was measured by Glycerol-3-phosphate Oxidase-Peoxidase (GPO-PAP), TC by Cholesterol Oxidase-Peroxidase (CHOD-PAP), and HDL-C by sedimentation method, LDL-C by Friedwald calculation and IL-6 by ELISA method. The serum values of each parameter were analyzed using one-way ANOVA at $P \leq 0.05$.

Results: In all experimental groups significant decrease of visceral fat was obvious as compared with control PCOS group. LDL-C, TC and IL-6 levels in experimental groups, particularly at dose of 50 mg/kg of GSE, were significantly decreased as compared with PCOS group. However, HDL-C levels were not significantly changed.

Conclusion: : According to the findings of this study, it can be concluded that GSE with its effects on serum TC, LDL-C and IL-6 could reduce the effects of dyslipidemia and inflammation in PCOS rats and improve systemic symptoms of PCOS.

Keywords: Dyslipidemia, Grape Seed Extract, IL-6, Polycystic Ovarian Syndrome, Wistar Rat

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Introduction

One of the most common endocrine disorders in women is polycystic ovarian syndrome (PCOS), affecting about 5 to 10% of women of reproductive age (15 to 45 years old) (1). PCOS was first described in 1935 by Stein and Leventhal (2). PCOS is a heterogeneous disease with a spectrum of endocrine protests such as polycystic ovarian morphology, ovarian follicular theca cell hyperplasia, chronic anovulation, menstrual disturbances and infertility. Common

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*Corresponding Address: P.O.Box: 15815/3587, Department of Animal Biology, Faculty of Biological Sciences, Kharazmi University, Tehran, Iran
Email: kouchesfehani@yahoo.com

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metabolic symptoms associated with this disease are obesity, hyperandrogenism, resistance to insulin and cardiovascular disorders. Women with PCOS demonstrate many features similar to metabolic syndrome, including dysfunction of the hypothalamic-pituitary-adrenal (HPA) axis, hyperinsulinemia, increase in cytokines and fat-derived factors and dyslipidemia (3).

Dyslipidemia in PCOS is characterized by increased triglycerides (TG) and decreased high density lipoprotein-cholesterol (HDL-C) (4). The classic criteria of atherogenic lipoprotein profile, characterized by elevated TG-rich lipoproteins, lower HDL levels, and higher low density lipoproteina (LDL)/HDL ratios, is the most distinctive characteristics of PCOS women, especially the obese ones (3). Besides conjunction of PCOS symptoms with those associated with metabolic syndrome, there is some evidence to present PCOS as a pro-inflammatory state (5). Blood levels of inflammatory markers, such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and C-reactive protein (CRP), are higher in women with PCOS than in controls matched for body mass index (BMI) and age (6). Most studies have reported a close relationship between levels of the inflammatory markers and insulin resistance/obesity, particularly central obesity (7). The location of *IL-6* gene in humans is on the short arm of chromosome 7, and in mice it is on the proximal region of chromosome 5 (8). IL-6, a major proinflammatory cytokine, is produced in a variety of tissues, including activated leukocytes, adipocytes, and endothelial cells (9).

Grape is a plant growing throughout the world, and its ingredients and properties have been widely examined. One of the most abundant ingredients of grapes are phenolic compounds which are present in large amounts (10). Grape seed is one of the richest sources of polyphenols (11), which exhibit antioxidant, free radical scavenging properties, and lipid lowering effects (12). The most common polyphenols of grape seeds are procyanidins ranging in size from monomers to long-chain polymers, such as catechin, epicatechin, and procyanidin B2 (13). Because of its different properties, grape seed extract (GSE) has been proposed to be a good nominee for decreasing metabolic and cardiovascular changes related to obesity and metabolic disorders (14). United States Food and Drug Administration (FDA) in 2011 recognized grape seed and skin extracts to be safe, due to their health food ingredients (15). Relying on the fact that the antioxidant effects of GSE are 20 times greater than vitamin E and 50 times greater than vitamin C, the aim of this study was to determine the impacts of GSE on lipid profile and one of the main inflammatory markers, IL-6, in PCOS rat model.

Materials and Methods

Grape seed extract preparation

Red grape (Vitis vinifera) was obtained from the city of Arak (Iran) and then washed and dried. Seeds were separated from grapes and were ground in a grinder (Shimaz, Iran). The powdered grape seeds (75 g) were added to 200 ml of 70% ethanol and were maintained in incubator (Fanazmagostar, Iran) for 24 hours at 40°C, rotated daily for three hours on a rotator device at 200 rpm and filtered by a filter paper Whatman No. 1. The solvent was then removed using a rotary evaporator (Hoilph, German). This procedure was repeated three times, and all collected samples were kept at -20°C. Shortly before each experiment 50, 75, 100 and 200 mg/kg of the dry extract was dissolved in 0.9% normal saline as solvent (Cytomatin gene, Iran).

Animals

In this experimental study, 84 female Wistar rats weighing 160 ± 20 g were used. Animals were kept in the animal maintenance and breeding center of Kharazmi University, in special cages under appropriate environmental conditions and desired temperature of 20-24°C, in 12-hours light/dark cycles and with free access to food and water. Rats with a 2-3 regular estrous cycles during the twelve to fourteen days of vaginal smear, and in the estrous phase of their reproductive cycle were chosen for experiments. To induce PCOS phenotype a variety of hormonal and non-hormonal techniques exist including treatments with testosterone, estradiol valerate (EV), dehydroepiandrosterone (DHEA), adrenocorticotropic hormone (ACTH) or long-term use of light. In this study hormonal induction of PCOS by EV (Aburaihan Co., Iran) was used. Rats were divided into 7 groups (n=12) including control (intact), sham (estradiol valerate solvent injection), control PCOS and 4 experimental PCOS groups. PCOS was induced by a single subcutaneous injection of 2 mg EV. Sham group received a similar dose of sesame oil as a solvent of EV and the control group had no

injections. Successful induction of the syndrome was achieved by eight weeks showing symptoms such as irregular estrous cycle and occurrence of the persistent vaginal cornification (PVC) phase. After ensuring that the syndrome was induced, PCOS rats were divided into 5 groups, named as control PCOS group, and 4 experimentals (n=12 each). The experimental groups received 50, 75, 100 and 200 mg/kg body weight (BW) GSE by intraperitoneal injections for 10 consecutive days. Five days after the last injection, rats of all groups were sacrificed with carbon dioxide inhalation and their blood was taken off from left ventricle and serum samples were separated by centrifugation at 6,000 rpm for five minutes. Samples were stored at -20°C prior to examining the expression of IL-6 and lipid profile.

Lipid profile measurements

After 10 hours of fasting, 5 ml of rat blood was collected in sterile bottles and allowed to clot for about an hour at 37°C. Then serum was separated and stored at -20°C. The serum level of TG were evaluated by the Glycerol-3-phosphate Oxidase-Peoxidase (GPO-PAP), as End Point Assay. Total cholesterol (TC) by Cholesterol Oxidase-Peroxidase (CHOD-PAP) and the HDL-C level was determined after lipoproteins were precipitated .The LDL-C level was by the Friedewald's equation (16).

VLDL=TG/5

LDL=TC-HDL-VLDL

IL-6 assay

The amount of IL-6 in blood serum was measured by ELISA using a rat IL-6 platinum ELISA kit (Bender Medsystems, Austria). The sensitivity of the assay for IL-6 was 12 pg/mL.

Statistical analysis

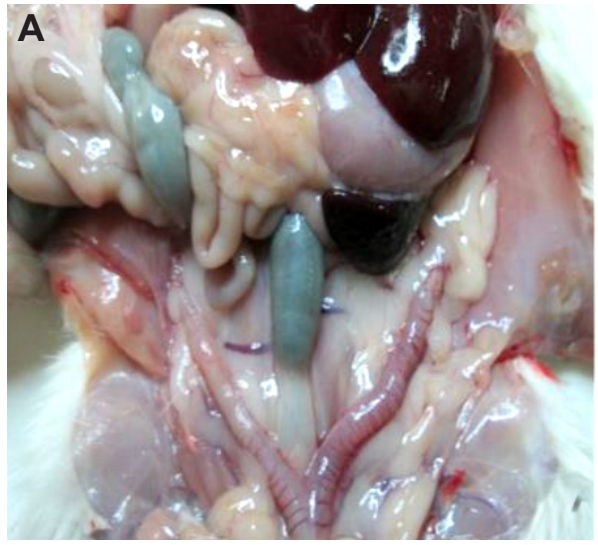
All data were presented as mean ± SE. Statistical significance was evaluated with one-way analysis of variance (ANOVA) using SPSS18. Significant differences between groups were measured using Tukey tests. $P \leq 0.05$ was considered significant and relevant histograms were drawn by the EXCEL program.

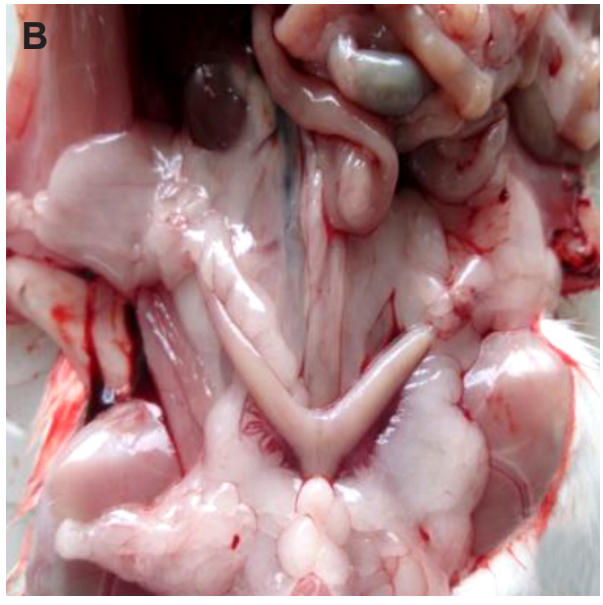
Ethical considerations

All research animals were treated in compliance with the guidelines for the care and use of animals approved by our institutions in accordance with the principles of laboratory animal care (NIH Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, Washington, D.C.) (code: No. 616.18)

Results

In all 4 experimental PCOS groups treated with GSE, notable reduction of visceral fat was observed as compared to sham, control and control PCOS groups (Fig.1). In the PCOS group treated with 200 mg/kg GSE, a severe inflammation of abdominal cavity and intensive changes in the appearance of liver and abdominal cavity was observed, indicating the destructive effects of the high dose applied.





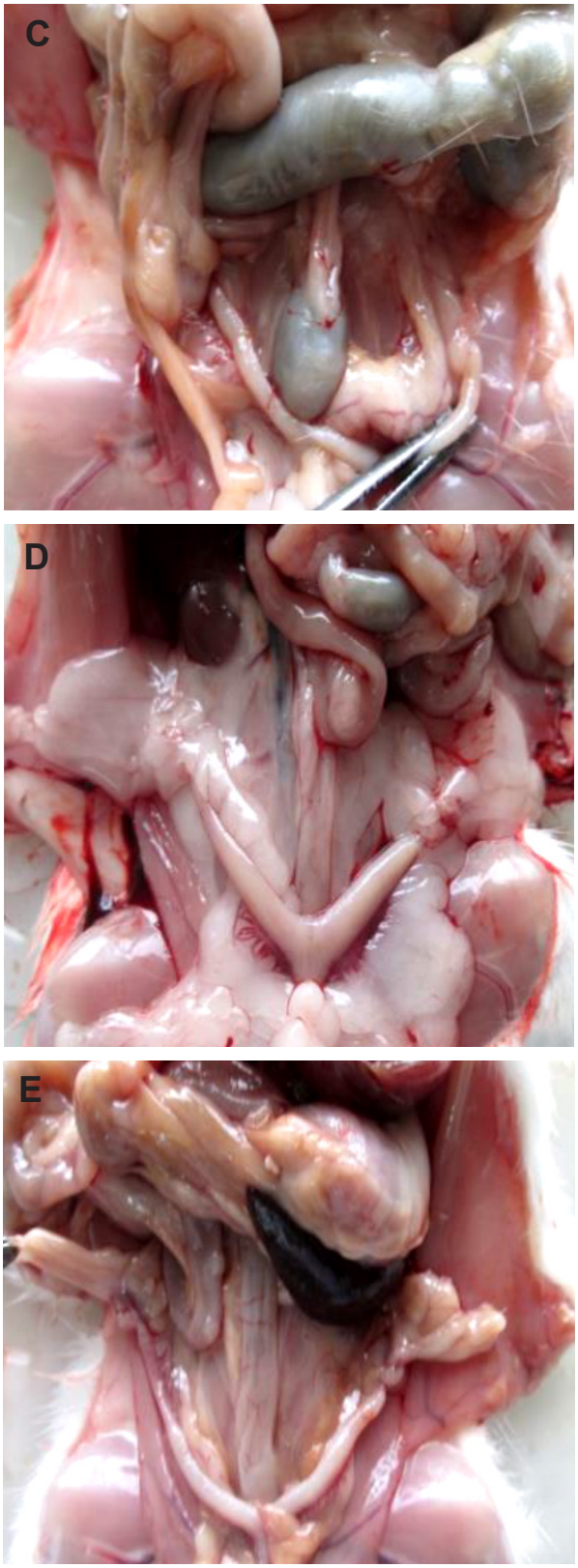


Fig.1: Ovary morphology showing decrease in visceral fat in the grape seed extract (GSE) treated groups compared with control and polycystic ovary syndrome (PCOS). Fat tissue in the abdominal cavity, particularly around the uterus and ovaries decreased in PCOS treatment groups. **A.** Control group, **B.** PCOS group, **C.** PCOS group treated with 50 mg/kg GSE, **D.** PCOS group treated with 75 mg/kg GSE, **E.** PCOS group treated with 100 mg/kg GSE, and **F.** PCOS group treated with 200 mg/kg GSE.

As shown in Figure 2, a significant increase in the serum levels of TC, TG and LDL-C, but not HDL-C, was observed in PCOS group as compared to the sham and control groups ($P \leq 0.05$).

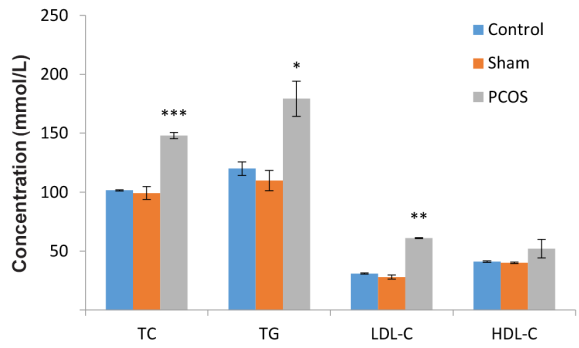


Fig.2: Comparison of lipid profile levels in polycystic ovary syndrome (PCOS) group as compared to the sham and control groups. The serum level of TC, TG and LDL-C in PCOS compared with sham and control groups have shown significant increases.
TC; Total cholestrol, TG; Triglyceride, LDL-C; Low-density lipoprotein-cholestrol, HDL-C; High density lipoprotein-cholestrol, *; $P<0.05$, **; $P<0.01$, and ***; $P<0.001$ compared with control and sham (treated with 0.9% normal saline).

Concentration of TC was significantly lowered in the GSE50 and GSE200 groups as compared with control PCOS group. On the other hand, TG plasma concentration was significantly decreased in the GSE100 and GSE200 groups compared to the control PCOS group. LDL-C level was significantly reduced in the 75 and 200 mg/kg GSE as compared with control PCOS group (P≤0 .001). Comparison of HDL-C levels did not show significant differences between GSE treated groups and PCOS group (Fig.3).

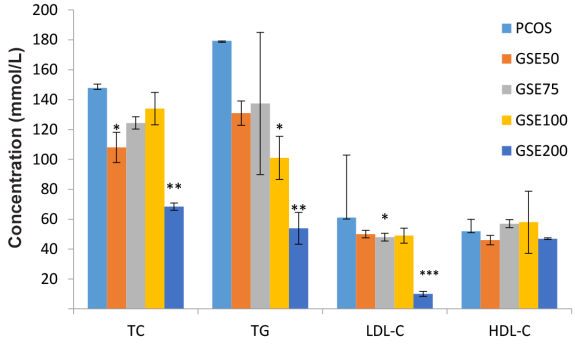
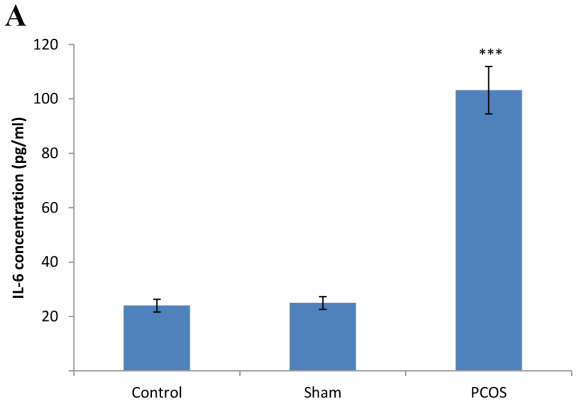


Fig.3: Comparison of the lipid profile levels in grape seed extract (GSE) treated groups with polycystic ovary syndrome (PCOS) group. Lipid profile showed decrease in GSE groups compared with PCOS.
TC; Total cholestrol, TG; Triglyceride, LDL-C; Low density lipoprotein-cholestrol, HDL-C; High density lipoprotein-cholestrol, GSE50; PCOS treated with a dose of 50 mg/kg GSE, GSE75; PCOS treated with a dose of 75 mg/kg GSE, GSE100; PCOS treated with a dose of 100 mg/kg GSE, GSE200; PCOS treated with a dose of 200 mg/kg GSE, *; P<0.05, **; P<0.01, and ***; P<0.001 compared with PCOS.

In Figure 4A a significant increase in the amount of IL-6 in control PCOS group compared to the control and Sham groups was observed (P<0.001). By comparing PCOS groups treated with different doses of GSE with control PCOS group, a significant decrease in IL-6 level was observed (Fig.4B).



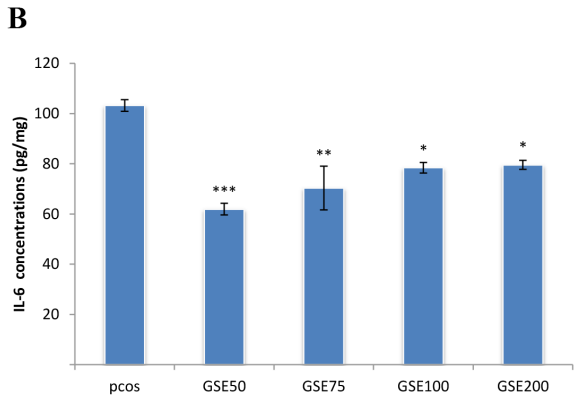


Fig.4: Comparison of interleukin-6 (IL-6) levels between groups. **A.** Serum interleukin-6 (IL-6) concentrations showed significant increase in polycystic ovary syndrome (PCOS) group compared to the control and sham groups and **B.** Comparison of IL-6 levels in grape seed extract (GSE) treated groups with PCOS group. A significant reduction in GSE treated groups were observed as compared with PCOS group.
GSE50; PCOS treated with a dose of 50 mg/kg GSE, GSE75; PCOS treated with a dose of 75 mg/kg GSE, GSE100; PCOS treated with a dose of 100 mg/kg GSE, GSE200; PCOS treated with a dose of 200 mg/kg GSE, *; P<0.05, **; P<0.01, and ***; P<0.001.

Discussion

PCOS is related to various patterns of dyslipidemia including reduced HDL-C, high levels of TG, TC and LDL-C (17). Our analysis of serum lipids showed increase in the TC, TG and LDL-C levels after the induction of PCOS by EV. Abdominal fat accumulation has been observed in about half of PCOS patients (18). Obesity is a classic characteristic of PCOS, with 30-60% of patients being overweight to some degree (19). Increased abdominal fat has been linked to insulin resistance and increased cardiovascular risk. Because many patients with PCOS present abdominal obesity, it may be the cause of insulin resistance seen in PCOS (20). In the present study, similar to previous studies, an increase in external visceral fat in PCOS rats was observed. IL-6 modulates the action of aromatase, a key regulatory enzyme for estrogen metabolism (21); The release of IL-6 into the systemic circulation and the fact that this release is greater in obese subjects support a possible novel role for IL-6 as a systemic regulator of BW (an adipostat) and a regulator of lipid metabolism. IL-6 receptors are present in the hypothalamus, which also supports the idea that this cytokine has direct central actions (22).

Dyslipidemia, type 2 diabetes and cardiovas-

cular disorders and the link between these conditions has been assumed to be chronic inflammation. Visceral obesity has been defined as a state of low-grade inflammation because visceral adipose tissue is able to produce cytokines (TNF-α, IL-6, and IL-1), chemokines (IP-10, IL-8, IL-18, monocyte chemotactic protein-1 (MCP-1), and regulated on activation normal T expressed and secreted (RANTES), and other adipokines, free fatty acid (FFA), plasminogen activator-1 (PAI-1, leptin, resistin, visfatin, and adiponectin) that act, directly or indirectly, as mediators of systemic inflammation (23). Linscheid et al. (24) showed that adipose tissue emerged as an important source of pro-inflammatory mediators including TNF-α, IL-6, and procalcitonin (ProCT). Results of IL-6 in the present study are in accordance with that of Kershaw and Linscheid.

Studies of Charradi et al. (14) showed that GSE is a safe anti-obesity and cardioprotective agent that should also have potential benefits in other inflammatory damaging conditions like stroke. Epidemiological studies report an inverse association between GSE consumption and mortality from cardiovascular diseases (25). In numerous studies, flavonoids and their derivatives have been reported to reduce LDL oxidation in both humans and animal models (26). Studies using flavonoids have also shown reductions in plasma lipids and multiple effects on lipoprotein metabolism (27). GSE prevents the differentiation of adipocytes *in vitro* (28). In the present study, all doses of 50, 75, 100, 200 mg/kg GSE decreased visceral fat in the treated rats. However, with lower doses of (50, 75 mg/kg) GSE, appearance of the ovary tissue was normal. Measuring the granulosa layer thickness in PCOS groups treated with GSE revealed significant increase as compared with the control PCOS group. The diameter of theca layer of antral follicles in PCOS groups treated with GSE at doses of 50 and 75 mg/kg showed significant decrease as compared with the control PCOS group.

We have previously shown that in doses of 50, 75, 100 mg/kg of GSE, the number of small follicles, antral and Graafian follicles, and in all 4 doses the number of corpus luteum has significantly increased, indicating a dramatic improvement in the polycystic ovaries (29). Since GSE at a dose of 200 mg/kg caused remarkable visceral inflammation, accumulation of fluid in the peritoneal cavity and severe damages to various organs (especially the liver), at this dose it was considered as toxic, and two doses of 50 and 75 mg/kg GSE due to their improving effects on systemic PCOS symptoms were considered as effective doses. Grape seeds possess cardioprotective effects by alleviating inflammatory conditions and reducing oxidative stress (30). Besides the free radical scavenging and antioxidant activity, pro-anthocyanidins exhibit vasodilatory, anti-carcinogenic, anti-allergic, anti-inflammatory, anti-bacterial, cardioprotective, immune stimulating, anti-viral and estrogenic activities, as well as being inhibitors of the enzymes phospholipase A2, cyclooxygenase and lipooxygenase (31).

Schewe et al. (32) showed that GSE has anti-inflammatory properties. Terra et al. (33) showed that orally ingested GSE helps preventing imbalanced cytokine patterns. Polyphenols in GSE could therefore be responsible for an anti-inflammatory effect in experimental studies (34). Terra et al. (33) demonstrated that induction of IL-6, CRP, and TNF-α expressions by high fat diet were reduced by adding procyanidins extract to the diet. They also showed that procyanidins reduced macrophage level. So, the inhibition of the cytokine expression in adipose tissue might be due to a decrease in the number of macrophages, but procyanidins mayalso directly affect the pro-inflammatory pathways in both adipocytes and macrophages. In any case, these findings demonstrate the potential effects of procyanidins on such low-grade inflammation-related diseases as obesity.

In an *in vitro* study by Moreno, GSE showed the inhibitory effects on fat metabolizing enzymes-pancreatic lipase and lipoprotein lipase activities and on lipolysis of 3T3-L1 murine adipocytes. This inhibiting activity suggests that GSE might be useful as a treatment to limit dietary fat absorption and the accumulation of fat in adipose tissue (35). Grape seeds contain numerous polyphenols including resveratrol and quercetin and have been used in an effort to treat conditions that comprise metabolic syndrome. Pigs fed by Resveratrol at a dose of 100 mg/kg per day for 7 weeks had lower serum glucose, cholesterol, LDL, systolic blood pressure, and BMI (36). In the present study, after induction of PCOS, an increase in the visceral fat and expression of IL-6 in animals was occurred,

while by using GSE, the level of IL-6, a marker of inflammation, has been significantly reduced.

Conclusion

According to the results of this study, it can be concluded that treatment with GSE causes significant decrease in visceral fat, cholesterol, TG, LDL-C and IL-6. Since the adipose tissue produces the IL-6, treating with GSE might be helpful for reducing adipose tissue, which is the main source of IL-6. By lowering the levels of IL-6, cholesterol, TG, LDL-C, dyslipidemia and inflammatory symptoms of PCOS will be improved.

Acknowledgements

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References

1. Franks S. Polycystic ovary syndrome. N Engl J Med. 1995; 333(13): 853-861.
2. Stein IF, Leventhal ML. Amenorrhoea associated with bilateral polycystic ovaries. Am J Obstet Gynecol. 1935; 29(2): 181-191.
3. Swetha R. Study of serum lipoprotein and lipid profile in polycystic ovarian syndrome. Bangalore: Ravi; 2013.
4. Berneis K, Rizzo M, Lazzaroni V, Fruzzetti F, Carmina E. Atherogenic lipoprotein phenotype and low-density lipoproteins size and subclasses in women with polycystic ovary syndrome. J Clin Endocrinol Metab. 2007; 92(1): 186-189.
5. Manneras Holm L. Polycystic ovary syndrome. Studies of metabolic and ovarian disturbances and effects of physical exercise and electro-acupuncture. Sweden: Hylte; 2010.
6. Ruan X, Dai Y. Study on chronic low-grade inflammation and influential factors of polycystic ovary syndrome. Med Princ Pract. 2009; 18(2): 118-122.
7. Puder JJ, Varga S, Kraenzlin M, De Geyter C, Keller U, Muller B. Central fat excess in polycystic ovary syndrome: relation to low-grade inflammation and insulin resistance. J Clin Endocrinol Metab. 2005; 90(11): 6014-6021.
8. Helnrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. Biochem J. 1990; 265(3): 621-636.
9. Stith RD, Luo J. Endocrine and carbohydrate responses to interleukin-6 in vivo. Circ Shock. 1994; 44(4): 210-215.
10. Rockenbach II, Gonzaga LV, Rizelio VM, Goncalves AS, Genovese MI, Fett R. Phenolic compounds and antioxidant activity of seed and skin extracts of red grape (Vitis vinifera and Vitis labrusca) pomace from Brazilian winemaking. Food Res Int. 2011; 44(4): 897-901.
11. da Silva JMR, Rigaud J, Cheynier V, Cheminat A, Moutonet M. Procyanidin dimers and trimers from grape seeds. Phytochemistry.1991; 30(4): 1259-1264.
12. Moreno DA, Ilic N, Poulev A, Brasaemle DL, Fried SK, Raskin I. Inhibitory effects of grape seed extact on lipases. Nutrition. 2003; 19(10): 876-879.
13. Cai Y, Yu Y, Duan G, Li Y. Study on infrared-assisted extraction coupled with high performance liquid chromatography (HPLC) for determination of catechin, epicatechin, and procyanidin B2 in grape seeds. Food Chem. 2011; 127(4): 1872-1877.
14. Charradi K, Sebai H, Elkahoui S, Ben Hassine F, Limam F, Aouani E. Grape seed extract alleviates high-fat diet-induced obesity and heart dysfunction by preventing cardiac siderosis. Cardiovasc Toxicol. 2011; 11(1): 28-37.
15. Charradi K, Elkahoui S, Karkouch I, Limam F, Hamdaoui G, Hassine F, et al. Grape seed and skin extract alleviates high-fat diet-induced renal lipotoxicity and prevents copper depletion in rat. Appl Physiol Nutr Metab. 2013; 38(3): 259-267.
16. Goyal S, C V, K S, Ch L. Serum lipid profile in patients with oral tobacco habits and oral precancer lesions and conditions. WebmedCentral ORAL MEDICINE. 2013; 4(2): WMC004034.
17. Talbott E, Clerici A, Berga SL, Kuller L, Guzick D, Detre K, et al. Adverse lipid and coronary heart disease risk profiles in young women with polycystic ovary syndrome: results of a case-control study. J Clin Epidemiol. 1998; 51(5): 415-422.
18. Faloia E, Canibus P, Gatti C, Frezza F, Santangelo M, Garrapa G, et al. Body composition, fat distribution and metabolic characteristics in lean and metabolic characteristics in lean and obes women with polycystic ovary syndrome. J Endocrinol Invest. 2004; 27(5): 424-429.
19. Dunaif A, Mandeli J, Flhur H, Dobrjansky A. The impact of obesity and chronic hyperinsulinemia on gonadotropin release and gonadal steroid secretion in the polycysti c ovary syndrome. J Clin Endocrinol Metab. 1988; 66(1): 131-139.
20. Carmina E, Bucchieri S, Esposito A, Del Puente A, Mansueto P, Orio F, et al. Abdominal fat quantity and distribution in women with polycystic ovary syndrome and extent of its relation to insulin resistance. J Clin Endocrinol Metab. 2007; 92(7): 2500-2505.
21. Purohit A, Ghilchik MW, Duncan L, Wang DY, Singh A, Walker MM, et al. Aromatase activity and interleukin-6 production by normal and malignant breast tissues. J Clin Endocrinol Metab. 1995; 80(10): 3052-3058.
22. Mohamed-Ali V, Goodrick S, Rawesh A, Katz DR, Miles JM, Yudkin JS, et al. Subcutaneous adipose tissue releases interleukin-6, but not tumor necrosis factor-a, invivo. J Clin Endocrinol Metab. 1997; 82(12): 4196-4200.
23. Repaci A, Gambineri A, Pasquali A. The role of low-grade inflammation in the polycystic ovary syndrome. Mol Cell Endocrinol. 2011; 335(1): 30-41.
24. Linscheid P, Seboek D, Schaer DJ, Zulewski H, Keller U, Muller B. Expression and secretion of procalcitonin and calcitonin gene-related peptide by adherent monocytes and by macrophage-activated adipocytes. Crit Care Med. 2004; 32(8): 1715-1721.
25. Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol Rev. 2000; 52(4): 673-751.
26. Hertog MG, Feskens EJ, Kromhout D. Antioxidant flavonols and coronary heart disease risk. Lancet. 1997; 349(9053): 699.
27. Borradaile NM, De Dreu LE, Barrett PHR, Huff MW. Inhibition of hepatocyte apo B secretion by naringenin: enhanced rapid intracel-lular degradation independent of reduced microsomal cholesteryl esters. J Lipid Res. 2002; 43: 1544-1554.
28. Baiges I, Palmfeldt J, Blade C, Gregersen N, Arola L. Lipogenesis is decreased by grape Seed proanthocya-

nidins according to liver proteomics of rats fed a high fat diet. Mol Cell Proteomics. 2010; 9(7): 1499-1513.
29. Mohseni Kouchesfahani H, Parivar K, Salmabadi Z. Effect of hydroalcoholic grape seed extract (Vitis vinifera L.) on polycystic ovarian syndrome in female Wistar rat. Journal of Cell and Tissue. 2015; 6(2): 153-164.
30. Sato M, Bagchi D, Tosaki A, Das DK. Grape seed proanthocyanidin reduces cardiomyocyte apoptosis by iinhibiting ischemia/ reperfusion-induced activation of JNK-1 and C-J UN. Free Radic Biol Med. 2001; 31(6): 729-737.
31. Rice-Evans CA, Miller NJ, Paganda G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med. 1996; 20(7): 933-956.
32. Schewe T, Kuhn H, Sies H. Flavonoids of cocoa inhibit recombinant human 5-lipoxygenase. J Nutr. 2002; 132(7): 1825-1829.
33. Terra X, Pallares V, Ardevol A, Blade C, Fernandez-Larrea J, Pujadas G, et al. Modulatory effect of grape-seed procyanidins on local and systemic inflammation in diet-induced obesity rats. J Nutr Biochem. 2011; 22(4): 380-387.
34. Danesh J, Wheeler JG, Hirschfield GM, Eda S, Eiriksdottir G, Rumley A, et al. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. N Engl J Med. 2004; 350(14): 1387-1397.
35. Arora P, Ansari SH, Nazish I. Bio-functional aspects of grape seeds-a review. International Journal of Phytomedicine. 2010; 2(3): 177-185.
36. Robich MP, Chu LM, Chaudray M, Nezafat R, Han Y, Clements RT, et al. Anti-angiogenic effect of high-dose resveratrol in a swine model of metabolic syndrome. Surgery. 2010; 148(2): 453-462.

Original Article

Predicting Implantation Outcome of *In Vitro* Fertilization and Intracytoplasmic Sperm Injection Using Data Mining Techniques

Pegah Hafiz, M.Sc.[1], Mohtaram Nematollahi, Ph.D.[2*], Reza Boostani, Ph.D.[3], Bahia Namavar Jahromi, M.D.[4, 5]

1. Department of Medical Informatics, School of Management and Medical Informatics, Shiraz University of Medical Sciences, Shiraz, Iran
2. Anesthesiology and Critical Care Research Center, Shiraz University of Medical Sciences, Shiraz, Iran
3. Department of Computer Science and Engineering and Information Technology, School of Electrical and Computer Engineering, Shiraz University, Shiraz, Iran
4. Department of Obstetrics and Gynecology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
5. Infertility Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Background: *In vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are two important subsets of the assisted reproductive techniques, used for the treatment of infertility. Predicting implantation outcome of IVF/ICSI or the chance of pregnancy is essential for infertile couples, since these treatments are complex and expensive with a low probability of conception.

Materials and Methods: In this cross-sectional study, the data of 486 patients were collected using census method. The IVF/ICSI dataset contains 29 variables along with an identifier for each patient that is either negative or positive. Mean accuracy and mean area under the receiver operating characteristic (ROC) curve are calculated for the classifiers. Sensitivity, specificity, positive and negative predictive values, and likelihood ratios of classifiers are employed as indicators of performance. The state-of-art classifiers which are candidates for this study include support vector machines, recursive partitioning (RPART), random forest (RF), adaptive boosting, and one-nearest neighbor.

Results: RF and RPART outperform the other comparable methods. The results revealed the areas under the ROC curve (AUC) as 84.23 and 82.05%, respectively. The importance of IVF/ICSI features was extracted from the output of RPART. Our findings demonstrate that the probability of pregnancy is low for women aged above 38.

Conclusion: Classifiers RF and RPART are better at predicting IVF/ICSI cases compared to other decision makers that were tested in our study. Elicited decision rules of RPART determine useful predictive features of IVF/ICSI. Out of 20 factors, the age of woman, number of developed embryos, and serum estradiol level on the day of human chorionic gonadotropin administration are the three best features for such prediction.

Keywords: *In Vitro* Fertilization, Intracytoplasmic Sperm Injection, Clinical Decision Support, Data Mining

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Introduction

Assisted reproductive technologies (ART) include all treatments that are used for *in vitro* handling of human oocytes and sperms or of the embryos to establish a pregnancy (1). Infertility is defined as a couple's inability to conceive after 12 months of regular unprotected intercourse (2). Among ART treatments, *In vitro* fertilization (IVF) and intracytoplasmic sperm

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*Corresponding Address: P.O.Box: 7187687599, Anesthesiology and Critical Care Research Center, Shiraz University of Medical Sciences, 5th Floor, Mohammad Rasool Allah Research Tower, Khalili Avenue, Shiraz, Iran
Email: mnemat@sums.ac.ir

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injection (ICSI) are well-known methods for infertility treatment. The process of IVF involves ovarian stimulation, oocyte retrieval, fertilization, embryo culture, and transferring embryos to the uterus (3). ICSI is another treatment used for infertile couples that includes injection of a selected sperm into the oocyte cytoplasm (4).

IVF and ICSI have almost similar variables in terms of demographical and clinical features. The latest study in Iran (5) demonstrates that the total average rate of infertility is about 10.9% of the population. This study states that among patients of several infertility clinics in the country, 78.4% had primary and 21.6% had secondary fertility factors. The results yield 34.0% of the average percentage for male factor, 43.5% for female factor, 17.1% for both factors, and 8.1% for unexplained infertility. Ovulatory dysfunction was the most frequent etiologic factor among female causes in that study.

Today, many couples suffering from infertility try ART to have a baby and ask about the probability of pregnancy due to several reasons. Firstly, due to the high cost of IVF and ICSI treatments in Iran, some couples cannot afford the cost of these treatments. Next, the probability of conception is 20 to 25% in a normal reproductive cycle (3), which by ART increases to about 30-40% in each cycle; however, it is still considered to be low. Lastly, ART consists of multiple steps that are time consuming and difficult to tolerate by infertile women. There are also three main clinical causes that make predicting pregnancy outcome necessary. First, there are many prognostic factors to this treatment that determine the chance of conceiving, which in turn make the decision difficult for clinicians. Second, using previous cases for this decision seems to be reliable, while it is a time-consuming task for clinicians. And last, there might be an alternative method to IVF and ICSI that a specialist proposes to couples with a very low chance of pregnancy, such as adoption, that causes them to call off infertility treatments. Data mining (DM) refers to using machine learning, pattern recognition, and statistical techniques to extract knowledge from data, in this case, patient information, and is a specific step in the process of knowledge discovery in databases (KDD) (6). In medical DM, classification system predicts the class to which the patient belongs by learning a model based on input dataset. Since DM methods perform data analysis and elicit valuable information from data, clinical obstetricians and gynecologists may use such information for diagnosis and treatment (7). According to Cios and Moore (8), medical DM can be beneficial for patients when finding a solution to analyze various types of clinical data.

In this study, five well-known classification techniques in DM are applied to our dataset along with 5-fold cross validation (CV) for training and testing. The main purpose of this research was to choose the best predictive model for calculating the probability of IVF/ICSI success for each couple, using a comparative study among various classifiers. Furthermore, we aimed to find the most effective factors for prediction of ART success in infertile couples. Note that classical predictive models could be used in this study; however, the methods used here are limited to DM approach to examine the effectiveness of artificial intelligence on the subject. In addition, DM discovers patterns from data and considers computational efficiency comparing to classical predictive models. There are several studies performed to predict IVF outcomes (9-12), where different methods have been used to predict IVF success with accuracies from 60.6% (9) to 84.4% (11).

In another similar study, unlike the attempts that solely consider accuracy, Güvenir et al. (11) utilized the area under ROC curve (AUC) as the performance criterion since it is practical in evaluating quality of the algorithm. Our dataset has 17 variables in common with the study of Guh et al. (10). Some of the features, like the information about the first and second stage culture medias, were not documented in our infertility center. In the study of Güvenir et al. (11), 19 variables similar to our database were used. Some of the variables such as anemia, which were used in their study were not considered as predictive features of IVF/ICSI by our infertility specialist as predictive features of IVF/ICSI, and therefore, were not used in our study. Finally, another similar study conducted by Chen et al. (12) used 9 variables in common with our dataset. The only variable that our infertility specialists considered a significant predictive feature, which was not seen in previous studies, was the number of gonadotropin ampules that were used for our patients.

Materials and Methods

A dataset of 486 labeled records along with 29

variables was gathered belonging to Infertility Research Center of Mother-and-Child Hospital in Shiraz, Iran, from 2009 to 2015. Each patient signed a consent form at the time of admission to the hospital and before entering the study. This study was approved by Ethics Committee of Shiraz University of Medical Sciences. The type of this study is cross-sectional and the method of sampling is census. This dataset contained 131 positive and 355 negative implantations. As far as the number of negative samples outnumbers positive ones, this dataset is highly imbalanced. Required variables for this study were extracted from paper-based medical records by our trained staff. In order to use these records for computer models, data entry process was performed. In this study, frozen embryo implantation results were excluded and only fresh embryo transfer was considered due to the diversity of some features between these two transferring methods. The name, type, and value of IVF/ICSI attributes are summarized (Table 1).

Table 1: IVF/ICSI attributes of our dataset

Attribute name	Attribute type	Attribute value
Age of woman	Numeric	18-47
Age of man	Numeric	23-70
Body mass index	Numeric	14.53-45.78
Secondary fertility	Text	Yes, no
Tubal factor	Text	Yes, no
Pelvic factor	Text	Yes, no
Ovulatory factor	Text	Yes, no
Uterine factor	Text	Yes, no
Male factor	Text	Yes, no
Infertility duration	Numeric	1-27
Experience of IVF treatment	Text	Yes, no
Sperm count	Numeric	0-513 (in million)
Sperm morphology	Numeric	0-95%
Sperm motility	Numeric	0-85%
Follicle stimulating hormone	Numeric	0.099-51.7
Anti-mullerian hormone	Numeric	0.01-93.93
Antral follicle counts	Numeric	2-57
Number of gonadotropin ampoules	Numeric	8-110 (in 75 units)
Number of follicles in ultrasound	Numeric	1-35
Serum E2 level on the day of hCG administration	Numeric	0.95-32840.8
Number of retrieved oocytes	Numeric	0-44
Number of oocytes of GV quality	Numeric	0-8
Number of oocytes of MI quality	Numeric	0-8
Number of oocytes of MII quality	Numeric	0-27
Type of treatment	Text	IVF, ICSI
Embryo grade	Text	A, B, C, D
Number of developed embryos	Numeric	0-26
Embryo transfer day	Numeric	2,3,4
Number of transferred embryos	Numeric	0-6

IVF; *In vitro* **fertilization, ICSI; Intracytoplasmic sperm injection, hCG; Human chorionic gonadotropin, E2; Estradiol, GV; Germinal vesicle, MI; Metaphase I, and MII; Metaphase II.**

Preparation of raw data is one of the most important steps in knowledge discovery. The importance of data preparation is discussed by Zhang et al. (13). This study asserts that almost 80% of the total efforts were spent on preparing data. The patients' records had missing values in some features; therefore, the power of classifiers declined in some cases. The most common methods in literature are case deletion, mean imputation, median imputation, and k-nearest neighbor (kNN) imputation (14).

Since the attributes with missing values in our dataset had skewed distribution, the missing values of numerical features are replaced with median and categorical attributes are filled with the mode of their corresponding column. Support vector machines (SVM), recursive partitioning (RPART), random forest (RF), Adaptive boosting (Adaboost), and 1NN are the state-of-art techniques employed in this research for intelligent decision making. These models are compared to each other for choosing the best option in order to predict IVF/ ICSI, as well as obtaining the probability of each decision rule. For implementation of the mentioned classifiers, we used R 3.2.3. and a five-fold stratified CV is utilized for the validation phase. K-fold CV (15) is a common technique for performance evaluation which reports the average output for classifiers. Since ROC is a good criterion for imbalance datasets, the AUC of ROC is selected as the performance measure instead of accuracy. Visualization of ROC curves is used frequently as performance graphing approach in medical decision making (16). Finally, sensitivity, specificity, positive predictive values (PPV), negative predictive values (NPV), positive likelihood ratio (LR+), and negative likelihood ratio (LR-) are also calculated (17).

Results

We applied the processed samples to each classifier to calculate AUC and accuracy over 5-fold CV, and represented them as mean values (Table 2). Each experiment is repeated 20 times to examine a comprehensive combination of data samples. The average over these experiments for each classifier is reported besides standard deviation. In addition, specificity, sensitivity, PPV, NPV, LR+, and LR- are also calculated for each classifier (Table 3). Our findings suggest that RF and RPART outperform other classifiers in terms of specificity, PPV, and NPV. RPART predicts positive cases better than RF; however, negative cases are classified by RF better than RPART. The higher value of PPV in RF is due to the lower number of false positives. Seemingly, the higher number of NPV in RPART is because of the lower number of false negatives in confusion matrices of both models. Adaboost has generally better values especially in terms of sensitivity comparing to SVM, and 1NN. While the specificity of SVM is 88.73% and higher than 1NN, its value for specificity (14.5%) is very low. Interestingly, given a positive pregnancy, the high positive likelihood ratio of RF shows a large increase in the likelihood of pregnancy, and the corresponding value for RPART implies a moderate increase. However, the rest of the models result in minimal increases. The negative likelihood ratios of all classifiers, which are almost between 0.5 and 1, represent minimal decrease in the probability of pregnancy.

Table 2: Experimental results of applying SVM, Adaboost, RPART, RF, and 1NN on our dataset. All values are rounded to two digitis after the decimal

	AUC (%)	Accuracy (%)
SVM	57.57 ± 1.51	68.3 ± 1.05
Adaboost	47.52 ± 4.5	66.99 ± 2.85
RPART	82.05 ± 2.34	83.56 ± 0.99
RF	84.23 ± 0.91	83.96 ± 0.62
1NN	50 ± 0	64.84 ± 1.46

SVM; Support vector machines, RPART; Recursive partitioning, RF; Random forest, 1NN; One-Nearest-Neighbor, Adaboost; Adaptive boosting, and AUC; Areas under the ROC curve.

Table 3: Sensitivity, specificity, PPV, NPV, LR+, and LR- of RF, RPART, Adaboost, SVM and 1NN for models. All values are rounded to two digits after the decimal

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	LR+	LR-
RF	48.85	98.03	90.14	83.86	24.78	0.52
RPART	59.54	91.83	72.90	86.02	7.29	0.44
Adaboost	54.96	70.42	40.68	80.91	1.86	0.64
SVM	14.5	88.73	32.20	73.77	1.29	0.96
1NN	35.88	73.52	33.33	75.65	1.35	0.87

PPV; Positive predictive values, NPV; Negative predictive values, LR+; Positive likelihood ratio, LR-; Negative likelihood ratio, RF; Random forest, RPART; Recursive partitioning, SVM; Support vector machines, 1NN; One-Nearest-Neighbor, and Adaboost; Adaptive boosting.

Among all tested classifiers in this study, RPART leads to the most usable information besides the probability of IVF/ICSI success. Therefore, we present the significance of the 20 features of IVF/ICSI using RPART (Table 4). The second column shows the scores of each feature. Note that only 11 features have specific values for positive pregnancy because these features were significant in RPART decision making. The other 9 variables are not considered in predicting IVF/ICSI outcome, as they did not have specific values for positive pregnancy. Figure 1 shows ROC curves for predictive models, using all of the data samples. As it is apparent, RF and RPART have higher AUC comparing to Adaboost, SVM, and 1NN, and the curve of SVM is closer to the top two classifiers than 1NN and Adaboost.

Table 4: Importance of IVF/ICSI variables using RPART

Variable	Score	Values for positive pregnancy
Age of woman	14	<38
Number of developed embryos	13	>3 and <16
Serum E2 level	12	<1040 and ≥1780
Embryo grade	9	A, B and C
Sperm motility	9	≥62%
Type of treatment	5	ICSI
Sperm count	5	>4.5 million
Embryo transfer day	4	3 and 4 days
AFC	4	<10
Infertility duration	3	<7.5 years
AMH	3	≥1.2
Number of transferred embryos	3	Not specific
Number of retrieved oocytes	3	Not specific
Number of Gonadotropin ampules	3	Not specific
Sperm morphology	3	Not specific
FSH	2	Not specific
Male factor	2	Not specific
Age of man	1	Not specific
Number of follicles	1	Not specific
Ovulatory factor	1	Not specific

IVF; *In vitro* fertilization, ICSI; Intracytoplasmic sperm injection, RPART; Recursive partitioning, E2; Estradiol, AFC; Antral follicle counts, AMH; Anti-Mullerian hormone, and FSH; Follicle stimulating hormone.

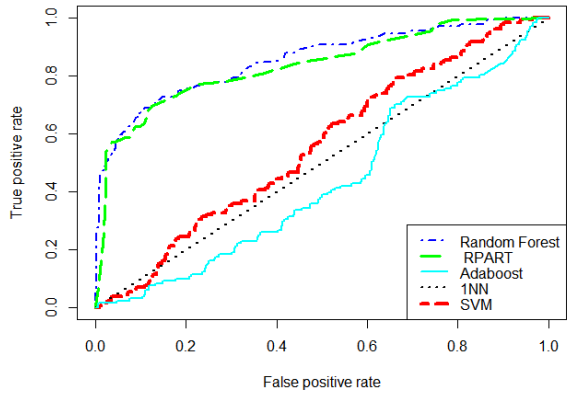

Fig.1: Receiver operating characteristic curves of all classifiers.

Discussion

DM methods used in this research involved a learning process, which utilizes previous IVF/ICSI records to predict the outcome of a new test case. This property improves the decision making of the physicians using previous cases. The low probability of success for a test case obtained by applying DM methods is practical for domain experts to prevent couples from choosing IVF/ICSI treatments. SVM, on the other hand, is suitable for binary classification tasks. It has been employed in many artificial intelligence fields, such as medical diagnosis. Since medical datasets are naturally imbalanced, SVM boundary will be biased in favor of the class with higher population, hence unsatisfactory results of SVM model obtained in this experiment are expected. KNN is a simple non-parametric distance-based method used in many applications. The complexity of kNN is highly dependent on the number of attributes and instances (18). In a study by Japkowicz and Stephen (19) the low performance of kNN when facing imbalanced dataset is demonstrated. Furthermore, kNN performance can be declined in noisy environments since the neighbors of each input take the decision about its label.

Although Adaboost is a strong ensemble learner that can construct a flexible boundary between the classes, it highly suffers from high sensitivity to noisy samples. This deficiency is due to the learning process of Adaboost in which learning of weak learners is performed sequentially; therefore, outlier and noisy samples are boosted in successive iterations and make the learners highly biased to these samples. The set of IVF/ICSI predictive

features in our findings indicates that the age of a women who is seeking IVF/ICSI treatment, plays the most important role in making a decision whether to proceed with thesetreatments. Features with the same score are considered to be equally significant, like infertility duration and anti-Mullerian hormone (AMH) testing features. In a study done by Lintsen et al. (20), they claimed that age of a woman is the key feature in the success of IVF/ICSI and those with the age of over 35 had a lower chance of pregnancy. The threshold obtained by the decision tree method is determined 38 years old. Another interesting finding is that AMH and antral follicle count features, which have close scores to each other, are considered to be accurate in predicting excessive response of ovarian hyperstimulation in IVF/ICSI treatment (21).

It has been previously demonstrated that AUC performs better than the accuracy index for comparing different learning algorithms (22, 23). Among former investigations, only Güvenir et al. (11) considered AUC as the main criterion. The mean AUC obtained in their study was 83.3%, which is close to the values obtained from RF and RPART in our study. The age of a woman is also indicated as the most remarkable feature for two out of three methods employed in the studies by Guh et al. (10); however, the set of features in their dataset differs from our dataset. One of the major limitations of this work was the number of IVF/ICSI records. This problem was mainly due to the number of incomplete patients' records available to us. In addition, the newly-established center from which our dataset was gathered didn't have enough considerable records of patients who did fresh embryo transfers. The other problem was missing values that affected the power of classifiers, since missing values decrease the accuracy of the classifiers. This issue affects the values of ranked features, providing positive value for pregnancy.

A restriction of the current study is that classical predictive models like Templeton, logistic regression, and Bayesian method are not considered for comparison since the focus of this study was only on a set of DM techniques. Note that logistic regression, for example, has a major limitation, which is the features of a dataset should be independent from each other. For example, follicle-stimulating hormone (FSH) and AMH are two features that have inverse relationship with each other. Also, a woman's age has proved correlations with AMH, FSH, the number of oocytes, and embryo quality. Nevertheless, in order to obtain a more comprehensive comparison, classical predictive models should have been used besides the DM models obtained in this study. Further studies should develop a suitable algorithm to tackle the problem of class imbalance for the classifiers that are sensitive to dissimilarity of the distribution of the classes. Ideally, it would be very helpful for such predictive analyses if healthcare institutes around the world would design a global database for IVF and ICSI, or ART in general. In that case, the results would be more generalized and comparable to each other. Presently, the variability in ART success among research centers provides different or in some cases contradictory results, which cannot be ignored.

Conclusion

According to the obtained results in the current study, RF and RPART outperformed the other methods for pregnancy prediction with AUC of 84.23 and 82.05%, respectively. Besides the issue of classifiers, knowledge in the form of selected features is extracted from RPART model. Age of a woman, number of developed embryos, and serum estradiol (E2) level on the day of human chorionic gonadotropin (hCG) administration are introduced as the best three predictive features for IVF/ICSI.

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References

1. Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, et al. International committee for monitoring assisted reproductive technology (ICMART) and the world health organization (WHO) revised glossary of ART terminology. Fertil Steril. 2009; 92(5): 1520-1524.
2. Practice Committee of the American Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss. Fertil Steril. 2008; 90(5 Suppl): S60.
3. Van Voorhis BJ. Clinical practice. In vitro fertilization. N Engl J Med. 2007; 356(4): 379-386.
4. Van Steirteghem A, Devroey P, Liebaers I. Intracytolasmic

sperm injection. Mol Cell Endocrinol. 2002; 186(2): 199-203.
5. Parsanezhad ME, Jahromi BN, Zare N, Keramati P, Khalili A, Parsa-Nezhad M. Epidemiology and etiology of infertility in Iran, systematic review and meta-analysis. J Womens Health, Issues Care. 2013; 2(6): 1-6.
6. Fayyad U, Piatetsky-Shapiro G, Smyth P. From data mining to knowledge discovery in databases. AI Magazine. 1996; 17(3): 37-54.
7. Wang YF, Chang MY, Chiang RD, Hwang LJ, Lee CM, Wang YH. Mining medical data: a case study of endometriosis. J Med Syst. 2013; 37(2): 9899.
8. Cios KJ, Moore GW. Uniqueness of medical data mining. Artif Intell Med. 2002; 26(1-2): 1-24.
9. Jurisica I, Mylopoulos J, Glasgow J, Shapiro H, Casper RF. Case-based reasoning in IVF: prediction and knowledge mining. Artif Intell Med. 1998; 12(1): 1-24.
10. Guh RS, Wu TCJ, Weng SP. Integrating genetic algorithm and decision tree learning for assistance in predicting in vitro fertilization outcomes. Expert Syst Appl. 2011; 38(4): 4437-4449.
11. Güvenir HA, Misirli G, Dilbaz S, Ozdegirmenci O, Demir B, Dilbaz B. Estimating the chance of success in IVF treatment using a ranking algorithm. Med Biol Eng Comput. 2015; 53(9): 911-920.
12. Chen CC, Hsu CC, Cheng YC, Li ST, editors. Knowledge discovery on in vitro fertilization clinical data using particle swarm optimization. 9th IEEE International Conference on Bioinformatics and BioEngineering; 2009 Jun 22-24; The Splendor Taichung,Taichung, Taiwan: IEEE; 2009.
13. Zhang S, Zhang C, Yang Q. Data preparation for data mining. Appl Artif Intell. 2003; 17(5-6): 375-381.
14. Acuna E, Rodriguez C. Classification, clustering, and data mining applications. 1st ed. Verlag Berlin Heidelberg: Springer; 2004; 639-647.
15. Forman G, Scholz M. Apples-to-apples in cross-validation studies: pitfalls in classifier performance measurement. SIGKDD Explorations. 2010; 12(1): 49-57.
16. Fawcett T. An introduction to ROC analysis. Pattern Recogn Lett. 2006; 27(8): 861-874.
17. Howlett B, Rogo E, Shelton TG. Evidence based practice for health professionals. 1st ed. Burlington: Jones & Bartlett Publishers; 2013; 235-245.
18. Sánchez JS, Mollineda RA, Sotoca JM. An analysis of how training data complexity affects the nearest neighbor classifiers. Pattern Anal Appl. 2007; 10(3): 189-201.
19. Japkowicz N, Stephen S. The class imbalance problem: a systematic study. Intelligent Data Analysis. 2002; 6(5): 429-449.
20. Lintsen A, Eijkemans M, Hunault CC, Bouwmans CA, Hakkaart L, Habbema JD, et al. Predicting ongoing pregnancy chances after IVF and ICSI: a national prospective study. Hum Reprod. 2007; 22(9): 2455-2462.
21. Broer SL, Dolleman M, Opmeer BC, Fauser B, Mol BW, Broekmans FJ. AMH and AFC as predictors of excessive response in controlled ovarian hyperstimulation: a meta-analysis. Hum Reprod Update. 2011; 17(1): 46-54.
22. Bradley AP. The use of the area under the ROC curve in the evaluation of machine learning algorithms. Pattern Recognit. 1997; 30(7): 1145-1159.
23. Ling CX, Huang J, Zhang H. AUC: a better measure than accuracy in comparing learning algorithms. Advances in Artificial Intelligence. 1st ed. Berlin Heidelberg: Springer; 2003; 329-341.

Original Article

Factors that Influence The Occurrence of Multiple Pregnancies after Intracytoplasmic Injection Cycles with Two or Three Fresh Embryo Transfers

Mahbubeh Abdollahi, Ph.D.[1], Reza Omani Samani, Ph.D.[2], Mandana Hemat, M.D.[3*], Arezoo Arabipoor, M.Sc.[3], Fatemeh Shabani, M.Sc.[2], Farzad Eskandari, Ph.D.[4], Masoud Salehi, Ph.D.[5]

1. Department of Biostatistics, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran
2. Department of Epidemiology and Reproductive Health, Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
3. Department of Endocrinology and Female Infertility, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
4. Department of Mathematical Statistics, Faculty of Economics, Allameh Tabatabai University, Tehran, Iran
5. Health Management and Economics Research Center, Department of Statistics and Mathematics, School of Health Management and Information Sciences, Iran University of Medical Sciences, Tehran, Iran

Abstract

Background: Multiple pregnancies are an important complication of assisted reproductive technology (ART). The present study aims to indentify the risk factors for multiple pregnancies independent of the number of transferred embryos.

Materials and Methods: This retrospective study reviewed the medical records of patients who underwent intracytoplasmic sperm injection (ICSI) cycles in Royan Institute between October 2011 and January 2012. We entered 12 factors that affected the number of gestational sacs into the poisson regression (PR) model. Factors were obtained from two study populations-cycles with double embryo transfer (DET) and cycles that transferred three embryos (TET). We sought to determine the factors that influenced the number of gestational sacs. These factors were entered into multivariable logistic regression (MLR) to identify risk factors for multiple pregnancies.

Results: A total of 1000 patients referred to Royan Institute for ART during the study period. We included 606 eligible patients in this study. PR analysis demonstrated that the quality of transferred embryos and woman's age had a significant effect on the number of observed sacs in patients who underwent ICSI with DET. There was no significant predictive variable for multiple pregnancies according to MLR analysis. Our findings demonstrated that both regression models (PR and MLR) had the same outputs. A significant relation existed between age and fertilization rate with multiple pregnancies in patients who underwent ICSI with TET.

Conclusion: Single embryo transfer (SET) should be considered with the remaining embryos cryopreserved to prevent multiple pregnancies in women younger than 35 years of age who undergo ICSI cycles with high fertilization rates and good or excellent quality embryos. However, further prospective studies are necessary to evaluate whether SET in women with these risk factors can significantly decrease multiple pregnancies and improve cycle outcomes.

Keywords: Multiple Pregnancy, Intracytoplasmic Injection Cycles, Risk Factors, Logistic Regression

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***Corresponding Address: P.O.Box: 16635-148, Department of Endocrinology and Female Infertility, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran**
Email: m.hemat@royaninstitute.org

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Introduction

At present, the use of assisted reproductive technology (ART) is expanding worldwide. A related challenge after ovarian hyperstimulation is multiple gestation and the health of children born by ART. Recently, the rate of multiple pregnancies has dramatically increased due to the widespread use of ART (1). Multiple pregnancies are associated with increased risk of maternal and fetal complications (2). The ideal of infertility therapy is to achieve one healthy baby at a time (3). Despite the attempts to limit the incidence of multiple pregnancies after ART by elective single embryo transfer (SET), the average *in vitro* fertilization (IVF) treatment includes transfer of two, three or sometimes more embryos into the uterus. SET, as a clinical practice, has not been executed in some countries.

Previous studies evaluated embryo and cycle-specific parameters associated with twin pregnancies after double embryo transfer (DET) (4-8). Niu et al. (4) found that four factors-the first treatment cycle, good ovarian response, higher number of top-quality embryos, and development stage score of the second-best embryo transferred had an independent association with twin pregnancies after DET. Xu et al. (5) reported that women's age and the number of high-quality embryos transferred were risk factors associated with twin pregnancies after IVF with DET. Groeneveld et al. (6) demonstrated that the height of the women and the number of oocytes retrieved were associated with an increased risk of twins after DET.

In a recent study, Kim et al. (8) concluded that younger age, higher body weight, and better quality of transferred embryos showed an association with increased chance for twin pregnancies after DET at cleavage stage. Identification of risk factors for multiple pregnancies can enable medical personnel to provide counseling and information to couples at high risk for multiple pregnancies and suggest SET in their infertility treatment program. The aim of present study is to evaluate the factors that affect the occurrence of multiple pregnancies after transfer of two or three embryos in intracytoplasmic sperm injection (ICSI) by two different regression analyses (poisson and Logistic).

Materials and Methods

This retrospective cohort study reviewed the records of patients referred to Royan Institute between October 2011 and January 2012. The Institutional Scientific Board of Royan Institute approved this study. Admitted patients gave written consent that stated which the treatment information would be used for scientific purposes without mentioning names or personal details.

The data related to infertile couples who underwent ICSI cycles and included: demographic information, medical records, and cycle characteristics. Excluded from the study were: women older than 40 years, uterine factor infertility (myoma, polyps, and congenital malformations), recurrent miscarriages, and stages II or III endometriosis. Cycles with donor oocytes or embryos, preimplantation genetics diagnosis, and blastocyst embryo transfer were also excluded. In this study we reviewed all ICSI cycles that had complete data. Embryo quality was recorded based on the fragmentation degree and regularity of blastomeres on day 3 after fertilization, as follows (9): excellent: 6-8 equal sized blastomeres with ≤10% frag mentation; good: 6-8 equal or unequal sized blastomeres with 10-20% fragmentation; and fair: uneven sized and few blastomeres with >20% fragmentation. We calculated the fertilization rate according to the number of fertilized oocytes per number of microinjected MII oocytes. Multiple pregnancies were considered as the observation of more than one gestational sac with heart beats by vaginal ultrasound evaluation six weeks after embryo transfer.

Statistical analysis

The findings were described as mean ± SD for quantitative variables and number (%) for qualitative variables. We compared the study population characteristics according to the number of embryos transferred, either DET or triple embryo transfer (TET) according to the student's t test and chi-square test for quantitative and qualitative variables, respectively. To investigate the relationship between factors and gestational sac, we used the statistical software STATA 11 program and the logistic and poisson regression (PR) models. On the basis of previous studies (5, 8, 10, 11), the possible variables that affected the num-

ber of gestational sacs included: women's age, body mass index (BMI), infertility type, infertility cause and duration, acquired uterine anomaly, endometrial thickness on the day of the human chorionic gonadotropin (hCG) injection, type of stimulation protocol, present cycle type, ovarian response type, total number of gonadotropin ampules, total number of retrieved oocytes, number of MII oocytes, fertilization rate, and quality of transferred embryos were listed in the PR model for both study populations (DET and TET). Multiple logistic regression (LR) model was used to determine significant variables related to multiple pregnancies. We considered P<0.05 to be statistically significant.

Results

A total of 1000 patients referred to Royan Institute for ART during the study period. We included 606 patients in this study. There were 336 patients with DET and 270 patients with TET. Table 1 lists the demographic and medical characteristics of patients according to group (DET or TET). The women's mean age, duration of infertility, and number of previous ART cycles in the TET group were greater than those in the DET group (P<0.001). The two groups showed no significant differences in terms of BMI, infertility type, endometrial thickness, and quality of transferred embryos. The DET group had a higher mean number of retrieved and MII oocytes. Despite a higher fertilization rate in the TET group, we observed clinical pregnancy rate between the two groups (P=0.1).

The distribution of the frequency of the number-of sacs in the DET and TET groups were shown in Table 2. The results showed that the mean of the observed gestational sac in the TET group (0.64 ± 0.88) was greater than the DET group (0.48 ± 0.72, P=0.01). We calculated incidence rate ratios (IRRs) for explanatory variables by the PR models to identify the related variables to the number of observed gestational sacs after the ICSI cycles. If an IRR is greater than 1, the incidence rate (IR, sac no.) increases as x (explanatory variable) increases. If an IRR is less than 1, the IR decreases as x increases.

Table 1: Study population characteristics in patients with double embryo transfer (DET) and three embryo transfer (TET)

Variable	DET n=336	TET n=270	P value
Women's age (Y)	29.3 ± 5.2	32.2 ± 4.2	<0.0001*
Body mass index (BMI)	25.2 ± 3.7	25.7 ± 3.8	0.1
Diagnosis infertility			0.09
Female	116 (34.5)	112 (58.5)	
Male	220 (65.5)	158 (41.5)	
Duration of infertility	5.9 ± 4.2	7.5 ± 5.3	<0.0001*
Type of infertility			0.5
Primary	270 (80.4)	212 (78.5)	
Secondary	66 (19.6)	58 (44.6)	
Previous ART attempts	1.09 ± 1.2	1.6 ± 2.0	<0.0001*
Number of used gonadotropin ampules	26.8 ±11.1	29.1 ±11.3	0.01*
Endometrial thickness on hCG day	10.3 ±1.8	10.3 ± 2.0	0.6
Total number of retrieved oocytes	9.9 ± 5.1	8.6 ± 3.5	0.001*
Number of MII oocytes	8.4 ± 4.5	7.6 ± 3.1	0.009*
Quality of transferred embryos			0.1
Fair	28 (8.4)	14 (5.2)	
Excellent	48 (14.3)	34 (12.6)	
Good	260 (77.3)	226 (82.2)	
Fertilization rate	0.59 ± 0.24	0.65 ± 0.22	0.002*
Clinical pregnancy rate	119 (35.4)	112 (44.6)	0.1

Results are presented as mean ± SD, n (%). ART; Assisted reproductive technology, hCG; Human chorionic gonadotropin, and *; Significant at the 0.05 level.

Table 2: Distribution of the frequency of observed gestational sacs in the double embryo transfer (DET) and triple embryo transfer (TET) groups

Number of sacs	DET n=336	TET n=270	P value
	Frequency (%)	Frequency (%)	
0	217 (64.6)	158 (58.5)	0.01*
1	78 (23.2)	62 (23)	
2	39 (11.6)	38 (14.1)	
3	3 (0.6)	12 (4.4)	

*; Significant at the 0.05 level.

The quality of the transferred embryos and woman's age significantly impacted the number of observed sacs in the DET group (Table 3). It means that the IR of multiple sac in excellent grade embroyos group was 9 times and meanwhile in good grade embroyos group was 6 times respect to fair grade embryos as refrence group. The results showed that the IRR for women's age was 0.96 (95% CI: 0.93-0.99). In other words, one year increases in female age showed an IR of the observed gestational sac that decreased 4%. The other explanatory variables did not impact the number of sacs.

In the TET group, women's age, type of infertility, and fertilization rate significantly impacted the number of sacs (Table 4). The IRR for maternal age was 0.94 (95% CI: 0.90-0.97) and IRR for the fertilization rate was 6.02 (95% CI: 2.96-12.22). The one year increase in female age showed an IR of the observed gestational sac that decreased 6%. The IR of the observed gestational sac increases 6 times when one unit increases in the fertilization rate. The results demonstrated that the IR of the observed gestational sac in patients with secondary infertility decreased 42% with respect to those with primary infertility (Table 4).

All the possible affecting variables that included women's age, BMI, infertility type, infertility cause and duration, acquired uterine anomaly, endometrial thickness on the day of the hCG injection, type of stimulation protocol, present cycle type, ovarian response type, total number of gonadotropin ampules, total number of retrieved oocytes, number of MII oocytes, fertilization rate, and quality of transferred embryos were entered in the multiple logestic regression (MLR) model. The result showed that women's age, duration of infertility, and number of transferred embryos were the most important variables related to multiple pregnancies in this population (Table 5). Therefore, we repeated the MLR analysis in the two separate populations (DET and TET). The results showed that none of the variables in the model had a significant association with multiple pregnancies in the DET group. Multiple LR showed that in the TET group, women's age and fertilization rate were significantly related variables (Table 6). Women with increased age had a 20% reduction in the odds for multiple pregnancies. When the fertilization rate increased one unit, the odds for a multiple pregnancy increased 19.7 times.

Table 3: Results of poisson regression for assessing the effect of explanatory variables on number of sac in patients with double embryos transfer

Variable	Coefficient	SE	P value	IRR (95% CI)
Women age	-0.05	0.02	0.004*	0.96 (0.93-0.99)
Quality of transferred embryos				
Excellent	2.20	0.73	0.003*	9.00 (2.16-37.50)
Good	1.79	0.71	0.012*	6.01 (1.48-24.37)
Fair	Reference	-	-	-

SE; Standard error, IRR; Incidence rate ratio; CI; Confidence interval, and * ; Significant at the 0.05 level.

Table 4: Results of poisson regression for assessing the effect of explanatory variables on number of sac in patients with three embryos transfer

Variable	Coefficient	SE	P value	IRR (95% CI)
Women age	-0.07	0.02	<0.001*	0.94 (0.90-0.97)
Type infertility				
Primary	Reference	-	-	0.58 (0.37-0.90)
Secondary	-0.55	0.23	0.016	
Fertilization rate	1.79	0.36	<0.001*	6.02 (2.96-12.22)

SE; Standard Error; IRR; Incidence rate ratio; CI; Confidence interval, and * ; Significant at the 0.05 level.

Table 5: Multiple logistic regression analysis by backward manner for predicting multiple pregnancy in all study population

Variable	OR	CI	P value*
Women age	0.93	(0.87-0.99)	0.04
Duration of infertility	1.07	(1.00-1.15)	0.03
No. of transferred embryos			
Three embryos	1.73	(0.96-3.10)	0.06
Two embryos	Reference		

OR; Odds ratio, CI; Confidence interval, and *; Significant at the 0.05 level.

Table 6: Multiple logistic regression analysis by backward manner for predicting multiple pregnancy in women with three embryos transfer in ICSI cycles

Variable	OR	CI	P value*
Women age	0.8	(0.79-0.99)	0.06
Fertilization rate	19.7	(2.6-100.6)	0.004

OR; Odds ratio, CI; Confidence interval, ICSI; Intracytoplasmic sperm injection, and *; Significant at the 0.05 level.

Discussion

In present study, on the basis of the PR model, we found that the quality of transferred embryos and women's age had a significant effect on the number of observed sacs in patients who underwent ICSI cycles with DET. However, based on the multiple LR model, we found no significant predictive variable for multiple pregnancy in these patients. Previous studies reported that maternal age (5, 8), body composition (6, 8), good ovarian response, cycle number, and the number of retrieved oocytes (4) had a relationship to multiple pregnancies after DET. The current study results showed no impact by body composition, cycle number, number of retrieved oocytes, and type of ovarian response. In agreement with previous studies (5, 8), we found that the quality of transferred embryos significantly influenced the IR of sac numbers. Kaser et al. (7) stated that there were six risk factor for twin live births after cryopreserved cleavage stage DET cycles: age <35 years, resumption of mitosis, 7-8 viable cells in the non-lead embryo, transfer of a lead embryo with ≥7 cells and a total of ≥14 viable cells.

Groeneveld et al. (6), in a large nationwide Dutch cohort, demonstrated that tall stature and increased number of retrieved oocytes independently increased the chance for dizygotic twins after IVF with DET. In contrast to this study, we did not find any relationship between multiple pregnancies and BMI or number of retrieved oocytes in ICSI cycles with DET.

In agreement with Niu et al. (4), we found that excellent and good quality transferred embryos independently increased the chances of multiple implantation after ICSI with DET. They suggested that it was advisable to perform SET when patients had high risk factors for twin pregnancies that included initial IVF-ET treatment, good or high ovarian response, more number of top-quality embryos, and development stage score of the second best embryo transferred. Fauque et al. (12) concluded that not only the implantation and pregnancy rates, but also the live birth rate depended on embryo quality.

On the basis of our knowledge, the present study was the first to evaluate risk factors for multiple pregnancies in patients who underwent ICSI with TET. Our findings demonstrated that both regression models (poisson and logistic) had the same outputs. In both models, women's age and fertilization rate showed significant relationships with multiple pregnancies in these patients. It could be taken as a measure of oocyte quality, which has been shown to be associated with increased implantation potential. A higher rate of fertilization would likely result in a higher number of MII oocytes and, consequently, a higher chance of good quality embryos would be associated with higher chances for multiple pregnancies. In agreement with previous studies we found a negative correlation between female age and multiple pregnancies in ICSI cycles with TET. Younger women had increased implantation potential and multiple pregnancies (10, 11).

In the present study, we have sought to evaluate the risk factors of multiple pregnancies after transfer of two or three embryos in ICSI cycles. If the risk factors of multiple pregnancies could be identified, we can give proper counseling to couples at risk for multiple pregnancies and suggest SET in their infertility treatment programs.

Currently an ideal target is considered in ART; a healthy baby is the success, not a positive pregnancy. The majority of twin or high order multiple pregnancies (HOMP) are not successful or associated with poor maternal and neonatal outcomes (13). De Neubourg and Gerris (14) have reported that the twin rate after IVF/ICSI dropped by at least 50% simply by transferring only one good-quality embryo in the first and second fresh IVF/ICSI cycles in young women, without a reduction in the overall pregnancy rate. They believed that preventing 'the second half' of IVF/ICSI twins constituted another, probably tougher challenge because the target

group was a heterogeneous mix consisting of patients in very different clinical situations. However, they suggested expanding the SET policy to women <38 years of age until the third cycle and to cryoperservation cycles. In many European countries the SET policy is the primary prevention method used to prevent multiple pregnancies. However, in Iran, the transfer of just one embryo, even with excellent morphology and quality, is taboo because it is feared that the pregnancy rate will decline (15). Despite warnings from physicians about the risks associated with multiple pregnancies, patients that have long-term infertility problems perceive twin or multiple pregnancies as blessings from God. In some cases they are not satisfied with multifetal pregnancy reduction. However, physicians are ethically obligated to inform the patients regarding the risks of multiple pregnancies.

A limitation of the present study is its retrospective nature; therefore, we could not evaluate the effect of some possible factors reported in previous studies.

Conclusion

We suggest the SET policy for prevention of multiple pregnancies in women younger than 35 years of age who undergo ICSI cycles with high fertilization rates and excellent or good quality embryos. The remaining embryos should be cryopreserved. However, further prospective studies are necessary to evaluate whether SET in women with these risk factors can significantly decrease the multiple pregnancy rate and improve cycle outcomes.

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References

1. Fauser BC, Devroey P, Macklon NS. Multiple birth resulting from ovarian stimulation for subfertility treatment. Lancet. 2005; 365(9473): 1807-1816.
2. Rao A, Sairam S, Shehata H. Obstetric complications of twin pregnancies. Best Pract Res Clin Obstet Gynaecol. 2004; 18(4): 557-576.
3. Practice Committee of American Society for Reproductive Medicine. Multiple gestation associated with infertility therapy: an American Society for Reproductive Medicine Practice Committee opinion. Fertil Steril. 2012; 97(4): 825-834.
4. Niu ZH, Feng Y, Zhang AJ, Zhang HQ, Sun YJ, Lu XW. Factors related to occurrence of twin pregnancy after double-embryo transfer in vitro fertilization cycles. Zhonghua Fu Chan Ke Za Zhi. 2009; 44(6): 413-417.
5. Xu WH, Tong XM, Zhu HY, Lin XN, Jiang LY, Zhang SY. Risk factors associated with twin pregnancy in double embryo transfer. Zhonghua Yi Xue Za Zhi. 2011; 91(37): 2615-2618.
6. Groeneveld E, Lambers MJ, Stakelbeek ME, Mooij TM, van den Belt-Dusebout AW, Heymans MW, et al. Factors associated with dizygotic twinning after IVF treatment with double embryo transfer. Hum Reprod. 2012; 27(10): 2966-2970.
7. Kaser DJ, Missmer SA, Correia KF, Ceyhan ST, Hornstein MD, Racowsky C. Predictors of twin live birth following cryopreserved double embryo transfer on day 3. J Assist Reprod Genet. 2013; 30(8): 1023-1030.
8. Kim MS, Kim JH, Jee BC, Suh CS, Kim SH. Factors affecting occurrence of twin pregnancy after double embryo transfer on day 3. J Obstet Gynaecol Res. 2015; 41(8): 1223-1228.
9. Ashrafi M, Karimian L, Eftekhari-Yazdi P, Hasani F, Arabipoor A, Bahmanabadi A, et al. Effect of oocyte dysmorphisms on intracytoplasmic sperm injection cycle outcomes in normal ovarian responders. J Obstet Gynaecol Res. 2015; 41(12): 1912-1920.
10. Tur R, Barri PN, Coroleu B, Buxaderas R, Martinez F, Balasch J. Risk factors for high-order multiple implantation after ovarian stimulation with gonadotrophins: evidence from a large series of 1878 consecutive pregnancies in a single centre. Hum Reprod. 2001; 16(10): 2124-2129.
11. Dickey RP, Taylor SN, Lu PY, Sartor BM, Rye PH, Pyrzak R. Risk factors for high-order multiple pregnancy and multiple birth after controlled ovarian hyperstimulation: results of 4,062 intrauterine insemination cycles. Fertil Steril. 2005; 83(3): 671-683.
12. Fauque P, Leandri R, Merlet F, Juillard JC, Epelboin S, Guibert J, et al. Pregnancy outcome and live birth after IVF and ICSI according to embryo quality. J Assist Reprod Genet. 2007; 24(5): 159-165.
13. Gerris J, De Neubourg D. Single embryo transfer after IVF/ICSI: present possibilities and limits. J Obstet Gynecol India. 2005; 55(1): 26-47.
14. De Neubourg D, Gerris J. What about the remaining twins since single-embryo transfer? How far can (should) we go? Hum Reprod. 2006; 21(4): 843-846.
15. Baruffi RL, Mauri AL, Petersen CG, Nicoletti A, Pontes A, Oliveira JB, et al. Single-embryo transfer reduces clinical pregnancy rates and live births in fresh IVF and Intracytoplasmic Sperm Injection (ICSI) cycles: a meta-analysis. Reprod Biol Endocrinol. 2009; 7: 36.

Original Article

Effect of Marital Relationship Enrichment Program on Marital Satisfaction, Marital Intimacy, and Sexual Satisfaction of Infertile Couples

Seyedeh Zahra Masoumi, Ph.D.[1], Somayeh Khani, B.Sc.[2], Farideh Kazemi, M.Sc.[3*], Fatemeh Kalhori, B.Sc.[2], Reyhaneh Ebrahimi, B.Sc.[2], Ghodratollah Roshanaei, Ph.D.[4]

1. Department of Midwifery, Mother and Child Care Research Center, Hamadan University of Medical Sciences, Hamadan, Iran
2. Department of Midwifery, Student Research Center, School of Nursing and Midwifery, Hamadan University of Medical Sciences, Hamadan, Iran
3. Department of Midwifery and Reproductive Health, School of Nursing and Midwifery, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Department of Biostatistics and Epidemiology, Modeling of Noncommunicable Diseases Research Center, School of Public Health, Hamadan University of Medical Sciences, Hamadan, Iran

Abstract

Background: Infertile couples only think of having children during their sexual intercourse, and their constant concern about this issue increases their stress level. Psychosocial and social stress leads to decreased life satisfaction, increased marital problems, and reduced sexual confidence. This study aims to determine the effect of enrichment program on marital and sexual satisfaction as well as marital intimacy among infertile couples.

Materials and Methods: This randomized controlled clinical trial was conducted on 50 infertile couples in 2013 in Hamedan. The marital relationship enrichment program was taught to the experimental group during seven 90 minutes sessions. Enrich marital satisfaction, Linda Berg sexual satisfaction, and marital intimacy questionnaires were completed by both groups in 3 pretest steps immediately after the end of training sessions, and 8 weeks later. The results were analyzed in STATA11 software using t test, Chi-square, ANCOVA, RM-ANOVA, and Bonferroni post-hoc test. To check the data normality, Kolmogorov-Smirnov test was used. $P<0.05$ was considered significant.

Results: Comparison of mean scores related to pretest on the one hand and immediately after the test in 8 week later on the other hand showed marital relationship enrichment program significantly increased marital and sexual satisfaction ($P<0.001$). Also, mean score of marital intimacy immediately after the test ($P=0.04$) and 8 weeks after the test ($P<0.001$) significantly increased in comparison with the pretest under the influence of the program.

Conclusion: Enrichment training can increase marital intimacy and also marital and sexual satisfaction in infertile couples (Registration Number: IRCT201604299014N97).

Keywords: Infertility, Training, Marital Therapy, Sexual Satisfaction

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*Corresponding Address: P.O.Box:1985717443, Department of Midwifery and Reproductive Health, School of Nursing and Midwifery, Shahid Beheshti University of Medical Sciences, Tehran, Iran
Email: faridehkazemi21@yahoo.com

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Introduction

Infertility fails to conceive after one year of sexual intercourse without using contraceptives methods (1). According to the World Health Organization's report, infertility affects approximately 8 million couples around the world and its rate varies from 5 to 30% in different countries (2). According to Systematic Review and Meta-Analysis conducted on the infertility in Iran, the prevalence of primary infertility has been reported to be 10.6% (3). Infertility is considered as a sign of failure and implies that the person is not perfect. Most people do not think that they are infertile, so an infertility diagnosis is a shock for them (4). Anxiety, loss of self-esteem, shame, and depression caused by infertility damage the infertile couples' sexual function. Also, diagnosis, investigation, and treatment of infertility may interfere with their sexual satisfaction (5). It is estimated that 80% of marital conflicts and incompatibilities are caused by couples' lack of sexual satisfaction (6).

According to the results of Iranian reports, many couples suffer from lack of satisfaction in their sexual relations and 50 to 60% of divorces as well as 40% of sexual infidelity are caused by this factor (7). Infertility can be adversely associated with relational, sexual and psychosocial wellbeing (8). It has been reported that infertile couples only think about having children during their intercourse; therefore, constant concern about the issue of facing another failure leads to their increased level of stress (9). Psychosocial and social stress leads to decreased life satisfaction, increased marital problems, and reduced sexual confidence among infertile couples (10). Based on Berg and Wilson (4) conclusions marital adjustment is reduced with increasing number of years of infertility and marital distress created. Marital distress is affected by intra couple coping method (11). Marital difficulties in infertile men and women cause the self-blame and detachment (12) and marital functioning is decreased in infertile couples with treatment process (13, 14).

For this reason, physical infertility treatments are not enough by themselves, and paying attention to the mental needs of infertile couples is an essential element in infertility treatment (15). Most therapists regard training couples in communication skills as the first step to improve the performance of couples, because communication problems are the most widespread complaints expressed by the couples who seeking (16). There are multiple approaches preventing marital difficulties or improving marital compatibility, one of which is the marital relationship promotion program known as "marital preparation and enrichment" that was first introduced by Olson and Olson (17). This program is one of the most successful ones whose efficacy has been reported in different works (16, 18). The Enrichment program is one that seeks to improve couples' relationships and could determine the factors and conditions upon which marital satisfaction and compatibility can be predicted after marriage. This program includes 4 preventive characteristics; first, it identifies the factors required by marital success. Second, it assists couples needing help to achieve growth and health criteria. Third, it requires feedback and training for the progress of couples. Finally, it provides some practices to couples that could affect their conflict resolution and communication skills. This program has 6 objectives and contains some exercises to help couples achieve these objectives with the purpose of encouraging them to do planning and cooperate with each other to deal with major issues (17).

Considering that a desirable sexual relationship can increase the chance of fertility (19). Infertility itself can be an important factor in marital dissatisfaction (20), and marital satisfaction can have a mutual influence on sexual satisfaction (21). This study was conducted to determine the effect of an enrichment program on marital and sexual satisfaction as well as marital intimacy of infertile couples using Enrich marital satisfaction, Linda Berg sexual satisfaction, and marital intimacy questionnaires in order to specify the effect of this preventive program on the satisfaction rate of infertile couples in terms of the expressed variables.

Materials and Methods

In this randomized controlled clinical trial participants were selected from the infertile couples referring to IVF Center of Fatemieh Hospital, Hamedan, for treatment in 2013. Using data from the study by Choobforoushzadeh et al. (22), considering the sample size at confidence interval (CI) 95%, statistical power of 0.90, and sample loss, finally, 50 couples were selected (25 couples in the intervention group and 25 couples in the control group).

First, the researcher prepared a list of the couples who had at least one history of failure in the use of assisted reproductive methods. After additional investigations, it was found that only 60 couples had all the inclusion criteria. From among these individuals, 50 couples who were willing to participate in the study were selected based on Helsinki principles (Fig.1). The random stratified sampling method was used to randomly assign these individuals into two experimental and control groups. For this purpose, the couples were first divided into two groups with monthly incomes of less than 5 million Rials and equal to or more than 5 million Rials. Then, they were divided into two subgroups in terms of infertility duration (less than 5 years and 5 years or more) and each of these subgroups was divided into three other subgroups according to education level (elementary, middle and high school, college). Finally, randomization was done based on drawing lots in the education sub-group and the participants were assigned into two studied groups with the ratio of 1:1. So, the assignment sequence was pre-determined. Assigning individuals to the groups was done by drawing lots and someone blind to the research, which led to proper concealment of assignment, but due to the intervention nature, blinding of the researcher and participants was not possible. Design, implementation, and reporting of the study were set based on the CONSORT statement (23).

Inclusion criteria included: i. Infertile couples with at least one failure history in infertility treatment using assisted reproductive methods, ii. Primary infertility, iii. Risk of infertility with female, male, both, and unknown factors, iv. Willingness to participate in the study, v. Having reading and writing literacy, and vi. Having less than 40 in marital satisfaction score in basis of Enrich marital satisfaction questionnaire. The participants were excluded from the study for the following reasons: lack of regular attendance at all sessions, attendance of only one of the marital partners, or pregnancy occurred during the treatment because the volunteers did not complete all of the survey instruments.

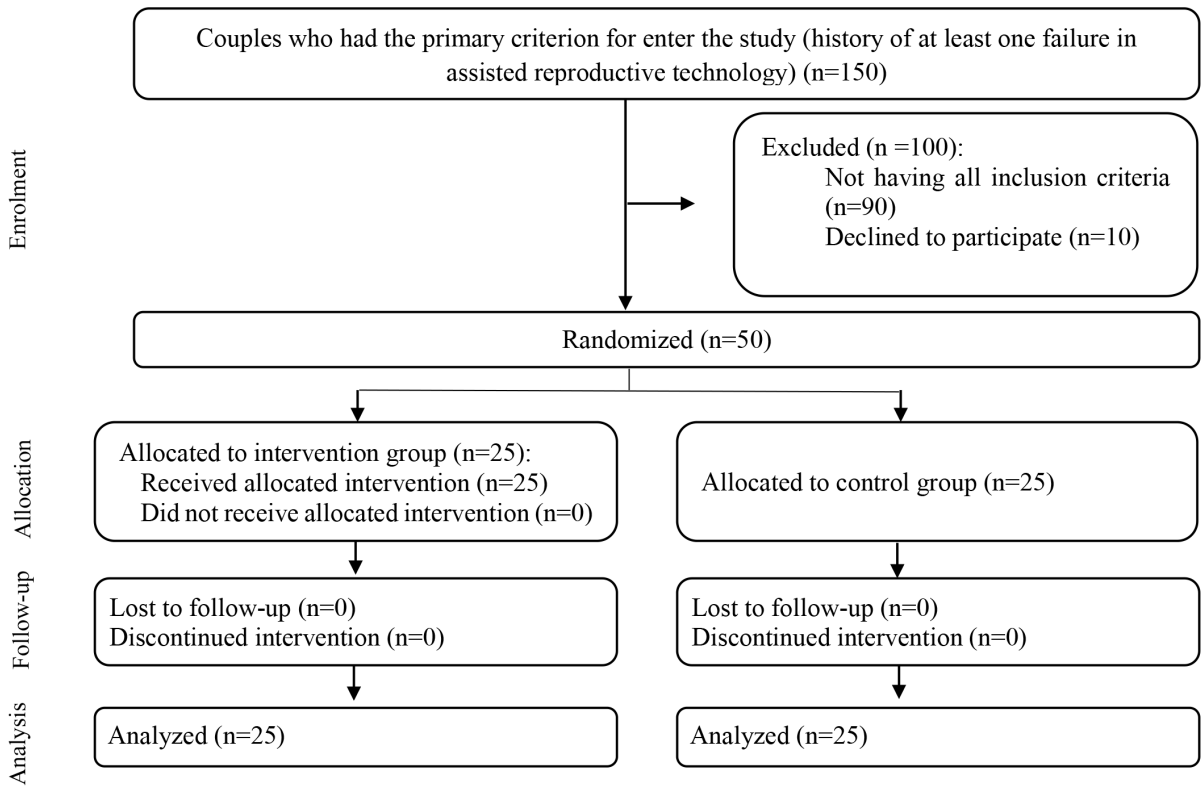

Fig.1: Flow diagram of the study.

The data collection tool included demographic characteristics, Persian Enrich marital satisfaction, Linda Berg sexual satisfaction, and marital intimacy questionnaires. Persian Enrich marital satisfaction questionnaire contained 47 questions, 5 of which were related to children. Since the questionnaire was used for infertile men and women, these 5 questions were removed in the expert panel formed based on the related specialists (2 Ph.D. holders in reproductive health, 2 epidemiologists, 2 Ph.D. holders in health education, and 1 Ph.D. holder in nursing) and the infertile couples answered 42 questions. The validity of this questionnaire is confirmed in previous studies conducted in this field (8). The Linda Berg sexual satisfaction questionnaire has 17 questions, whose validity was confirmed by Salehi Fedardi (24). The marital intimacy questionnaire's content validity was confirmed the Oulia et al. (25) study. Regarding reliability of each of the employed questionnaires, we obtained acceptable reliability for all tools examined by the Cronbach's alpha coefficient analysis (Persian Enrich marital satisfaction, 0.87; Linda Berg sexual satisfaction, 0.91; and marital intimacy questionnaire, 0.85).

The participants in the study were contacted and asked to return to the center in order to complete the pretest questionnaires after assigning the individuals into two above-mentioned groups. The questionnaires were given to 100 people (50 men and 50 women) as intervention (25 men and 25 women) and control (25 men and 25 women) groups. The provisional norm of these questionnaires were calculated separately for the interventional and control groups. Before performing the research, written informed consent was obtained from all participants. One session of the expert panel was held, in which the respective professors attended (1 social prevention specialist, 1 statistician and epidemiologist, 1 psychiatrist, and 1 reproductive health specialist) to determine the best intervention method to promote the marital relationship of infertile couples. Finally, it was decided to use the couples' relationship enrichment model. For the experimental group and based on the couples' relationship enrichment model, training classes seven 90 minutes sessions as a group with couples (men and women at the same time) were held twice per week, which included indeed, the fourth session (training on sexual relationship promotion) was held separately for men and women (Table 1).

Table 1: Marital relationship enrichment program

First session
Objective: Familiarity with the members and expression of the logic and objectives of the training sessions
Educational content
Acquaintance with the participants
Expression of goals
Conclusion of a contract and getting a commitment for regular participation
Second session
Objective: Open cognitive interpretation training
Educational content
Studying the problem from the viewpoint of each infertile couple
Making couples informed about kinds of irrational beliefs on infertility
Training A-B-C principles in infertility Methods to deal with irrational beliefs on infertility
Third session
Objective: Training intimacy between couples
Educational content
Defining intimacy and its dimensions
Training how to establish intimacy
Practicing intimacy methods
Feedback on the implementation of solutions
Fourth session
Objective: Training on the improvement of sexual relationship
Educational content
Expressing importance of sexual relationship
Expressing cycle of sexual issues
Factors hindering proper sexual relationship
Diagnosis and intervention
Training about wrong sexual myths
Fifth session
Objective: Evaluating conflict resolution methods
Educational content
Conceptual definition of marital conflict in infertility
Understanding the normality of conflict between couples
Extracting common ways of dealing with conflict among participants
Training correct principles and practices of conflict resolution on infertility
Practicing proper way of conflict resolution on infertility
Sixth session
Objective: Conflict resolution via teaching problem solving
Educational content
Effect of having self-attitude on the manner of infertility problem solving
Identifying infertility problem solving process
Steps of problem solving process
Hindering factors of problem solving
Seventh session
Objective: Home management training
Educational content
Training how to deal with infertility problem
Training how to deal with main families
Training how to deal with financial problems of infertility
Training how to deal with gender roles

In other words, six training sessions were held for men and women together, and the fourth session was held separately. The educational program was done by the researcher with Ph.D. degree in reproductive health along with a psychologist. Immediately after the end of the training sessions and 8 weeks later, sexual and marital satisfaction as well as marital intimacy questionnaires were given by someone who was not aware of the content of the training sessions and completed by both groups. A pamphlet containing the instructional materials of enrichment relations was presented to the control group after the follow-up completion in order to comply with the ethical issue.

Statistical analysis

Results were analyzed in STATA 11 software using t test, Chi-square, ANCOVA, Repeated-Measure ANOVA, and Bonferroni post-hoc test. To check data normality, Kolmogorov-Smirnov test was used. $P<0.05$ was considered significant.

Ethical considerations

This study was approved by the Medical Ethics Committee of Hamedan University and all participants gave an informed consent before commencing the study (code: IR.UMSHA.REC.1395.10).

Results

Participant recruitment and follow-up began in September and ended in December 2013. Fifty patients (25 couples) participated in each one of the group. None of the participants were excluded from the study during the training and follow-up periods. Characteristics of the participants are compared in Table 2; no significant difference was found between the two groups.

Table 2: Baseline characteristics of participants by intervention and control groups

Variable	Intervention group	Control group	P value
Age (Y), mean ± SD	30.0 ± 4.9	28.3 ± 4.4	0.89*
Gender, n (%)			
Male	25 (50)	25 (50)	1.00**
Female	25 (50)	25 (50)	
Education level, n (%)			
Primary	5 (10)	4 (8)	0.37**
Secondary	6 (12)	12 (24)	
High school and diploma	23 (46)	17 (34)	
College	16 (32)	17 (34)	
Employment status, n (%)			
Employed	27 (54)	25 (50)	0.63**
Unemployed	23 (46)	25 (50)	
Residence, n (%)			
City	40 (80)	40 (80)	0.34**
Village	10 (20)	10 (20)	
Duration of marriage (Y), mean ± SD	6.7 ± 4.1	5.5 ± 2.1	0.08*
Duration of infertility (Y), mean ± SD	4.5 ± 3.9	4.1 ± 2.4	0.58*
The number of previous IVF, n (%)			
1	43 (86)	40 (80)	0.13**
2	5 (10)	2 (4)	
3	2 (4)	4 (8)	
6	-	4 (8)	
Cause of infertility, n (%)			
Female	17 (34)	10 (20)	0.53**
Male	17 (34)	16 (32)	
Female-male	16 (32)	24 (48)	
Monthly income (1 million Rial), mean ± SD	6.8 ± 3.7	6.4 ± 1.7	0.42*

*; Independent t test, **; Chi-square test, and IVF; *In vitro* fertilization.

In this study, the P value for the Mauchly's test of sphericity was not significant (P=0.62). So repeated measure test was used to compare the mean scores of marital and sexual satisfaction as well as marital intimacy at different times of investigation in both groups. Findings showed that the mean score of marital satisfaction had a significant difference (P<0.001) at different times between the two groups. The Bonferroni post-hoc test demonstrated statistically significant difference in marital satisfaction scores between immediately after completing the training courses and the pretest (P<0.001) as well as 8 weeks after completion of the training courses and the pretest (P<0.001). These results were repeated in the case of sexual satisfaction. Investigating the marital intimacy mean scores showed statistically significant difference between the experimental and control groups (P<0.001). The Bonferroni post-hoc test demonstrated that, immediately after the completion of the courses, the marital satisfaction score was significantly increased compared with the pretest (P=0.04). Also, the investigation conducted 8 weeks after the course completion showed significant increase of marital intimacy scores in comparison with the pretest (P<0.001, Table 3).

ANCOVA was used to eliminate the effect of pretest on the results obtained in the posttest. The findings showed that, by controlling for the pretest effect, the intervention significantly increased the marital and sexual satisfaction immediately after the intervention and 8 weeks later (P<0.001, Table 4).

Table 3: Comparing the intervention and control groups in terms of mean scores of marital satisfaction, sexual satisfaction and marital intimacy

	Before intervention (mean ± SD)	Immediately after intervention (mean ± SD)	2 months after intervention (mean ± SD)	P value*
Marital satisfaction				
Control group	48.1 ± 8.4	43.4 ± 10.6	40.6 ± 10.3	<0.001
Intervention group	42.9 ± 9.3	71.0 ± 1.0	62.6 ± 0.8	
P value**	<0.001	<0.001	<0.001	
Sexual satisfaction				
Control group	26.4 ± 11.0	39.3 ± 18.0	40.0 ± 19.4	<0.001
Intervention group	57.8 ± 18.0	84.0 ± 1.2	83.4 ± 1.6	
P value**	<0.001	<0.001	<0.001	
Marital intimacy				
Control group	226.4 ± 43.2	224.9 ± 43.7	223.9 ± 44.3	<0.001
Intervention group	301.4 ± 76.0	318.0 ± 77.2	423.2 ± 2.0	
P value**	<0.001	<0.001	<0.001	

*; Repeated-Measure ANOVA and **; Independent t test.

Table 4: Results of ANCOVA investigating the relationship between grouping on marital satisfaction and sexual satisfaction

Measuring tool	Statistical indicators of variables	P value
Immediately after intervention		
Marital satisfaction	Pretest	0.06
	Grouping	<0.001
Sexual satisfaction	Pretest	<0.001
	Grouping	<0.001
Two months after intervention		
Marital satisfaction	Pretest	0.01
	Grouping	<0.001
Sexual satisfaction	Pretest	0.005
	Grouping	<0.001

Discussion

The present study was performed to determine the effect of enrichment program on marital as well as sexual satisfaction and marital intimacy of infertile couples. As far as the effect of the program on marital satisfaction was concerned, individuals in the control group attained a higher mean score of marital satisfaction before the intervention, but after the test and two months later, these scores were decreased, which was probably due to the marital satisfaction effect of time and continued duration of infertility. The mean score in the individuals of the experimental group was higher immediately after the intervention in comparison with the pretest. Two months after the intervention, although this mean score was higher than the pretest, it was decreased compared with the study conducted immediately after the intervention.

A drop after 2 months proved the necessity of continuing the enrichment trainings. Providing continuous training either in person, through mass communication means, or by family, and friends, and other relatives could have a major role in increasing marital satisfaction. Maintenance and skills help couples maintain and apply what they have learned during the sessions and expand them to other areas of their life such as work environments (26). Laub et al. (27) concluded that the longer-term the enrichment program and the more emphasis on the formation of skills, the higher and more stable its positive effect on the couples and their life satisfaction would be. As mentioned, among infertile couples undergoing infertility treatment, couples relationship enrichment program could increase the level of marital satisfaction in the experimental group. The findings of this study were consistent with the results of other works (16, 26); in the study by Isanezhad et al. (26) conducting on 36 couples in Comprehensive Medical and Counseling Center in Isfahan, the relationship enrichment program could significantly improve the total score of couples' marital quality, including marital agreement, satisfaction, and marital cohesion. This effect persisted in the follow-up carried out 1 month later. Ghasemi Moghadam et al. (16) in their study which was conducted on married women in Tehran during 2010 to 2011 observed that marital relationship enrichment training using Olson's method could significantly increase the mean score of overall marital satisfaction, even when husbands did not participate in the training program.

The results of the current study showed that the marital relationship enrichment training was effective in increasing the infertile couples' intimacy in the pretest and 2 months later. The finding that satisfaction with marital relationship could have an effect on marital intimacy was consistent with Etemadi's finding in 2005 on the application of cognitive-behavioral techniques and increased marital intimacy (28). In another hypothesis by these authors representing the role of applying therapeutic communication techniques to increase marital intimacy, it was found that use of the therapeutic communication techniques could increase affective, psychological, intellectual, spiritual, social, and entertainment intimacy. This finding was consistent with our research results.

Another objective of the present study was to determine the effect of this program on the couples' sexual satisfaction. The marital relationship enrichment training increased sexual satisfaction in infertile couples. In support of this finding, Shams Mofarahe et al. (29) also demonstrated that the sexual satisfaction of women, 1 month after consultation, was higher in the intervention group than the control group. In fact, if sexual relations between spouses are not satisfactory, this leads to feelings of deprivation, frustration, insecurity and lack of happiness. Dissatisfaction with sexual relationship might cause problems such as depression (30) or divorce (31). Satisfactory sexual relations could contribute to family strengthening and become the basis for acquiring and consolidating a solid relationship (32). Laub et al. (27) investigated the effectiveness of a relationship enhancement program on couples' relationships in the long run and concluded that the spouses attained higher sexual and physical intimacy as well as more communication stability than the control group. To prevent sample loss due to the large number of training sessions, the sessions were held twice per week.

Conclusion

Considering the positive impact of the enrichment program at posttest and follow-up stages on marital and sexual satisfaction and sexual intimacy in the infertile couples, it can be concluded that the program can be appropriately used in infertile cou-

ples with sexual problems. Enrichment skills are the skills that help satisfy the strongest desires of families (sexual desires) and are used in almost all cultures. Considering the fact that enrichment training is a preventive and non-invasive program and can prevent deterioration into marital conflict, establishing and developing a center to provide such training is recommended, especially for vulnerable groups such as infertile couples who need special attention.

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References

1. Johnson JV. Infertility. Danforth's obstetrics and gynecology. London: Lippincott Williams&Wilkins; 2008; 685-695.
2. Vayena E, Rowe P, Griffin P. Current practices and controversies in assisted reproduction. Geneva, Switzerland: World health Organization; 2002; 355-362.
3. Parsanezhad MB, Jahromi BN, Zare N, Keramati P, Khalili A, Parsa-Nejad M. Epidemiology and etiology of infertility in Iran, systematic review and meta-analysis. J Womens Health, Issues Care. 2013; 2(6): 1-6.
4. Berg BJ, Wilson JF. Psychological functioning across stages of treatment for infertility. J Behav Med. 1991; 14(1): 11-26.
5. McInnes RA. Chronic illness and sexuality. Med J Aust. 2003; 179(5): 263-266.
6. Sharyati M, Ghamarani A, Solati Dehkordi SK, Abbasi Molid H. The study of relationship between alexithymia and sexual satisfaction among female married students in Tabriz University. Journal of Family Research. 2010; 6(1): 59-70.
7. Honar Parvaran N, Tabrizi M, Navabi Nezhad Sh, Shafi Abadi A. Efficacy of emotion-focused couple therapy training with regard to increasing sexual satisfaction among couples. Andisheh va Raftar. 2010; 4(15): 59-70.
8. Keramat A, Masoomi SZ, Mousavi SA, Poorolajal J, Shobeiri F, Hazavhei SM. Quality of life and its related factors in infertile couples. J Res Health Sci. 2014; 14(1): 57-63.
9. Boivin J, Appleton TC, Baetens P, Baron J, Bitzer J, Corrigan E, et al. Guidelines for counselling in infertility: outline version. Hum Reprod. 2001; 16(6): 1301-1304.
10. Chachamovich J, Chachamovich E, Fleck MP, Cordova FP, Knauth D, Passos E. Congruence of quality of life among infertile men and women: finding from a couple-based study. Hum Reprod. 2009; 24(9): 2151-2157.
11. Benazon N, Wright J, Sabourin S. Stress, sexual satisfaction, and marital adjustment in infertile couples. J Sex Marital Ther. 1992; 18(4): 273-284.
12. Slade P, Raval H, Buck P, Lieberman BE. A 3-year follow-up of emotional, marital and sexual functioning in couples who were infertile. J Reprod Infant Psychol. 1992; 10(4): 233-243.
13. Pepe MV, Byrne TJ. Women's perceptions of immediate and long-term effects of failed infertility treatment on marital and sexual satisfaction. Family Relations. 1991; 40(3): 303-309.
14. Drosdzol A, Skrzypulec V. Evaluation of marital and sexual interactions of Polish infertile couples. J Sex Med. 2009; 6(12): 3335-3346.
15. Hart VA. Infertility and the role of psychotherapy. Issues Ment Health Nurs. 2002; 23(1): 31-41.
16. Ghasemi Moghadam K, Bahmani B, Asgari A. Effectiveness of group education of marital enrichment program (olson style) on improvement of married women's satisfaction. Clinical Psychology and Personality. 2013; 2(8): 1-10.
17. Olson D, Olson A. Preventive approaches in couple therapy. New York: Talor, Francis pub; 1999.
18. Fowers BJ, Olson DH. Enrich marital inventory: a discriminant validity and cross-validation assessment. J Marital Fam Ther. 1989; 15(1): 65-79.
19. Sargolzaee MR, Moharreri F, Arshadi HR, Javadi K, Karimi S, Fayyazi-bordbar MR. Psychosexual and depression disorders in infertile female referring to Mashhad Infertility Treatment Center. J Reprod Infertil. 2001; 2(4): 46-51.
20. Orji E, Ogunniyi SO. Sexual behaviuor of infertile nigerian women. J Obstet Gynaecol. 2001; 21(3): 303-305.
21. Litzinger S, Gordon KC. Exploring relationships among communication, sexual satisfaction, and marital satisfaction. J Sex Marital Ther. 2005; 31(5): 409-424.
22. Choobforoushzadeh A, Kalantari M, Molavi H. The effectiveness of cognitive behavioral stress management therapy on marital satisfaction in infertile women. Journal of Fundamentals of Mental Health. 2010; 12(3): 596-603.
23. Schulz KF, Altman DG, Moher D. CONSORT 2010 statement: updated guidelines for reporting parallel group randomized trials. Ann Intern Med. 2010; 152(11): 726-732.
24. Salehi Fedardi J. The development and validation of marital satisfaction questionnaire on a sample of students of Ferdowsi University. Psychotherapical Novelties. 1999; 4(13): 84-93.
25. Oulia N, Bahrami F, Fatehizadeh M. Marital enrichment training. Tehran: Danzheh; 2009.
26. Isanezhad O, Ahmadi SA, Etemadi O. Effectiveness of relationship enhancement on marital quality of couples. Journal of Behavioral Sciences. 2010; 4(1): 9-16.
27. Laub JH, Nagin DS, Sampson RJ. Trajectories of change in criminal offending: good marriage and the desistance process. Am Sociol Rev. 1998; 63(2): 225-238.
28. E'temedi O, NavvabiNezhad S, Ahmadi SA, Farzad V. A study on the effectiveness of cognitive-behaviour couple therapy on couple's intimacy that refer to counselling centres in Esfahan. Psychological Studies. 2006; 2(1): 69-87.
29. Shams Mofareheh Z, Shahsiah M, Mohebi S, Tabaraee Y. The effect of counseling on sexual satisfaction of couples in Shiraz city. Health System Research. 2010; 6(3): 417-424.
30. Boyd M. Psychiatric nursing contemporary practice. Philadelphia: Lippincott Williams & Wilkins; 2002.
31. Dyer S, Abrahams N, Mokoena NE, van der Spuy Z. You are a man because you have children': experiences, reproductive health knowledge and treatment-seeking behaviour among men suffering from couple infertility in South Africa. Hum Reprod. 2004; 19(4): 960-967.
32. Jahanfar S, Molaeenezhad M. Textbook of sexual disorders. Tehran: Salami Press; 2001.

Original Article

Correlation of The Etiology of Infertility with Life Satisfaction and Mood Disorders in Couples who Undergo Assisted Reproductive Technologies

Behnaz Navid, M.Sc.[1], Maryam Mohammadi, M.Sc.[1], Samira Vesali, M.Sc.[1], Marzieh Mohajeri, M.A.[2], Reza Omani Samani, M.D.[1*]

1. Department of Epidemiology and Reproductive Health, Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
2. Department of Psychology, Faculty of Educational Sciences and Psychology, University of Al Zahra, Tehran, Iran

Abstract

Background: This study compared common psychological symptoms and life satisfaction in husbands and wives according to infertility diagnosis.

Materials and Methods: We conducted this cross-sectional study on 248 infertile couples between November 1, 2014 and February 28, 2015 at Royan Institute, Tehran, Iran. Participants answered three questionnaires. First, they completed a demographic questionnaire followed by the Hospital Anxiety and Depression Scale (HADS, 14-item self-report instrument) composed of two sub-scales: anxiety (HADS-A) and depression (HADS-D). Participants also completed the Satisfaction with Life Scale (SLWS) comprised of 5 items. Both our questionnaires were validated for the Iranian population.

Results: In couples with male factor infertility, wives had a significantly higher mean score for anxiety compared to their husbands (P<0.001). When the cause of infertility was female factor, the wives appeared significantly more anxious (P<0.001) and depressed (P=0.004) than their husbands. Male patients, those with unknown and female factors, expressed greater satisfaction with life compared to other male patients (P=0.022). Significantly greater depression existed among the couples in which the wives' educational levels was above their husbands (P=0.045).

Conclusion: Our findings showed that when the infertility etiology was male factor, female factors or unexplained, wives showed significantly higher anxiety than their husbands. In couples diagnosed with female factor infertility, wives showed significantly more depression than their husbands.

Keywords: Anxiety, Depression, Reproduction, Infertility

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Introduction

Childbearing is an important, valuable issue in marital relationships, especially in traditional societies because it stabilizes the family and increases marital satisfaction (1). After attempts at pregnancy over an extended period of time, the inability to have a child may cause marital problems (2). Most infertile couples report loss of self-esteem, sexual stress, depression, anxiety, guilt, frustration, emotional distress, tension in their marital status, and reduced life satisfaction (3). Most commonly reported forms of infertility related mood disorders are anxiety and depression. These disorders are influenced by a number of factors such as gender, cause of infertility, uncertain treatment duration, finan-

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***Corresponding Address: P.O.Box: 16635-148, Department of Epidemiology and Reproductive Health, Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran**
Email: samani@royaninstitute.org

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cial stress, and pressure from others (4). Several studies have reported the psychosocial impacts of an infertility diagnosis on men and women (1). Infertile couples showed higher rates of psychological symptoms when they had female factor and unexplained causes for infertility. Women have been shown to experience more stress and depression, along with less marital satisfaction compared to their partners. This finding is most probably due to increased involvement in therapeutic procedures, which affects women more (5). Men diagnosed with male factor infertility have more negative emotional responses than other infertile men. They have expressed feelings of stigma and loss of self-esteem (6). It is reported that individuals diagnosed with male factor infertility have greater stress and sexual concern than those with either idiopathic or female factors (7).

Life satisfaction is defined as an assessment of feelings and attitudes about a person's life at a particular time that ranges from negative to positive and it is a cognitive, judgmental process based on a comparison of individual circumstances to an appropriate standard (8). Research has found that infertile women expressed less satisfaction with their lives compared to fertile women (9). Some studies have focused on marital adjustment, sexual functioning and marital satisfaction in infertile patients (10, 11). Relatively little is known about the influencing factors on life satisfaction in women and men who undergo assisted reproductive technology (ART). We have conducted this study to investigate anxiety, depression, and life satisfaction in Iranian infertile couples who experienced ART.

Materials and Methods

We conducted this cross-sectional study at Royan Institute, a referral infertility clinic in Tehran, Iran. The sample size was 280 couples from which we gathered 248 completed questionnaires from these couples, for a response rate of 88.5%. Inclusion criteria were: 18 years of age or older, first time undergoing ART, no history for treatment of psychological disorders or chronic diseases, and ability to read and write in Persian. The Ethical Committee of Royan Institute approved this study. Participants received clear explanations about the study aims and data confidentiality. Eligible individuals were assured that acceptance or refusal to participate in the research was voluntarily had no influence on their treatment procedures. Voluntary completion of the questionnaires was considered as consent.

Participants completed three questionnaires. The demographic and fertility information questionnaire included age (years), sex, educational levels (illiterate/under diploma/diploma/academic), duration of infertility (years), and cause of infertility (male factor/female factor/both/unknown). The second questionnaire, the Hospital Anxiety and Depression Scale (HADS) was published in 1983 by Zigmond and Snaith (12). This is a 14 item self-report instrument composed of two subscales: anxiety (HADS-A) and depression (HADS-D). Both consist of 7 items scored on a 4-point Likert scale (0 to 3). In both subscales, 8 items require reverse scoring; then, the anxiety part (HADS-A) and depression (HADS-D) totals can be summed. Both HADS-A and HADS-D scores range from 0 to 21 where higher scores indicate a higher level of anxiety and depression.

The Persian version of HADS has been validated with a Cronbach's alpha coefficient of 0.78 for HADS-A and 0.86 for HADS-D (13). In the current study, we have determined Cronbach's alpha scores to be 0.88 for HADS-A and 0.77 for HADS-D. The Satisfaction with Life Scale (SWLS) was published in 1985 by Diener et al. (8). This scale measures global life satisfaction and subjective well-being, and does not tap related constructs such as positive affect or loneliness. It includes 5 items scored on a 7-point Likert scale, that ranges from 1 to 7. The Iranian version of SWLS as determined by Maroufizadeh et al. (14), has a Cronbach alpha coefficient of 0.887. The current study Cronbach alpha coefficient was determined to be 0.85.

Statistical analysis

We used SPSS version 16.0 (SPSS Inc, Chicago, IL, USA) for statistical analyses. Continuous variables were expressed as mean ± SD and categorical variables as number (%). The relationship between individual independent variables (demographic and fertility char-

acteristics), and dependent variables (anxiety, depression, and SWLS) were assessed by the Pearson correlation coefficient, paired t test (between wives and husbands), independent samples t test (between men and women), and one-way analysis of variance (ANOVA) between causes of infertility followed by the Duncan post hoc test. $P<0.05$ was considered statistically significant. After paired and pulled analyses, as the mood of husbands and wives influenced each other, we defined a new variable which considered a single, mean score for each couple. We called this analysis "couple analysis" (CA).

Results

Participants had a mean age of 33.25 ± 5.70 years for men and 29.15 ± 5.28 years for women. We observed no significant difference in depression between men (5.50 ± 3.63) and women (6.65 ± 4.09). Women (8.96 ± 5.00) had significantly more anxiety compared to men (6.04 ± 3.86, $P<0.001$). No significant difference existed between men (24.46 ± 7.07) and women (23.64 ± 7.06) in terms of SWLS. CA showed that SWLS had a value of 24.05 ± 6.07, anxiety of 7.50 ± 3.58, and depression was 6.07 ± 3.22 for couples. Only 97 (39.1%) men and 95 (38.5%) women had academic educations. In 25% of our couples, wives had a higher educational level compared to their husbands; 24% of husbands had a higher educational level compared to their wives; and the remaining 51% had the same educational level. No significant difference existed between the different educational levels among husbands and wives in SWLS and anxiety ($P>0.05$). However, there was significantly greater depression among couples in which the wives had a higher educational level than their husbands ($P=0.045$) or their educational levels were same ($P=0.018$) compared to those in which the husbands had higher educational levels. According to the cause of infertility, 98 (39.5%) couples had male factor infertility, 76 (30.6%) had female factor, 23 (9.3%) had both, and 51 (20.6%) were unknown. The mean duration of infertility was 4.82 ± 3.50 years in couples. Table 1 lists the participants' demographic and fertility characteristics.

Table 1: Demographic and fertility characteristics of participants (n=248 couples)

	Men **Mean ± SD or n (%)**	**Women** **Mean ± SD or n (%)**	**P value**
Age (Y)	33.25 ± 5.70	29.15 ± 5.28	<0.001
Education			0.899
Illiterate	2 (0.8)	2 (0.8)	
Under diploma	65 (26.2)	59 (23.9)	
Diploma	84 (33.9)	91 (36.8)	
Academic	97 (39.1)	95 (38.5)	
Location			-
City	224 (90.3)		
Village	24 (9.7)		
Cause of infertility			-
Male factor	98 (39.5)		
Female factor	76 (30.6)		
Both	23 (9.3)		
Unknown	51 (20.6)		
Duration of infertility (Y)	4.82 ± 3.50		-

Gender differences in anxiety, depression, and the Satisfaction with Life Scale in male factor group

In couples with male factor infertility, wives had a significantly greater mean score for anxiety compared to their husbands (P<0.001). No significant difference existed between husbands and wives for the HADS-D (P=0.960) and SWLS (P=0.594, Table 2).

Gender differences in anxiety, depression, and the Satisfaction with Life Scale in the female factor group

In couples with female factor infertility, the wives had significantly higher mean scores for anxiety (P<0.001) and depression (P=0.004) compared to their husbands, but no significant difference was seen in SWLS (P=0.094, Table 2).

Gender differences in anxiety, depression, and the Satisfaction with Life Scale in the both factor group

In this group, no significant differences existed in anxiety, depression, and SWLS between wives and husbands (Table 2).

Gender differences in anxiety, depression and the Satisfaction with Life Scale in the unknown factor group

In couples with unexplained infertility, there was no significant difference in depression and SWLS between husbands and wives. Wives had a significantly higher mean score for anxiety than their husbands (P=0.041, Table 2).

The influence of infertility etiology on anxiety, depression and the Satisfaction with Life Scale

The Kruskal-Wallis test was used separately for males and females for anxiety, depression, and SWLS to identify differences according to the diagnosis of infertility. No significant difference existed among the male patients in anxiety and depression between different causes of infertility (Table 2). In contrast, males with unknown factor fertility had significantly greater SWLS compared to other males (P=0.022). No significant differences were apparent among females in HADS-A, HADS-D, and SWLS between the different etiology groups of infertility (P=0.729, P=0.397, P=0.122 respectively). In CA, we observed no significant differences between couples with different infertility etiologies in SWLS, anxiety, and depression.

Association between the two questionnaires

Anxiety had a positive, significant association with depression (P<0.001). A significant, negative correlation existed between anxiety and depression with SWLS (P<0.001). These correlations were the same when we analyzed them separately in women and men (Table 3).

Table 2: Anxiety, depression, and the Satisfaction with Life Scale (SWLS) in couples and groups

		Male (Mean ± SD)	Female (Mean ± SD)	Both (Mean ± SD)	Unknown (Mean ± SD)	P value*
Anxiety						
	Male	6.15 ± 4.11	5.91 ± 3.96	6.13 ± 4.38	6.41 ± 3.74	0.834
	Female	8.36 ± 4.60	9.09 ± 5.11	8.35 ± 4.07	8.04 ± 4.16	0.729
	P value**	<0.001	<0.001	0.071	0.041	
Depression						
	Male	5.94 ± 3.75	5.41 ± 4.28	6.04 ± 4.29	5.24 ± 3.25	0.603
	Female	6.29 ± 4.28	6.91 ± 4.12	6.52 ± 3.96	5.65 ± 3.25	0.397
	P value**	0.690	0.009	0.671	0.508	
SWLS						
	Male	24.40 ± 6.73	25.51 ± 6.28	21.35 ± 7.39	26.31 ± 6.31	0.022
	Female	24.12 ± 7.05	23.89 ± 6.62	21.48 ± 7.25	25.63 ± 5.91	0.122
	P value**	0.594	0.094	0.807	0.578	

*; Test for several independent groups and **; Paired test

Table 3: Correlation between anxiety, depression, and the Satisfaction with Life Scale (SWLS)

	Anxiety		Depression		SWLS	
	R	**P value**	**R**	**P value**	**R**	**P value**
Anxiety	1	-	0.536*	<0.001	-0.348*	<0.001
Depression	0.536*	<0.001	1	-	-0.431*	<0.001
SWLS	-0.348*	<0.001	-0.431*	<0.001	1	-

R; Pearson correlation and *; P<0.05.

Discussion

To the best of our knowledge this was the first study on life satisfaction in both husbands and wives (couples) who underwent ART. It appeared that when men have superiority in the family, the family had a lower mean depression score. Our results showed that when the husbands' educational levels were higher compared to their wives, the couples had a significantly lower mean depression score.

Most studies investigated psychological moods in infertile patients and showed that infertile women experienced higher psychological problems especially for anxiety, depression, and stress (15-17). Our results showed no difference between males and females for depression, also a meta-analysis conducted in Iran showed same results about difference between males and females in depression (18). However, in other studies, infertile women reported more depression than men (19, 20). We found significantly more anxiety in females compared to males which supported results from previous studies (3, 19). An Iranian study showed a greater prevalence of anxiety in infertile women that increased with a history of treatment failure. This finding agreed with the current study results (21). We observed no significant difference between males and females in SLWS.

In 2001, Wischmann et al. (22) reported more satisfaction with life in women compared to men which contradicted to findings of the current study. In this study used a questionnaire on life satisfaction which covered more social factors that included vocational life, financial situation, leisure, marriage, self-esteem, sexuality, and living situation along with health but we have used the SWLS introduced by Diener et al. (8). This questionnaire consists of two major components: i. The emotional or affective component and ii. The judgmental or cognitive component. The difference between our study and Wischmann et al. (22) was possibly due to the different type of the questionnaires and cultures.

We could not find any paper on the relationship between etiology of infertility and satisfaction with life, but we found papers on a similar factor-marital satisfaction. In a Chinese study, wives with both male and female factors expressed less marital satisfaction than their husbands (1). Another study in Iran showed that women with female factor infertility had less marital satisfaction than their infertile counterparts (11). Although our study did not evaluate marital satisfaction, we could not find any significant difference in satisfaction with life between the different infertility etiologies among females.

We found that when the infertility etiology was male factor, female factors or unexplained, wives also showed strongly significantly higher anxiety than their husbands. The reason was thought to be caused by the idea that conception and childbirth have been considered women's responsibility, especially in traditional societies and some developed countries (15). Ramezanzadeh et al. (23) have explained that in Islamic and Middle Eastern countries, such as Iran, childbearing is very important and leads to family stabilization and increased marital satisfaction. They also reported that in these countries negative attitudes toward infertility exist such as stigmatization, marital instability, divorce, and abuse for infertile women. Therefore, involuntary childlessness may be associated with enhanced psychological problems. In our study, in couples who suffered from female factor infertility, wives showed significantly more depression than their husbands.

Wischmann et al. (22) found that anxiety and depression in couples with unexplained factor infertility were higher than couples with other factors, and in women more than men. A study reported that less anxiety and depression in females whose male partners were infertile (24), but we did not observe these results in the current study. Ogawa et al. (2) reported that females with knowledge of their male partner's

infertility had lower anxiety scores on the HADS test than infertile women who did not have such knowledge. Our study could not confirm this finding.

The current study had several limitations. First, we have relied on infertile couples who presented to a single center. However this is a referral clinic for infertility treatment that treats patients from all of Iran. Second, the cross-sectional nature of the study only reveals a correlation, but no conclusion on causality. Additional longitudinal studies are required to explore the direction of causality and to determine how the study variables may change over the course of the infertility treatment.

Conclusion

Infertile women with female and male factor showed higher anxiety and depression compared to their husbands. Couples with unknown factor showed no significant differences in depression and SWLS between husbands and wives. SWLS was higher in men with unknown factor compared to other males. Women with female factor expressed more anxiety and depression than women with male factor infertility, but SWLS did not differ among these women. It has been suggested to add psychological counseling before and during treatment cycles.

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References

1. Lee TY, Sun GH, Chao SC. The effect of an infertility diagnosis on the distress, marital and sexual satisfaction between husbands and wives in Taiwan. Hum Reprod. 2001; 16(8): 1762-1767.
2. Ogawa M, Takamatsu K, Horiguchi F. Evaluation of factors associated with the anxiety and depression of female infertility patients. Biopsychosoc Med. 2011; 5(1): 15.
3. Chen TH, Chang SP, Tsai CF, Juang KD. Prevalence of depressive and anxiety disorders in an assisted reproductive technique clinic. Hum Reprod. 2004; 19(10): 2313-2318.
4. Boivin J, Griffiths E, Venetis CA. Emotional distress in infertile women and failure of assisted reproductive technologies: meta-analysis of prospective psychosocial studies. BMJ. 2011; 342: d223.
5. Franco JG Jr, Razera Baruffi RL, Mauri AL, Petersen CG, Felipe V, Garbellini E. Psychological evaluation test for infertile couples. J Assist Reprod Genet. 2002; 19(6): 269-273.
6. Nachtigall RD, Becker G, Wozny M. The effects of gender-specific diagnosis on men's and women's response to infertility. Fertil Steril. 1992; 57(1): 113-121.
7. Newton CR, Sherrard W, Glavac I. The fertility problem inventory: measuring perceived infertility-related stress. Fertil Steril. 1999; 72(1): 54-62.
8. Diener E, Emmons RA, Larsen RJ, Griffin S. The satisfaction with life scale. J Pers Assess. 1985; 49(1): 71-75.
9. Callan VJ, Hennessey JF. The psychological adjustment of women experiencing infertility. Br J Med Psychol. 1988; 61(Pt2): 137-140.
10. Valsangkar S, Bodhare T, Bele S, Sai S. An evaluation of the effect of infertility on marital, sexual satisfaction indices and health-related quality of life in women. J Hum Reprod Sci. 2011; 4(2): 80-85.
11. Jafarzadeh F, Golzari M, Jomehri F, Poursamar SL, Sahraian K. The comparison of coping strategies with stress and marital satisfaction in women on the basis of infertility factor. Women's Health Bull. 2015; 2(2): e25227.
12. Zigmond AS, Snaith RP. The hospital anxiety and depression scale. Acta Psychiatr Scand. 1983; 67(6): 361-370.
13. Montazeri A, Vahdaninia M, Ebrahimi M, Jarvandi S. The Hospital Anxiety and Depression Scale (HADS): translation and validation study of the Iranian version. Health Qual Life Outcomes. 2003; 1: 14.
14. Maroufizadeh S, Ghaheri A, Omani Samani R, Ezabadi Z. Psychometric properties of the satisfaction with life scale (SWLS) in Iranian infertile women. Int J Reprod Biomed (Yazd). 2016; 14(1): 57-62.
15. El Kissi Y, Romdhane AB, Hidar S, Bannour S, Ayoubi Idrissi K, Khairi H , et al. General psychopathology, anxiety, depression and self-esteem in couples undergoing infertility treatment: a comparative study between men and women. Eur J Obstet Gynecol Reprod Biol. 2013; 167(2): 185-189.
16. Wirtberg I, Möller A, Hogström L, Tronstad SE, Lalos A. Life 20 years after unsuccessful infertility treatment. Hum Reprod. 2007; 22(2): 598-604.
17. Volgsten H, Skoog Svanberg A, Ekselius L, Lundkvist O, Sundström Poromaa I. Prevalence of psychiatric disorders in infertile women and men undergoing in vitro fertilization treatment. Hum Reprod. 2008; 23(9): 2056-2063.
18. Noorbala AA, Ramazanzadeh F, Malekafzali H, Abedinia N, Forooshani AR, Shariat M, et al. Effects of a psychological intervention on depression in infertile couples. Int J Gynaecol Obstet. 2008; 101(3): 248-252.
19. Drosdzol A, Skrzypulec V. Depression and anxiety among Polish infertile couples--an evaluative prevalence study. J Psychosom Obstet Gynaecol. 2009; 30(1): 11-20.
20. Chiaffarino F, Baldini MP, Scarduelli C, Bommarito F, Ambrosio S, D'Orsi C, et al. Prevalence and incidence of depressive and anxious symptoms in couples undergoing assisted reproductive treatment in an Italian infertility department. Eur J Obstet Gynecol Reprod Biol. 2011; 158(2): 235-241.
21. Maroufizadeh S, Karimi E, Vesali S, Omani Samani R. Anxiety and depression after failure of assisted reproductive treatment among patients experiencing infertility. Int J Gynaecol Obstet. 2015;130(3): 253-256.
22. Wischmann T, Stammer H, Scherg H, Gerhard I, Verres R. Psychosocial characteristics of infertile couples: a study by the Heidelberg Fertility Consultation Service. Hum Reprod. 2001; 16(8): 1753-1761.
23. Ramezanzadeh F, Aghssa MM, Abedinia N, Zayeri F, Khanafshar N, Shariat M, et al. A survey of relationship between anxiety, depression and duration of infertility. BMC Womens Health. 2004; 4(1): 9.
24. Fassino S, Pierò A, Boggio S, Piccioni V, Garzaro L. Anxiety, depression and anger suppression in infertile couples: a controlled study. Hum Reprod. 2002; 17(11): 2986-2994.

Original Article

Desired Numbers of Children, Fertility Preferences and Related Factors among Couples Who Referred to Pre-Marriage Counseling in Alborz Province, Iran

Razieh Lotfi, Ph.D.[1, 2*], Masoumeh Rajabi Naeeni, M.Sc.[3*], Nasrin Rezaei, M.Sc.[4], Malihe Farid, M.D.[5], Afsoon Tizvir, M.D.[6]

1. Social Determinants of Health Research Center, Alborz University of Medical Sciences, Karaj, Iran
2. Department of Midwifery and Reproductive Health, School of Nursing and Midwifery, Alborz University of Medical Sciences, Karaj, Iran
3. Department of Midwifery and Reproductive Health, School of Nursing and Midwifery, Shahid Beheshti University of Medical Sciences, Tehran, Iran
4. Alborz University of Medical Sciences, Karaj, Iran
5. Department of Community Medicine, School of Medicine, Alborz University of Medical Sciences, Karaj, Iran
6. Deputy of Chancellor for Health, Alborz University of Medical Sciences, Karaj, Iran

Abstract

Background: The Islamic Republic of Iran has experienced a dramatic decrease in fertility rates in the past three decades. One of the main issues in the field of fertility is the couple's preferences and the desire to bear children. This study aimed to determine desired number of children, fertility preference, and related factors among people referring pre-marriage counseling to clarify their presumed behavior in case of fertility.

Materials and Methods: This study was a descriptive analytic cross-sectional survey, conducted during 8 months. The participants were 300 couples came to pre-marriage counseling centers of two health centers of Karaj and asked to complete a 22 items questionnaire about of demographic characteristics, participants' interest, preference about fertility, and economic situation.

Results: Majority of the males were between the ages of 20-30 years (66.6%) while majority of the females were below 25 years of age (57%). About 17 percent of men and 22.3 percent of women stated that they want to have 1 child and equally 52.7 percent of men and 52.7 percent of women wanted to have 2 children. The only factor that contributed to the female participant's decision for a desirable number of children was the number of siblings that they have. In male participants with an increasing age at marriage and aspiration for higher educational level, the time interval between marriage and the birth of the first child has increased. There was a convergence in desired number of children in male and female participants.

Conclusion: Majority of the participants express their desire to have only one or two children in future but in considering the fact that what one desires does not always come into reality, the risk of reduced fertility is generally present in the community. Appropriate policies should be implemented in order to create a favorable environment for children.

Keywords: Fertility, Fertility Preferences, Time to Pregnancy, Family Size

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*Corresponding Addresses: P.O.Box: 3146883811, Social Determinants of Health Research Center, Alborz University of Medical Sciences, Golestan 1, Boulevard Eshteraki, Baghestan, Gohardasht, Karaj, Iran
Department of Midwifery and Reproductive Health, School of Nursing and Midwifery, Shahid Beheshti University Of Medical Sciences, Tehran, Iran
Emails: lotfi_razieh@yahoo.com, rajabishirin@yahoo.com

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Introduction

Changes in fertility rates are seen throughout the world. The Islamic Republic of Iran has experienced a dramatic decrease in fertility rates in the past three decades. Population studies showed that total fertility rate (TFR) has decreased from 7.7 in 1966 to 6.3 in 1976. Years after the Islamic Revolution, there was a slight increase in fertility rate to about 7 in 1980 but with initiation of the country's national family planning program, this figure has declined to 5.5 (1). The fertility rate in the Islamic Republic of Iran fell dramatically from around 7.0 births per woman in the early 1980s to 1.9 births per woman in 2006. The total fertility rate was below replacement level from 2006 onwards. Based on the report given by the world bank, Iran's population growth rate will be below 1% by 2025 (2).

Also based on the 2006 census, the National Statistical Center of Iran has reported a TFR of 1.9. Postponing of childbearing is one of the reasons of fertility decline (3). Also childbearing has been postponed due to several reasons; advanced maternal age at marriage, higher education and the aim to secure economic stability before conception (4). In Iran, fertility rate is influenced by factors such as education, number of children, age of partner and age at first marriage while age and income are not considered important factors (5). It is the fact that, the preference of family size and women's social background has great relevance. A study conducted in China showed that factors contributing in family's preference for smaller family size include; young age, preference to reside in the city, and pursue higher education (6). Lower education was associated with more number of children.

In Japan, younger women desire to have fewer children while women in rural areas prefer to have more numbers of children than those residing in urban areas. The mean desired number of children was 2.55 which were significantly more than the mean actual number of children 1.77 in all generations (7). One of the most important issues in relation to fertility is the age of mother at the time of attempting conception. Results for study conducted in the United States of America to assess the attitudes and awareness of male and female students regarding conception, indicated that although 90% of these students showed desire to have children and highly valued parenting as an important part of their future; lack of understanding between age related fertility decline and the complications associated with higher maternal age and delayed conception proved to be extensively high (3). Due to Iran's declining population, the government and policymakers have expressed great concern regarding the sharp decline and the negative growth in the next 40 years. Accordingly, policies on population control that have been implemented in Iran for 25 years have been abandoned and those policies encouraging birth in the country's 6th development program are very much emphasized. It seems that socio-economic changes that have been shaped in Iran's society would make it difficult to reverse the trend of the country's fertility rate. Delayed fertility is related to factors including; economic, social, and cultural which can be affected by the couple's intention for childbearing and their concept of a desired family size. Education, access to health care services, awareness, and authority to decide on the number of children have important role in fertility preferences (4, 8-10). Evidences suggest that in many parts of the world, fundamental change has taken place in an individual's attitude towards marriage and childbearing (2).

One of the main issues in the field of fertility and in assessing the factors related to fertility behavior is the couple's preferences-fertility and the desire to bearing child. Aside from this, the number of desired children is considered a very important and serious issue in predicting the actual number of children that couple would desire as emphasized by various studies conducted by Habbema et al. (11), and Günther and Harttgen (12). After three years of policymaking on the population of Iran, few studies have been conducted specially on childbearing trends. This study aimed to determine desired number of children, fertility preference, and related factors among people refer to pre-marriage counseling to clarify their presumed behavior in case of fertility.

Materials and Methods

The present descriptive analytic cross sectional study was conducted in the Province of Alborz, located in the neighboring capital of Iran (with a distance of 45 km) having an area of 5121 square kilometers and a population of about 3 million, between May 2014 and March 2015. This province, due to its nearness to Tehran is the reason behind the number of migration taking place in different parts of

the country and due to the diversity of ethnicity has been coined a name "small Iran". The study populations were women and men who planned to marry in near future. The mentioned couples referred to the pre-marriage counseling centers of Alborz province. These centers are two university pre-marriage counseling centers that one of them is referral.

This paper was a part of the larger study with 60 variables (items) and we have selected 10 samples for each variable. As a result, 600 women and men were recruited. Based on convenience sampling method, during 8 months all the women and men who came to two pre-marriage counseling centers including 300 couples (600 individual) aged between 15 to 45 years participated in this study and were asked to complete a questionnaire. Exclusion criteria were lack of consent to participate in the study or any other nationality except being Iranian. A written informed consent was obtained from each participant after explaining the aims of the study. None of the participants were excluded. The self-administered anonymous questionnaire was included 13 items; 7 items about demographic characteristics, 6 items related to participants' interest and preference related to fertility. The questionnaire included variables such as: age, education, occupation, condition of house, predicted status of future residence, monthly family income, number of brother (s) or sister (s), desired number of children, number of children based on the child's gender, suitable years of interval for conception of the first baby after marriage, suitable age of marriage for females, suitable age of marriage for males, level of interest to become a father/mother and suitable years of interval between pregnancies.

This questionnaire was designed and implemented by the Ministry of Health and Medical Education, Tehran throughout the country in order to assess the decision making patterns of childbearing intentions. Validity and reliability of the questionnaire was evaluated. Content validity was analyzed by experts in the field of fertility and demographics and minor corrections implemented. Reliability of the questionnaire was determined as 0.88. Statistical analysis was done using SPSS software version 19. Also descriptive and analytical statistics were implemented to data analysis. This study was a small part of a larger study designed by MOH of Iran. Ethic Committee of Alborz University of Medical Sciences approved the study (ABZUMS.Rec.1393.45).

Results

The age range of male participants was between 19-54 years (mean ± SD: 28.36 ± 5.8) while the in female participants was between 13-45 years (mean ± SD: 24.8 ± 5.9). Majority of the males were between the 20-30 years of age (66.6%) while females were below 25 years of age (57%). A higher percentage of men than women had education level less than diploma. A large percentage of men (87%) were employed, while only 35% of women employed. Both, men and women thought that they are going to live in a rental house. About half of the male participants and 37.3% of females earned less than 10 million Rials (280 US $) respectively. The vast majority of the participants were anticipated that they will live in a house with size of 50 to 100 square meters. More than 70% of samples had 1 to 4 siblings (Table 1).

In item of the participants' desire to be parent, more than 80% of men and 70% of women stated that they are interested much and/or very much to be parent. Less than 2 percent of both genders were not interested to be parent. The male participants believe that appropriate age for marriage is 26.3 for men and 21.6 for women and the female participants mention 27.2 and 22.9 respectively for men and women. Appropriate time for attempting the first conception after marriage was 2.7 years from opinions of men and women. About 17 percent of men and 22.3 percent of women stated that they want to have 1 child and equally 52.7 percent of men and women wanted to have 2 children. The only factor that contributed to the female participant's decision for a desirable number of children was the number of siblings that they have. There was a convergence in desired number of children in male and female participants. About 10% of men and 11% of women want to have 2 children no difference in gender. Three percent of men and none of the women have decided to have no child. Demographic characteristics and desired number of children according to gender were analyzed. Bivariate test showed that there was significant difference between numbers of women's sibling and desired numbers of children ($P<0.001$). Other demographic characteristics were not related to desired numbers of children. With the increase in the number of siblings, desired numbers of children was increased.

Table 1: Demographic characteristic of participants

Variable	Men		Women		Total	
	n	%	n	%	n	%
Age						
<20	12	4	83	27.3	95	15.8
20-24	88	29.3	88	29.3	176	29.3
25-39	112	37.3	78	26	190	31.7
30-34	56	18.7	36	12	92	15.3
35-39	20	6.7	11	3.7	31	5.2
40-44	7	2.3	4	1.3	11	1.8
≥45	5	1.7	0	0	5	0.9
Education						
Less than diploma	169	56.3	148	49.3	317	52.8
Diploma and higher	131	43.7	152	50.7	283	47.1
Job						
Employee	261	87	105	35	366	61
Unemployed	21	7	170	56.0	191	31.8
Job seeker	18	6	25	8.4	43	7.2
Opinion about housing condition in future						
Renter	139	46.4	120	40	259	43
Landlord	106	35.3	111	37	217	36
Belong to father	55	18.3	69	23	124	21
Income						
No income	7	2.3	138	46	145	24.2
< 1 million tomans	148	49.3	114	37.3	262	43.6
1 to 2 million tomans	107	35.7	41	13.7	148	24.7
2 to 3 million tomans	26	8.7	5	1.7	31	5.2
> 3 million tomans	12	4	2	0.7	14	2.3
Opinion about house size in future						
<50 m^2	15	5	9	3	24	4
51-75 m^2	113	37.3	108	36	221	36.8
76-100 m^2	113	37.7	119	39.7	232	38.7
101-150 m^2	40	13.3	56	18.6	96	16
>151 m^2	19	6.3	8	2.7	27	4.5
Number of siblings						
None	10	3.3	19	6.3	29	4.8
1 to 4	213	71	231	77	444	74
5 to 8	67	22.3	56	15	112	18.7
> 8	10	3.3	5	1.7	15	2.5

Table 2 shows demographic characteristic and the first birth interval according to gender. There was a statistically significant relationship between the age of marriage and the first birth interval (P<0.001). Also education (P<0.001) and numbers of siblings of the participants (P=0.007) were related to the first birth interval. With the increase in marriage age and education level, the first birth interval was increased, but having more siblings led to decrease in the first birth interval marriage age, education, income and numbers of women's siblings were related to proper age for male marriage (Table 3). These relationships were positive for all mentioned variables. The same variables were related to proper age of male marriage that had relationship to proper age of female marriage (Table 4). There was a statistically significant relationship between income (P=0.01), house size of male participants (P=0.01) and pregnancy intervals (Table 5). Increasing the number of siblings was associated with pregnancy intervals inversely (P<0.05). In term of interest to be parents, there was a significant relationship with ages of the male participants (P=0.01). Indeed older men had lower interest to be parent (Table 6).

Table 2: Demographic characteristic and the first birth interval according to gender

Demographic variable	Gender	The first birth interval	P value
Marriage age	Male	Krus-kalwalis	<0.001*
	Female	Krus-kalwalis	<0.001*
Education	Male	Chi-square	<0.001*
	Female	Chi-square	<0.001*
Job	Male	Chi-square	0.2
	Female	Chi-square	0.91
Housing condition	Male	Chi-square	0.07
	Female	Chi-square	0.47
Income	Male	Chi-square	0.19
	Female	Chi-square	0.4
House size	Male	Chi-square	0.4
	Female	Chi-square	0.22
Number of sisters and brothers	Male	Spearman	0.007*
	Female	Spearman	0.01*

*; Significant at P<0.05.

Table 3: Demographic characteristic and proper age for male marriage according to gender

Demographic variable	Gender	Proper age for male marriage	P value
Marriage age	Male	Spearman	<0.001*
	Female	Spearman	<0.001*
Education	Male	Mann-withney	0.001*
	Female	Mann-withney	0.002*
Job	Male	Mann-withney	0.88
	Female	Mann-withney	0.19
Housing condition	Male	Krus-kalwalis	0.001*
	Female	Krus-kalwalis	0.19
Income	Male	Krus-kalwalis	0.001*
	Female	Krus-kalwalis	0.005*
House size	Male	Mann-withney	0.31
	Female	Mann-withney	0.43
Number of sisters and brothers	Male	Spearman	0.37
	Female	Spearman	0.004*

*; Significant at P<0.05.

Table 4: Demographic characteristic and proper age for female marriage according to gender

Demographic variable	Gender	Proper age for female marriage	P value
Marriage age	Male	Spearman	<0.001*
	Female	Spearman	<0.001*
Education	Male	Mann-withney	<0.001*
	Female	Mann-withney	<0.001*
Job	Male	Mann-withney	0.62
	Female	Mann-withney	0.4
Housing condition	Male	Krus-kalwalis	0.01*
	Female	Krus-kalwalis	0.53
Income	Male	Krus-kalwalis	0.01*
	Female	Krus-kalwalis	0.001*
House size	Male	Mann-withney	0.68
	Female	Mann-withney	0.54
Number of sisters and brothers	Male	Pearson	0.05
	Female	Pearson	0.001*

*; Significant at P<0.05.

Table 5: Demographic characteristic and pregnancy intervals according to gender

Demographic variable	Gender	Pregnancy intervals	P value
Marriage age	Male	Krus-kalwalis	0.06
	Female	Krus-kalwalis	0.11
Education	Male	Chi-square	0.19
	Female	Chi-square	0.3
Job	Male	Chi-square	0.68
	Female	Chi-square	0.3
Housing condition	Male	Chi-square	0.62
	Female	Chi-square	0.28
Income	Male	Chi-square	0.01*
	Female	Chi-square	0.47
House size	Male	Chi-square	0.01*
	Female	Chi-square	0.6
Number of sisters and brothers	Male	Pearson	0.007*
	Female	Pearson	0.01*

*; Significant at P<0.05.

Table 6: Demographic characteristic and interested to be parents according to gender

Demographic variable	Gender	Interested to be parents	P value
Marriage age	Male	Krus-kalwalis	0.01*
	Female	Krus-kalwalis	0.23
Education	Male	Fishers exact test	0.53
	Female	Fishers exact test	0.64
Job	Male	Fishers exact test	0.87
	Female	Fishers exact test	0.33
Housing condition	Male	Fishers exact test	0.07
	Female	Fishers exact test	0.8
Income	Male	Fishers exact test	0.9
	Female	Fishers exact test	0.77
House size	Male	Fishers exact test	0.22
	Female	Fishers exact test	0.36
Number of sisters and brothers	Male	Krus-kalwalis	0.28
	Female	Fishers exact test	0.04*

*; Significant at P<0.05.

The only factor that contributed to the female participant's decision for a desirable number of siblings was associated with the number of siblings that they have. In male participants, no association was observed between the demographic information and their desire in a number of children. With an increasing age at marriage and aspiration for higher educational level, the time interval between marriage and the birth of the first child has increased. But this gap has decreased with the increasing number of siblings. With increasing age of marriage, education, income and type of residence, male participants have believed that the age of marriage of the male gender should be higher. In female participants, age of marriage, education, income, and the number of sister(s) or brother (s) were associated to appropriate age of men for marriage.

The same relationship exists where women considered a certain age to be suitable for marriage. With the increase in men's income, the preference for bigger houses and higher intervals for pregnancies have increased but with the increasing number of siblings both female and male groups stated less number of intervals between desired pregnancies. With the increasing age of men at marriage, the desire to become a father has decreased but this issue has not been observed in women. With the increasing number of siblings in women, their desire to become a mother has increased but this case yield an opposite results for male participants. There exist an association among an increasing desire for parenthood, increasing number of desired children and also a lesser interval between marriage and the first conception. For fathers, this desire was associated with a desire to decrease the interval between pregnancies.

Discussion

In this study, we presented viewpoints of young Iranian people and explored related factors. About 50% of the female and male participants have expressed that the desired number of children for a desired family is 2 (one girl, one boy). In this study, the desired number of children per woman from the perspective of the participants was calculated to be approximately 1.91 which is below replacement level. Some sociologists and policy makers strongly believed that fertility rate is mostly influenced by the demands and preferences of families especially women, regarding the desired number of children. Also the study conducted by Günther and Harttgen (12) indicated a strong correlation between the desired number of children and the actual number of children. The desired number of children per generation could be affected by

the context in which an individual grows (9). Although some studies have not confirmed this issue, the actual number of children for a family to the desired number can be completely different (13). They believed that between the number of children expected, and the number of actual children, there was a slight difference in developed and developing countries. Developing countries with a high fertility rate usually expects a lower number of children, lower than the actual number but in developed countries the opposite is true. In a study conducted in Japan, the actual number of children was 1.77 and the number of desired one has been reported 2.55 which is significantly lower than the number of children considered desired (7). The desired number of children not necessarily will be concordant with the reality of fertility behavior of families that named "fertility gap". The reason of this gap can be due to socio-economic factors such as divorce, financial problems, higher education, employment and aspiration for higher income (4).

The number of desired children as expressed by 83.1% of the participants in this study was between 1 to 2. About 20 % favors one child while 63.3% favors 2 children. On the other hand, despite the immense interest of the couple to become parents as observed in this study the desire for less conception can serve as a warning sign for an increasing decline of fertility in the country resulting to an aging population and a reduction of the productive younger generation. It seems that the overall population policies must be directed towards more favorable conditions for economic security and welfare for the society especially the women's need in nurturing her child and transform these policies to the stage of implementation in order to increase fertility to a satisfactory condition.

Although in some western countries the desire to be childless is an ordinary issue, studies have indicated that European and especially Asian countries desire to have the number children of that everyone wants which, is the foundation of living (3, 7). In Japan, 58.4% of individuals aging 20-29 years old have expressed that 2 children is desired for a family, in South Korea 58.7, in the United States of America 42.2% and in France 56.6% have expressed 2 children as a desired number in a family (14). Interestingly, there exist a similar tendency in the desired number of children in most countries and it seems that there is a convergence of opinions in this issue in most developed countries and also in Iran.

In the present study, none of the demographic factors were related to the men's choice for a desired number of children while in women, the increased number of siblings has been reported to affect the choice for a desired number. In some studies, the relationship between age at marriage, education, income and financial situation justifies childbearing behavior (8, 13). In fact, motivation to higher education and higher incomes decreases the motivation for childbearing (4, 15). The result of this study about relation between the participants' age at marriage and desired number of children was in line with that of another study in Iran (5).

The results of a study conducted to assess fertility desire of women in Tehran showed that poor income was related to fertility disinterest (16). Although some researchers argued that income might not purely interpret child birth behaviors of couples, it is necessary to assess the different kinds of social support that people receive (10). One related reason that higher education levels may lead to less childbearing is balancing between education affairs and mother roles. Moreover, more- educated women may attain to a better career than other women. Also more income and authority, may provide more control on childbearing for educated women (17). The possible reason for the difference in the results of the present study in comparison to other studies might be due to the fact that only 12.7% of male participants have monthly income of 600 US dollars and approximately 50% have an income of 300$ monthly. In women, these figures have been less. An important point in this study is that there is a unanimous agreement among individuals with different demographic characteristics in the desired number of children a couple should have.

In this study, advanced age of men and women during marriage and the pursuit of higher education has increased the interval between marriage and the conception of the first child and also the increased age considered appropriate for marriage in men and women. With improved income, the age considered appropriate for marriage in both genders have also increased. Findings in this study correspond to the findings of the study conducted by Ericsson et al. (18) in Sweden which is a qualitative study on professional men and women post-

poning conception in favor to acquire higher education. Participants expressed that the reason for delayed childbearing in men and women having higher education is to cope with adapting social changes and the new life style. Hence; a change in priorities. Another reason is that nowadays children are expensive and then parents decide to postpone their childbearing until they feel they achieve to more stable economic position. Therefore it is predictable that most of the couples will have their first child at later age. Advanced maternal age can pose a lot of problems in considering the sociocultural changes in Iran's present society in which a little less than half of the female population have university education. With increasing age of marriage and time interval between marriage and conception of the first child, decline in female fertility will occur (19). Furthermore; advanced maternal age during pregnancy may be associated with more medical and obstetrical complications for mother and fetus that could create a negative effect on population growth, health and dynamics respectively (20-22).

Accordingly; as indicated by results of some studies conducted on Iranian society, there is an increasing trend between the interval of marriage and the conception of the first child which can generally affect the rate of fertility (2). Some studies have shown that there were misconceptions regarding fertility in a way that couples presumed that with the emergence of the modern methods of fertility treatments, age associated infertility problems will be completely resolved. Based on the conducted by Virtala et al. (23) more than 50% of male and 1/3 of female college students have believed that decline in fertility would only occur at the age of 45 in women. Also, in another study conducted on non-medical students, they believed that women's fertility can still be preserved even with increasing age. Therefore, they can plan to have their pregnancy at ages when fertility declines (24).

Community policy-makers must be aware of this issue and should address significant issues in their policies regarding childbearing to support families. Some studies have shown that policies designed and implemented to resolve conflict between work and study have played an important role in strengthening the couples desire for childbearing and helping couples to counter their decision for postponement of conception on their first child (4). Although the availability of contraceptive methods is considered to be one of the factors affecting reproductive behavior, the most important factor in order to achieve success in changing the community's behavior towards fertility is more understanding in order to reduce conflicts between maternal and paternal roles, education and employment (4).

In this study, having more siblings was related to higher number of desired children in women, the shorter first birth intervals, and pregnancy intervals in men and women. One key point in this regard is the effect that someone may receive from his family background variables. It may be related to common values of individuals in a family. Several studies have emphasized the essential role of social interaction for fertility behaviors (4, 25). The present study has several strengths. It was a part of the first national study about fertility preferences and desired numbers of children. The mentioned topic that has also not been studied among the general population in Iran. Convenience sampling was a limitation of our study. The sample was a part of the large study that has been conducted by MOH of Iran. The response rate was very good and neat to all of the couples answered to the questionnaire, therefore the study results reflect their opinions reliably. Further studies with a larger number of samples, or nationally representative studies are suggested to achieve to more precise findings. In addition, future studies are proposed to assess attitude of couples about fertility and their fertility awareness to obtain more interpretable findings.

Conclusion

In this study, preferences and desires related to the reproductive behavior on couple's prior to marriage were evaluated and one of the strong points that can be pointed out is that, these couples will serve as representatives for the whole province for the reason that all couples would refer to these 2 clinics for pre-marriage counseling. Majority of the participants of the present study express their desire to have one or two siblings. In considering the fact that what one desires does not always come into reality, the risk of reduced fertility is generally present in the community and in order to create a favorable environment for childbearing, appropriate policies should be implemented.

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References

1. Abbasi-Shavazi MJ, McDonald P. Fertility decline in the Islamic Republic of Iran, 1972- 2000. Asian Popul Stud. 2006; 2(3): 217-237.
2. Abbasi-Shavazi MJ, Morgan SP, Hossein-Chavoshi M, McDonald P. Family change and continuity in iran: birth control use before first pregnancy. J Marriage Fam. 2009; 71 (5): 1309-1324.
3. Peterson BD, Pirritano M, Tucker L, Lampic C. Fertility awareness and parenting attitudes among American male and female undergraduate university students. Hum Reprod. 2012; 27(5): 1375-1382.
4. Mills M, Rindfuss RR, McDonald P, te Velde E; ESHRE Reproduction and Society Task Force. Why do people postpone parenthood? Reasons and social policy incentives. Hum Reprod Update. 2011; 17(6): 848-860.
5. Shiri T, Bidarian S. Economic factors affecting the fertility of women 15-49 years old population employed in the school districts 22 of Tehran. Journal of Social Sciences. 2009; 3(3): 93-107.
6. Ding QJ, Hesketh T. Family size, fertility preferences, and sex ratio in China in the era of the one child family policy: results from national family planning and reproductive health survey. BMJ. 2006; 333(7564): 371-373.
7. Matsumoto Y, Yamabe S. Family size preference and factors affecting the fertility rate in Hyogo, Japan. Reprod Health. 2013; 10: 6.
8. Pham DT, Stephens EH, Antonoff MB, Colson YL, Dildy GA, Gaur P, et al. Birth trends and factors affecting childbearing among thoracic surgeons. Ann Thorac Surg. 2014; 98(3): 890-895.
9. Berrington A, Pattaro S. Educational differences in fertility desires, intentions and behaviour: a life course perspective. Adv Life Course Res. 2014; 21: 10-27.
10. Rindfuss RR, Guilkey D, Morgan SP, Kravdal O, Guzzo KB. Child care availability and first-birth timing in Norway. Demography. 2007; 44 (2): 345-372.
11. Habbema JD, Eijkemans MJ, Leridon H, te Velde ER. Realizing a desired family size: when should couples start? Hum Reprod. 2015; 30(9): 2215-2221.
12. Günther I, Harttgen K. Desired fertility and number of children born across time and space. Demography. 2016; 53(1): 55-83.
13. Goldstein J, Lutz W, Testa MR. The emergence of sub-replacement family size ideals in Europe. Popul Res Policy Rev. 2003; 22(5): 479-496.
14. Morita M, Ohtsuki H, Sasaki A, Hiraiwa-Hasegawa M. Factors affecting the number of children in five developed countries: a statistical analysis with an evolutionary perspective. Letters on Evolutionary Behavioral Science. 2012; 3(1): 7-11.
15. Kravdal Ø. Education and fertility in sub-Saharan Africa: individual and community effects. Demography. 2002; 39(2): 233-250.
16. Tavousi M, Motlagh ME, Eslami M, Haerimehrizi A, Hashemi A, Montazeri A. Fertility desire and its correlates: a pilot study among married citizens living in Tehran, Iran. Payesh. 2015; 14(5): 597-605.
17. Amuedo-Dorantes C, Kimmel J. The motherhood wage gap for women in the United States: the importance of college and fertility delay. Rev Econ Househ. 2005; 1: 17-48.
18. Eriksson C, Larsson M, Skoog Svanberg A, Tydén T. Reflections on fertility and postponed parenthood-interviews with highly educated women and men without children in Sweden. Ups J Med Sci. 2013; 118(2): 122-129.
19. Yoldemir T. Fertility in midlife women. Climacteric. 2016; 19(3): 240-246.
20. Carolan M, Frankowska D. Advanced maternal age and adverse perinatal outcome: a review of the evidence. Midwifery. 2011; 27(6): 793-801.
21. Kenny LC, Lavender T, McNamee R, O'Neill SM, Mills T, Khashan AS. Advanced maternal age and adverse pregnancy outcome: evidence from a large contemporary cohort. PLoS One. 2013; 8(2): e56583.
22. Balasch J, Gratacós E. Delayed childbearing: effects on fertility and the outcome of pregnancy. Curr Opin Obstet Gynecol. 2012; 24(3): 187-193.
23. Virtala A, Vilska S, Huttunen T, Kunttu K. Childbearing, the desire to have children, and awareness about the impact of age on female fertility among Finnish university students. Eur J Contracept Reprod Health Care. 2011; 16(2): 108-115.
24. Sahib Khalil N, Israa TH, Hussam DS. Fertility awareness among medical and non- medical undergraduate university students in Al-Iraqia University, Baghdad, Iraq. American Journal of Medical Sciences and Medicine. 2015; 3(6): 74-78.
25. Regnier-Loilier A. Influence of Own sibship size on the number of children desired at various times of life: the case of France. Population. 2006; 61(3): 165-194.

Original Article

Tehran Survey of Potential Risk Factors for Multiple Births

Reza Omani Samani, M.D.[1], Amir Almasi-Hashiani, M.Sc.[1], Samira Vesali, M.Sc.[1], Fatemeh Shokri, M.Sc.[1], Rezvaneh Cheraghi, M.Sc.[1], Farahnaz Torkestani, M.D.[2], Mahdi Sepidarkish, M.Sc.[1*]

1. Department of Epidemiology and Reproductive Health, Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
2. Department of Obstetrics and Gynecology, Shahed University of Medical Sciences, Tehran, Iran

Abstract

Background: The multiple pregnancy incidence is increasing worldwide. This increased incidence is concerning to the health care system. This study aims to determine the frequency of multiple pregnancy and identify factors that affect this frequency in Tehran, Iran.

Materials and Methods: This cross-sectional study included 5170 mothers in labor between July 6-21, 2015 from 103 hospitals with Obstetrics and Gynecology Wards. The questionnaire used in this study consisted of five parts: demographic characteristics; information related to pregnancy; information related to the infant; information regarding the multiple pregnancy; and information associated with infertility. We recruited 103 trained midwives to collect data related to the questionnaire from eligible participants through an interview and medical records review. Frequencies and odds ratios (OR) for the association between multiple pregnancy and the selected characteristics (maternal age, economic status, history of multiple pregnancy in first-degree relatives, and reproductive history) were computed by multiple logistic regression. Stata software, version 13 (Stata Corp, College Station, TX, USA) was used for all statistical analyses.

Results: Multiple pregnancy had a prevalence of 1.48% [95% confidence interval (CI): 1.19-1.85]. After controlling for confounding variables, we observed a significant association between frequency of multiple pregnancy and mother's age (OR=1.04, 95% CI: 1.001-1.09, P=0.044), assisted reproductive technique (ART, OR=6.11, 95% CI: 1.7-21.97, P=0.006), and history of multiple pregnancy in the mother's family (OR=5.49, 95% CI: 3.55-9.93, P=0.001).

Conclusion: The frequency of multiple pregnancy approximated results reported in previous studies in Iran. Based on the results, we observed significantly greater frequency of multiple pregnancy in older women, those with a history of ART, and a history of multiple pregnancy in the mother's family compared to the other variables.

Keywords: Multiple Pregnancy, Pregnancy, Labor, Cross-Sectional Study, Prevalence Rate

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Introduction

The occurrence of twin and multiple pregnancies has increased in developed countries (1) and is associated with concern in the health care system. Multiple pregnancy results in premature delivery, underweight newborns, and increased congenital anomalies. The worst outcome is maternal and neonatal mortality (2). The existing evidence shows a significantly lower one-year survival in multiple infants compared to singletons. The frequency of growth disorders, as well as physical and mental disabilities

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*Corresponding Address: P.O.Box: 16635-148, Department of Epidemiology and Reproductive Health, Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
Email: Mahdi.sepidarkish@gmail.com

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is higher in multiple newborns, if the infants survive (3, 4). A few studies conducted in Iran have reported a frequency of twin pregnancy from 1.5 to 8% (5-7). However, these studies were frequently conducted in one or several hospitals. Most were retrospective studies that reviewed the records. Inconsistency in reporting the frequency of multiple pregnancy could be due to structural differences in the populations studied and design effect and systematic errors (selection or information bias), in addition to changes in the frequency of the interested outcome over time (8). Hence, it is necessary to accurately identify the frequency of multiple pregnancy and impacting factors which lead to identification of high-risk groups and increased care for these groups, and assists authorities and policy makers in evidence-based decision making to increase cost effectiveness of the interventions. The aim of this study is to determine the frequency of twin and multiple pregnancy and to identify factors that affect the frequency of this phenomenon in Tehran Province, one of the main provinces in Iran.

Materials and Methods

We conducted a cross-sectional study in Tehran Province, Iran which included the twenty-fifth most populated city worldwide-the capital of Iran (9). Participants comprised 5170 mothers in labor between July 6 to 21, 2015 who referred to the Obstetrics and Gynecology Wards of 103 hospitals. These hospitals are affiliated with Tehran, Beheshti, and Iran medical universities, which oversee and mange 19 (Tehran), 43 (Beheshti), and 41 (Iran) hospitals. We included all women in this study regardless of the type of delivery (natural or cesarean section) and the pregnancy outcome (live birth, stillbirth, or spontaneous abortion).

The Ethical Committee of Royan Institute approved this study (EC/92/1097). All participants received complete explanations about the study aims and data confidentiality, which mentioned their complete freedom to participate. Eligible individuals were also assured that acceptance or refusal to participate in the research had no influence on their treatment procedures. Completion of the questionnaire was considered as written informed consent.

According to the 2% prevalence of multiple pregnancy in the population (10), the effect size of 0.006 and a design effect of approximately 2, we estimated the required minimum sample size to be approximately 4181 pregnant women (α=0.05). The dependent variable studied was multiple pregnancy (twin or higher). The questionnaire used in the study included five parts: demographic characteristics (13 items); information related to pregnancy (26 items); information related to the infant (15 items); information regarding the multiple pregnancy (18 items); and information associated with infertility (7 items).

Face and content validity

A total of 10 experts in gynecology, sexology, and methodology assessed face and content validity of the questionnaire. The validity index for each question and total validity were calculated (11). To equalize the experts' perceptions of content validity indices (relevancy, clarity, and comprehensiveness of the tool), we sent the definitions of these indices with the questionnaire. Relevancy, clarity, and comprehensiveness were defined as follows. Relevancy was the ability of selected questions in order to reflect the content, lucidity of the questions concerned their wording, concept was clarity, and the instrument's ability to include all content domains or areas was considered comprehensiveness. The experts were asked to review clarity and relevancy of each item, and comprehensiveness of the total questionnaire. Scores were given as: 1 (inappropriate), 2 (somewhat appropriate), 3 (appropriate), and 4 (quite appropriate). Experts' responses were gathered within 1 to 3 weeks (12).

Data related to the questionnaire was collected from eligible participants through interviews conducted by 103 trained midwives. If pain was a barrier to mothers' responses to the questionnaire, data were taken after childbirth at the time of admission in the hospital, which took 24 hours. To ensure valid and reliable data collection, the following actions were taken. We conducted three training sessions for midwives who collected the data. In these sessions, the correct way to collect data, definition of variables, and creation of a common perception among midwives were considered. A pilot study for operational feasibility and identification of implementation problems and difficulties related to

the questionnaire was conducted in five hospitals. A visit to hospitals without previous coordination for examining how to complete the questionnaires.

Statistical analysis

Categorical and continuous variables were summarized as number (%) and mean (SD). The frequency of multiple pregnancies was calculated as the percentage of multiple pregnancies by mother's age, history of infertility, assisted reproductive technique (ART), history of multiple pregnancy in the mother's family, history of multiple pregnancy in the father's family, the mother born of multiple pregnancy, and the father born of multiple pregnancy. Crude odds ratios (OR) for the association between the selected characteristics (maternal age, economic status, a history of multiple pregnancy in first-degree relatives, and reproductive history) and multiple pregnancy were computed by univariate logistic regression. In the analysis we considered hospitals as a cluster. Multivariate logistic regression was used to adjust OR simultaneously for the aforementioned variables. Criteria for model building was based on the Hosmer-Lemeshow method (13). Results were presented as OR with 95 % confidence intervals (CI) (14). The Hosmer-Lemeshow test was used for goodness of fit of the model (15). We used Stata version 13 (Stata, College Station, TX, USA) for statistical analysis.

Results

In this study, the IRA (Inter Rater Agreement) relevancy of the questions was 78.34% with a clarity of 92.78%. The questionnaire had a total relevancy of 86.23%, clarity of 87.48%, and comprehensiveness of 82%. In this survey we examined 5170 eligible pregnant women. Among the examined pregnancies, there were 5093 single cases and 77 multiple cases. Multiple pregnancy had a frequency of 1.48% (95% CI: 1.19-1.85). Mothers had a mean age of 29.23 years (95% CI: 29.08-29.38). Mothers with single pregnancy had a significantly lower mean (SD) age of 29.20 (5.46) compared to 30.98 (5.86) for mothers with multiple pregnancy (P=0.004, Table 1).

Table 1: Association between multiple pregnancy and potential predictors

Variable	Multiple pregnancies		Crude OR	95% CI for OR
	Yes	No		
Mother's age (Y)				
Mean (SD)	30.98 (5.86)	29.20 (5.46)	1.05	1.01-1.10
Type of pregnancy				
Wanted	63 (1.56)	4069 (98.48)	1	-
Unwanted	14 (1.37)	1006 (98.63)	0.89	0.50-1.61
History of infertility				
No	55 (1.15)	4719 (98.85)	1	-
Yes	22 (5.70)	364 (94.30)	5.18	3.12-8.59
Received assisted reproductive technique (ART)				
No	57 (1.16)	4876 (98.84)	1	-
Yes	20 (8.44)	217 (91.56)	7.88	4.65-13.35
History of multiple pregnancy in mother's family				
No	24 (0.62)	3832 (99.38)	1	-
Yes	53 (4.05)	1257 (95.95)	6.73	4.13-10.94
History of multiple pregnancy in father's family				
No	50 (1.20)	4100 (98.80)	1	-
Yes	27 (2.66)	989 (97.34)	2.23	1.39-3.59
Mother as the outcome of multiple pregnancy			1	
No	74 (1.45)	5014 (98.55)		-
Yes	3 (3.85)	75 (96.15)	2.71	0.83-8.79
Father as the outcome of multiple pregnancy				
No	75 (1.47)	5012 (98.53)	1	-
Yes	2 (2.53)	77 (97.47)	1.73	0.41-7.19

OR; Odds ratio and CI; Confidence interval.

There were 237 (4.58%) cases treated with ART. The frequency of multiple pregnancy was 1.15% in women who did not receive ART (95% CI: 0.08-1.49), while the frequency of multiple pregnancy was 8.44% in women who received ART (95% CI: 5.5-12.72). Using logistic regression analysis, we estimated the OR for the association between ART and multiple pregnancy to be approximately 7.88 (95% CI: 4.65-13.35, P<0.001). Hence, the frequency of multiple pregnancy in women who received ART was 7.88 times greater.

As seen in Table 2, a significant association existed between variables such as mother's age (OR=1.04, 95% CI: 1.001-1.09, P=0.044), ART (OR=6.11, 95% CI: 1.7-21.97, P=0.006), and history of multiple pregnancy in the mother's family (OR=5.49, 95% CI: 3.55-9.93, P=0.001) with the frequency of multiple pregnancy after controlling for other variables in this table. No significant association existed between the frequency of multiple pregnancy and other variables. The goodness of fit test was performed for the final version, which showed a good fit of the model (Hosmer-Lemeshow chi2=5.57, P=0.695).

Table 2: Demographic characteristic and the first birth interval according to gender

Variable	Adjusted OR	95% CI	P value
Mother's age (Y)	1.04	1.01-1.09	0.044
ART	6.11	1.70-21.97	0.006
History of multiple pregnancy in mother's family	5.94	3.55-9.93	0.001
Type of pregnancy	0.81	0.44-1.51	0.518
History of infertility	0.94	0.27-3.25	0.929
History of multiple pregnancy in father's family	1.31	0.79-2.17	0.293
Mother as the outcome of multiple pregnancy	1.30	0.38-4.47	0.669
Father as the outcome of multiple pregnancy	1.88	0.43-8.24	0.399

ART; Assisted reproductive technique, OR; Odds ratio, and CI; Confidence interval.

Discussion

After remarkable reduction in multiple births during the second half of the twentieth century, most recently a steady increase exists in multiple births and its adverse subsequent consequences worldwide (16). Studies have shown that the majority of this increase is due to the increased age at pregnancy and the emergence of ART. In the United States from 1972 to 1999, there were 6 times more triplets and 12 times more multiples than the past. If women who became pregnant at an older age were considered in the calculation, the above prevalence would increase approximately 50-60 times (17). Iran, like other developing countries, has experienced major changes in the structure of its population. The socio-economic development and establishment of health care networks caused major changes in indicators of population health and epidemiology in Iran (18, 19). Demographic information, mainly derived from the census10 years once in Iran, along with health indicators confirmed the above mentioned. Fertility indicators (birth and total fertility rate) showed that since 2000, Iran has experienced a downward trend and the population growth rate has been close to one. By taking into account the age composition of the community, we have found that the population is increasing in age, whereas the relative frequency of marriage has decreased and the age of marriage increased in both men and women (9). During the last 10 years, no study has evaluated the frequency of multiple pregnancy and its trend. With regard to information obtained in a few studies, the results have suggested a subtle increase in multiple births in Iran. The highest frequency reported was 2% estimated from the last study conducted in 2005 in three large teaching hospitals in Tehran (10). In the current study, multiple pregnancy had a frequency of 1.48 (95% CI: 1.19-1.85), which approximated the frequency reported in previous studies. The rate has been affected by Genetics agents and ART. Therefore, it differs in various regions of the world. Bortolus et al. (20) carried out a systematic review on the epidemiology of multiple births. The results showed a higher frequency of multiple births in African countries and the black race compared with other countries. The lowest frequency was reported from Japan and Southeast Asian countries. Our results showed a moderate rate in Iran.

An international committee for monitoring ART suggested that one embryo should be transferred per cycle (21). Saraswat et al. (22) conducted a systematic review in 2010. The results indicated that infertility centers increased the number of embryos transferred (sometimes up to 4 embryos) according to domestic law and patient preference. In the current study, the OR for an association be-

tween ART and multiple pregnancy was estimated at 7.88 (95% CI: 4.65-13.35), which confirmed findings of other studies (23). Another systematic review on studies from 1950 to 2010 in the United States revealed that 20% of twins, 40% of triplets, and 71% of other types of multiple pregnancy were caused by ovarian stimulation whereas 16% of twins, 45% of triplets, and 30% of other types were the result of IVF (16).

Martikainen et al. (24) conducted a study in four centers in 2001. The results showed the clinical pregnancy rate per transfer was 32.4% in the one embryo transfer group and 47.1% in the two embryo transfer group. The relative risk for twin birth was 10.18. Mclernon et al. (25) reported a relative risk for twin birth of approximately 24.4 (95% CI: 3.42-173.8), which indicated a very high risk for twin pregnancy after the transfer of two or more embryos. A review study conducted in 2002 by De Sutter et al. (26) compared double embryo transfer (DET) to single embryo transfer (SET) according to the results of the 7407 cycles in 6 cohort studies. Overall, the pregnancy success rate had no significant difference between the two procedures (SET: 33.9%-DET: 35%) Twins were 1% in SET which increased to 32.6% in DET. In the current study, we have observed a direct association between the mother's age and multiple pregnancy. Age is one of the risk factors for multiple pregnancy. The trend for this type of pregnancy exactly depended on the pattern of change in women's age at marriage (27, 28).

Adashi et al. (29), in a research conducted in the United States, found that 20% of the increase in twin births was attributed to the reproductive age of women. From the remaining 80%, ovulation with fertility drugs comprised 40 and 40% was attributed to IVF. In Denmark, an increase in multiple births was seen exclusively in women 30 years of age and older; most of the pregnancies were dizygotic. Blondel and Kaminski (30) have reported a one-quarter to one-third increase in twin pregnancies attributable to an increase in reproductive age in women. The increase in reproductive age is effective in twin birth of dizygotic, but the rate of monozygotic pregnancies is constant with changes in maternal age. This is due to the increase in gonadotropin levels with increasing age (31). Maximum follicle stimulation occurs between the ages of 35-39 years, after which ovarian function declines. Another finding of our study was the strong association between multiple pregnancy and a positive history of multiple births in the mother's family. This association was not seen between multiple pregnancy and a positive history of multiple birth in the father's family. In a case-control study in Italy the OR in women who had a history of multiple pregnancy in their first-degree relatives were 2.4 for dizygotes and 2.7 for monozygotes (14). Baldwin (32), observed these findings only in dizygotic twins. In a systematic review by Bortolus et al. (20) on factors that affected multiple births, an increase existed in the risk of multiple pregnancy in those with a history of multiple pregnancy in their first-degree relatives. The findings were confirmed for dizygotic twins. The mechanism of this association was explained in 1970 by Bulmer (33).

Our study was the first survey conducted with the large sample sizes from both public and private hospitals that had no selection bias (response proportion: 100%). We attempted to hold the same training session for interviewers (midwives) to minimize information bias. A pilot study carried out at the beginning of the study during over one week detected operational problems, and examined reliability and validity of the questionnaire. Our study has several limitations. First, the cross-sectional nature of the study did not allow for conclusions on causality due to the because of temporality between the exposure and outcome. Second, in this study we only assessed multiple pregnancy without considering the type of multiple pregnancy (i.e., monozygote or dizygote).

Conclusion

Based on the our study results, frequency of multiple pregnancy in older women, women with history of ART, and a history of multiple pregnancy in the mother's family had a significant relationship with increased frequency of multiple pregnancy. We observed no significant relationship between the frequency of multiple pregnancy and other included variables.

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References

1. Collins J. Global epidemiology of multiple birth. Reprod Biomed Online. 2007; 15 Suppl 3: 45-52.
2. Multiple gestation pregnancy. The ESHRE Capri Workshop Group. Hum Reprod. 2000; 15(8): 1856-1864.
3. Daltveit AK, Vollset SE, Skjærven R, Irgens LM. Impact of multiple births and elective deliveries on the trends in low birth weight in Norway, 1967-1995. Am J Epidemiol. 1999; 149(12): 1128-1133.
4. Joseph KS, Kramer MS, Marcoux S, Ohlsson A, Wen SW, Allen A, et al. Determinants of preterm birth rates in Canada from 1981 through 1983 and from 1992 through 1994. N Engl J Med. 1998; 339(20): 1434-1439.
5. Khademi A, Alleyassin A, Amini M, Ghaemi M. Evaluation of sexual dysfunction prevalence in infertile couples. J Sex Med. 2008; 5(6): 1402-1410.
6. Safarinejad MR. Infertility among couples in a population-based study in Iran: prevalence and associated risk factors. Int J Androl. 2008; 31(3): 303-314.
7. Vahidi S, Ardalan A, Mohammad K. Prevalence of primary infertility in the Islamic Republic of Iran in 2004-2005. Asia Pac J Public Health. 2009; 21(3): 287-293.
8. Kazem M, Ali A. An overview of the epidemiology of primary infertility in Iran. J Reprod Infertil. 2009; 10(3): 213-216.
9. Davatchi F, Jamshidi AR, Banihashemi AT, Gholami J, Forouzanfar MH, Akhlaghi M, et al. WHO-ILAR COPCORD study (stage 1, urban study) in Iran. J Rheumatol. 2008; 35(7): 1384.
10. Kavehmanesh Z, Torkaman M, Haghiri M. Frequency of multiple pregnancy and its complications in three educational hospitals, tehran. Iran J Pediatr. 2007; 17(Suppl 2): 261-267.
11. Polit DF, Beck CT, Owen SV. Is the CVI an acceptable indicator of content validity? Appraisal and recommendations. Res Nurs Health. 2007; 30(4): 459-467.
12. Asadi-Lari M, Ahmadi Pishkuhi M, Almasi-Hashiani A, Safiri S, Sepidarkish M. Validation study of the EORTC information questionnaire (EORTC QLQ-INFO25) in Iranian cancer patients. Support Care Cancer. 2015; 23(7): 1875-1882.
13. Hosmer Jr DW, Lemeshow S, Sturdivant RX. Applied logistic regression. 3rd ed. John Wiley & Sons; 2013.
14. Parazzini F, Tozzi L, Bocciolone L, Molteni E, Moreschi C, Fedele L. Risk factors for multiple births. Acta Obstet Gynecol Scand. 1993; 72(3): 177-180.
15. Lemeshow S, Hosmer DW Jr. A review of goodness of fit statistics for use in the development of logistic regression models. Am J Epidemiol. 1982; 115(1): 92-106.
16. Black M, Bhattacharya S. Epidemiology of multiple pregnancy and the effect of assisted conception. Seminars in Fetal and Neonatal. 2010; 15(6): 306-312.
17. Martin JA, Park MM. Trends in twin and triplet births: 1980-97. Natl Vital Stat Rep. 1999; 47(24): 1-16.
18. Aghajanian A, Mehryar AH. Fertility transition in the Islamic Republic of Iran: 1976-1996. Asia Pac Popul J. 1999; 14(1): 21-42.
19. Dhonte P, Bhattacharya R, Yousef T. Demographic transition in the middle east-implications for growth, employment, and housing. International Monetary Fund. 2000.
20. Bortolus R, Parazzini F, Chatenoud L, Benzi G, Bianchi MM, Marini A. The epidemiology of multiple births. Hum Reprod Update. 1999; 5(2): 179-187.
21. Mansour R, Ishihara O, Adamson GD, Dyer S, de Mouzon J, Nygren KG, et al. International Committee for Monitoring Assisted Reproductive Technologies world report: assisted reproductive technology 2006. Hum Reprod. 2014; 29(7): 1536-1551.
22. Saraswat L, Bhattacharya S, Maheshwari A, Bhattacharya S. Maternal and perinatal outcome in women with threatened miscarriage in the first trimester: a systematic review. BJOG. 2010; 117(3): 245-257.
23. Wilcox LS, Kiely JL, Melvin CL, Martin MC. Assisted reproductive technologies: estimates of their contribution to multiple births and newborn hospital days in the United States. Fertil Steril. 1996; 65(2): 361-366.
24. Martikainen H, Tiitinen A, Tomás C, Tapanainen J, Orava M, Tuomivaara L, et al. One versus two embryo transfer after IVF and ICSI: a randomized study. Hum Reprod. 2001; 16(9): 1900-1903.
25. McLernon DJ, Harrild K, Bergh C, Davies MJ, de Neubourg D, Dumoulin JC, et al. Clinical effectiveness of elective single versus double embryo transfer: meta-analysis of individual patient data from randomised trials. BMJ. 2010; 341: c6945.
26. De Sutter P, Gerris J, Dhont M. A health-economic decision-analytic model comparing double with single embryo transfer in IVF/ICSI. Hum Reprod. 2002; 17(11): 2891-2896.
27. Dickey RP, Taylor SN, Lu PY, Sartor BM, Rye PH, Pyrzak R. Risk factors for high-order multiple pregnancy and multiple birth after controlled ovarian hyperstimulation: results of 4,062 intrauterine insemination cycles. Fertil Steril. 2005; 83(3): 671-683.
28. Gleicher N, Oleske DM, Tur-Kaspa I, Vidali A, Karande V. Reducing the risk of high-order multiple pregnancy after ovarian stimulation with gonadotropins. N Engl J Med. 2000; 343(1): 2-7.
29. Adashi EY, Barri PN, Berkowitz R, Braude P, Bryan E, Carr J, et al. Infertility therapy-associated multiple pregnancies (births): an ongoing epidemic. Reprod Biomed Online. 2003; 7(5): 515-542.
30. Blondel B, Kaminski M. Trends in the occurrence, determinants, and consequences of multiple births. Semin Perinatol. 2002; 26(4): 239-249.
31. Blickstein I, Keith LG. Multiple pregnancy: epidemiology, gestation, and perinatal outcome. UK: The Parthenon Publishing Group; 2005.
32. Baldwin VJ. Pathology of multiple pregnancy. New York: Springer Science & Business Media; 2012.
33. Bulmer M. The twinning rate in Europe and Africa. Ann Hum Genet. 1960; 24: 121-125.

Ethics, Legal, Social, Counseling Article

Personhood and Moral Status of The Embryo: It's Effect on Validity of Surrogacy Contract Revocation according to Shia Jurisprudence Perspective

Saeid Nazari Tavakkoli, Ph.D.

Department of Jurisprudence and Principles of Islamic Law, Faculty of Theology and Islamic Studies, Tehran University, Tehran, Iran

Abstract

Background: One of the most controversial issues related to the human embryo is the determination of the moment when an embryo is considered a human being and acquires a moral status. Although personhood and moral status are frequently mentioned in medical ethics, they are considered interdisciplinary as concepts that shape the debate in medical law (fiqh) since their consequences are influential in the way which the parents and other individuals behave towards the embryo.

Materials and Methods: This analytical-descriptive research gathered relevant data in a literature search. After a description of the fundamentals and definitions, we subsequently analyzed juridical texts and selected one of the viewpoints that regarded the surrogacy contract revocation.

Results: The surrogacy contract is a contract based upon which two sides (infertile couple and surrogate mother) involved in making the contract are obligated to fulfill its terms. Therefore, contract revocation can be surveyed from three perspectives: mutual revocation (iqala), legal unilateral wills (khiar al-majlis, khiar al-ayb), and contractual wills (khiar al-shart).

Conclusion: Revocation of a surrogacy contract either by the genetic parents, surrogate or the fertility clinic is allowed by Muslim jurists only when the embryo lacks personhood. Based on Islamic teachings, the termination of a surrogacy contract in and after the sixteenth week of pregnancy, when the embryo acquires a human soul (ensoulment), is not allowed. However religious thought emphasizes the moral status of the fetus before the sixteenth week and states that optional termination of the surrogacy contract is not permitted while the fetus becomes a human being.

Keywords: Personhood, Moral Status, Embryo

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Introduction

The desire to have children is associated with the creation of human beings. Surrogacy is one method used by infertile couples who wish to have a baby. The first legal surrogacy agreement was enacted in the mid-1970s. The first paid traditional surrogacy arrangement was conducted in the United States in 1980. In 1983, the first successful pregnancy occurred via egg donation. This event later led to the first gestational surrogacy in the United States in 1985 (1, 2). In a study reported the first surrogate gestational pregnancy that resulted

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*Corresponding Address: P.O.Box: 15766-1-3111, Department of Jurisprudence and Principles of Islamic Law, Faculty of Theology and Islamic Studies, Tehran University, Motahari Street, Tehran, Iran
Email: sntavakkoli@ut.ac.ir

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in heated debates in the UK. In 1985, the UK established the Surrogacy Law (3) which permitted surrogacy under special circumstances. Therefore, the UK became one of the few European countries where a surrogacy contract was permitted under specific circumstances. In 1959, Patrick Steptoe and Professor Robert Edwards initially introduced the surrogacy contract in Europe. Finally, in 1990, the UK passed the Human Fertilization and Embryology Act (the 1990 Act), which was revised in 2008 (4). However, since the United States has legally allowed commercial surrogacy contracts, the majority of these contracts reportedly occur in this country.

In Iran, the first infertility center was inaugurated in Yazd in 1989. The Gamete and Embryo Donation law (5) was passed in 2003 which has permitted gestational surrogacy for infertile couples. Gestational surrogacy is when *in vitro* fertilization (IVF) is performed after aspiration of the husband's sperms and wife's eggs. The zygote or embryo is subsequently transferred into the womb of the surrogate mother to naturally spend his early development. After birth, the surrogate then gives the child to the intended (genetic) parents. Use of a surrogate and surrogacy during pregnancy is divided into several classes based on the presence or absence of a genetic link between the surrogate and the intended parents. The most common type of surrogacy is "full surrogacy" that has no genetic link (6). Full surrogacy is used when there is a fertility disorder or dysfunction such as habitual abortion or another unknown problem. In this case, the ovum is fertilized by husband's sperm in vitro and the embryo is subsequently transferred into the surrogate uterus (7, 8).

Materials and Methods

This analytical-descriptive research gathered the relevant data as a literature search. After describing the jurisprudents' fundamentals, we subsequently analyzed the Shiāat fiqh sources. This was not a clinical trial, hence no need existed for patient consent or Ethical Committee approval.

Validity of the surrogacy contract

The surrogacy contract is an arrangement between an infertile couple and a woman who agrees to conceive with reproductive methods using their embryo. Based on this contract, the surrogate agrees to accept transfer of the embryo from the genetic parents to her uterus. The surrogate accepts responsibility to maintain the pregnancy and perform conventional measures for fetal growth until the child is born. She agrees to refrain from risky, harmful behaviors that affect fetal growth and consents to return the child to the infertile (genetic) couple after its birth. There are two, legal surrogacy contracts: commercial surrogacy and altruistic surrogacy. If the genetic parents (with the surrogate's agreement) guarantee compensation for her involvement, this contract is termed a commercial surrogacy. However, altruistic surrogacy is when the surrogate accepts to become pregnant and deliver the baby to the parents without any financial compensation and only for charity motives. The surrogacy contract has three essential participants: surrogate mother, infertile couple (sperm owner and ovule owner) and the fertility clinic. The fertility clinic is the therapist center in which necessary actions for fertilization of the infertile couple's sperm and ovule is performed which results in its subsequent placement into the surrogate mother's uterus by a necessary medical procedure.

This agreement is legal according to article 10 of the civil law, "freedom of contracts", and permitted (ebahah principle) (9) since it is not contrary to the law. This contract is valid and effective, and Commercial and Altruistic of this contract has not effect on its being lawful (javaz) or validity (sehhat). Although the principle of autonomy sovereignty and freedom of contracts can prove the legality of a surrogacy contract, of note, such an argument may not suffice since the sanctity of using reproductive organs outside the context of marriage can be the primary obstacle to the effect of the autonomy sovereignty. The need to respect the precautionary principle (asle ehtiat) in such cases is an obstacle to perform freedom of contracts principle (ebahah principle). Thus, the validity of a surrogacy contract faces a fundamental problem (10).

Contract revocation due to cancellation by the parties of the contract

Based on the principles of Islamic Law and jurisprudence, the contract can be revoked (11). It is possible only due to the application of one of the legal wills (khiar; khiar al-majlis, khiar al-ayb) or

contractual wills (khiar al-shart). A surrogacy contract is a contract based upon which the two sides involved in making the contract are obligated to fulfill its terms unless a health risk or problem occurs for the surrogate mother. Therefore, contract revocation can be surveyed from two perspectives:

Mutual revocation

Iqala is one of the ways to terminate the commitments whereby the contract can be annulled by mutual agreement of the parties (12) and avoid its sequence effects in the future. Annulment of a surrogate contract by iqala is examined in three assumptions.

Mutual revocation before starting the process

If the genetic parents, surrogate mother, and fertility clinic agree upon creation of the embryo and its transfer to the surrogate's uterus and they sign a contract with the mediation of the fertility clinic but no embryo has yet formed *in vitro*, can the contract be revoked by cancellation of each of the parties involved? There is no doubt that the surrogacy contract is a continuous contract whereby, before the process (the issue of contract), mutual revocation (iqala) exists (13). However, regarding the fact that mutual revocation (iqala) is permissible according to mutual agreement of the parties (tarazi), can either of the parties involved (the genetic parents, surrogate mother or fertility clinic) alone dissolve the contract or is it dependent on mutual agreement of all the members?

If, after signing the contract, the genetic parents are not inclined to give their sperm and egg to the fertility clinic to form the embryo during the process of fertilization, then the fertility clinic and the surrogate mother have no commitment to the terms of the contract. Furthermore, if the fertility clinic is not willing to perform IVF and its transfer to the surrogate's uterus, the surrogate's commitment to the contract is meaningless. Finally, if the surrogate refrains from pursuing the contract and she does not allow the fertility clinic to implant an infertile couple's IVF embryo into her uterus, no issue will remain for the surrogacy contract. Ethically she has a duty for the contract's execution, but she cannot be forced to uphold the contract. There is a longitudinal relationship between the effective parties' demands and their commitment to this contract. Each party's commitment is an essential condition for contract execution and no action can be considered a sufficient condition, however the commitment of all parties toward the terms of the contract is a sufficient condition for its performance. In this case when one of the parties is not inclined to continue the surrogacy contract and decides to terminate the contract, it will automatically be terminated due to the loss of its subject. It is obvious that this revocation results from failure to commit from each of the parties, and is not due to iqala. Therefore, each party's will in the surrogacy contract is alone sufficient for contract revocation, either with iqala that includes the mutual consent of all parties or not to commit to contract terms that would lead to termination of the contract.

Mutual revocation after egg cell formation and before implantation in the surrogate's uterus

If the fertility clinic performs IVF after the request and consent of the genetic parents and agreement of the surrogate, the contract revocation can be surveyed from three perspectives: i. The decision made by one or both genetic parents about not having a child, ii. Refusal of the fertility clinic to continue the process, or iii. Refusal of the surrogate to lend her uterus for the embryo's implantation. The common point in these three assumptions is that cancellation of each of the parties involved will destroy the human embryo. If the created embryo is not used for scientific research or donated to other infertile couples or frozen for the genetic parents' future use, will this be embryo destruction or prevention of its life. Is this permissible or not? In the first and second cases, the request of contract revocation from the genetic parents or fertility clinic, and lack of commitment to contract's terms results in destruction of the embryo. However, the third case that the surrogate refuses to lend her uterus does not prevent the embryo from surviving because the embryo can survive in vitro before being implanted in another uterus. Although, finding other appropriate surrogates takes time and the golden time for implantation could be lost.

Permitting or not permitting the destruction of a human embryo is based upon two issues: the personhood of the embryo and the condition of embryo placement in the uterus. If we consider the zygote as a human being after its formation, then we should

accept that it possesses human dignity and therefore each action which leads to its destruction will be unlawful. Moreover, if we accept the validity of embryo placement in the uterus, not in terms of its subject matter, but only in terms of its method (14), the refusal of genetic parents, the fertility clinic, or both to follow surrogacy contract terms is still not allowed since it will destroy a human being. However if before ensoulment, the fetus or human embryo is not considered to be a human being or we consider its placement in the uterus as a subject, it will be allowable. Because the prevention to perform the contract's terms and it's termination does not cause the destruction of a human being and this beings doesn't place in surrogate's uterus. The Qur'an considers two stages of embryo development: before and after ensoulment (15).

Thus, human life begins with its ensoulment, as its revocation occurs with separation of the soul from the body (16, 17). There is no doubt that a human embryo after ensoulment is considered a human being; any action that leads to its death (abortion) is the killing of a living creature (manslaughter) and this is an unlawful action. Whoever performs this action, regardless of whether it is the mother, father or any other person must pay blood money for killing a full-grown man in addition to eternal punishment. Nevertheless, this judgment does not imply that the human embryo lacks personhood before ensoulment (i.e., before the sixteenth week, from the 4th month onwards) and therefore it will ethically and religiously be allowed to perish since the Qur'an considers beginning of human creation from the moment of egg cell (Notfa) creation (18).

The egg cell does not refer only to male sperm but it refers to the fertilized male sperm and female egg (zygote) (Notfa Amshaj) (19). Since the egg cell will fail to humanize before it is implanted in the uterine wall, God considers implantation in the uterine wall as the precondition for human creation (according to the author's understanding of this verse) (20). Accordingly, after implantation, after the second week onwards, the human being will undoubtedly be formed. The question is whether any human person exists before this period? The simplest interpretation is to say that an egg cell is not considered a human before reaching this stage (i.e., from the baseline week until the second week). In other words, when the egg cell is only an egg cell (21).

However, a human can be defined since the formation of the egg cell, that is, shortly before reaching the implantation stage. A proof of this claim, in addition to narratives of the infallible Imams (22), is the understanding of Muslim jurists (foqaha). Therefore, the request for surrogacy contract dissolution is an unlawful (haram) act since it causes the death of a potential life. In the third case where the genetic parents and the fertility clinic are committed, the parents provide sperm and ovule to the institution and the institution creates a germ cell and embryo, but the surrogate has requested to terminate the contract. She does not lend her uterus for implantation despite the previous agreement. What should be done?

According to the principle of autonomy sovereignty and a human's inherent genetic ownership of his/her body's integrity (23, 24) as well as the lack of legal hindrance to terminate the contract in terms of self-preservation, the contract is terminated by the request of the surrogate and consent of the other parties of the contract. Because the duty of self-preservation, is not a duty that only the surrogate should notice; and the other women could not have a mediating role and they could not perform it until when the surrogate cancel it, potential human is destroyed. But if there is no consent by the other parties of the contract, there will not be iqala and the surrogate must fulfill her commitment. Until the surrogate does not lend her uterus, it cannot act on the contract terms and create a human. On the other hand, there is not a binding force of the surrogate to the nine months of pregnancy acceptation and the surrogacy contract is automatically terminated. It is clear that the surrogate mother is responsible to compensate for the losses imposed on the genetic parents or fertility clinic since contract revocation by the person who has requested it does not negate compensation for the losses that result from revocation. As in the previous two cases, contract revocation by the genetic parents or the fertility center does not negate the compensation for the losses imposed on the surrogate or the other party.

Mutual revocation after implantation in the surrogate's uterus

If the genetic parents, the fertility clinic, and the surrogate fulfill their obligations based on the surrogacy contract and the *in vitro* formed embryo is

injected into the surrogate's uterus can the parties involved refuse to fulfill contractual provisions (terms) afterwards? Contract revocation by the genetic parents or the surrogate means that they are not inclined to continue the process of pregnancy. As a result, the process of pregnancy should be terminated. The existence of the right to revocation for each in this assumption is subject to determining the time of the embryo's personhood, since the embryo has been settled in the surrogate's uterus. If we consider the human embryo as a person even before the sixteenth week, neither the genetic parents nor the surrogate has the right to annul the contract because taking into consideration their rights for revocation conflicts with the right of the embryo's life. However, if we do not consider the human embryo as a person until ensoulment, then we can possibly consider the right of revocation for both the genetic parents and surrogate.

Following these explanations, it is clear that no right of revocation exists for the parties involved in the contract after ensoulment because the embryo in the surrogate's uterus, though not a property, belongs to all three parties involved: genetic parents, surrogate, and the embryo itself (although the fetus is not directly a party for the contract, but by performance of the contract terms and its creation by the fertility clinic, it will have the right to life). Therefor neither the mother nor father can use their hypothetical right to annul the contract. In addition, their agreement upon revoking the contract, assuming the agreement of the surrogate, will not be a reason for revocation since it is in contrast with the right of an embryo's life. The right of life precedes any other right that negate that life, except in cases where not allowing the embryo to live is permitted, such as the priority of the mother's life over the embryo's life if the mother's life is saved by an abortion (25, 26). Hence, one can claim that malformed or patient fetus abortion is not lawful after ensoulment or 16 weeks when the possibility exists for a viable birth or survival. Accordingly, use of mutual revocation (iqala) by any of the parties involved to annul the surrogacy contract is permitted only when the obligation to contractual provisions has not yet started or if it has started, the embryo has not been implanted in the surrogate's uterus.

Applying option

Option (khiar) in Islamic texts refers to the ability (governance) of one or two parties involved in contract revocation (27). The existence of the option of revocation (khiar al-faskh) for each of the effective parties involved in a surrogacy contract can be investigated from four perspectives:

Mention of the right to revocation in the contract

Mentioning the right to revocation in the contract and its application does not differ from what has been previously mentioned about mutual revocation (iqala) according to legal-jurisprudent (fiqh) texts.

Option of violation from a conduct's condition

The conduct's condition refers to the obligation of either one party of the contract, both parties or a person outside the contract to do something which can be either within the regulations of the contractual provisions or beyond its provisions (28). Within the contract each of the effective parties is obligated to do something. For example, the obligation of the genetic parents to financially support the surrogate and pay for medical expenses related to surrogate's pregnancy; the surrogate's obligation to use essential nutrients necessary for growth of the fetus and refrain from alcohol and smoking (quitting the conduct), and have regular visits to a health center for checkups; or the obligation of the fertility center to monitor the health status of the surrogate and her embryo, and timely detection of fetal genetic diseases. However, if one side does not observe the contractual provisions, can the other side annul the surrogacy contract due to a contract violation?

Undoubtedly, violation from a conduct mentioned in the contract will lead to the option of revocation (khiar al-faskh) for the other side; however, as far as the surrogacy contract is concerned, the issues of embryo's life and his personhood distinguish this contract from the others. If a violation from the condition mentioned in the surrogacy contract occurs before starting the procedure the possibility of revocation exists for other sides as well; however, if it occurs after implantation of the embryo (egg cell) in the surrogate's uterus and if we consider personhood for the embryo, then the surrogacy contract cannot be annulled due to option of violation from the condition. This type of

revocation means revocation of pregnancy and killing a human embryo which is an unlawful conduct based on Islamic regulations. However, it can force the violator to follow the condition by setting up some regulations; if any loss occurs, the person who has suffered a loss can force the violator to compensate for their loss. In cases where the condition is beneficial for the embryo, the genetic father, due to his right of custody of the embryo can act on behalf of the embryo and its rights.

Option of violation from a characteristic condition

A characteristic condition (shart e sefat) is one of the correct conditions in contracts that refer to one of the qualitative or quantitative features of the contract (12). The option of violation from a characteristic condition in the surrogacy contract refers to the clinic's obligation to create a child with a specific attribute not realized in the child (embryo). For example, the clinic's obligation to create a specific sex (boy or girl) or specific physical attributes by using genetic changes (specific eye color, ear and nose shape, or hair type, or a defined genetic trait) or to create specific number of children (single or twins). The fertility clinic's commitment for creating an embryo with special characteristics is unmoral and illegal. However, if we assume that the fertility clinic is committed to this action, in those characteristics that their understandings are possible after the embryo's growth in the surrogate mother's uterus. In case of non-fulfillment of that characteristic, the surrogacy contract cannot be terminated due to a violation of the characteristic condition (shart e sefat). The prerequisite required to terminate the contract by the genetic parents is abortion or abandoning the child by entrusting it to the surrogate, and not accepting responsibility for the embryo-none of which are acceptable.

Option of deficiency

The fertility clinic is obligated to prevent creation of an embryo with physical abnormalities (paralyzed or disabled) in a timely manner by using diagnostic techniques based on previously accepted medical science and to prevent embryo development or at least tell the genetic parents about the condition of the embryo when such abnormalities occur. Then, the genetic parents can make a decision about the future of their child. However, if the fertility clinic, intentionally or mistakenly, does not fulfill its responsibility despite having the knowledge or necessary equipment and this oversight results in an abnormal embryo and at any stage of the embryo development, the genetic parents are not informed about the embryo's defect, can the genetic parents based on the option of deficiency (khiar al-ayb) annul their contract with the surrogate?

Contract revocation based on option of deficiency (khiar al-ayb) is possible in two cases. The abnormality exists at the time of signing the contract; however, the other side is not aware of this abnormality or that another abnormality has occurred due to the previously existing abnormality after signing the contract. Accordingly, a surrogacy contract can be annulled based on option of deficiency (khiar al-ayb) when the genetic abnormality existed prior to signing the surrogacy contract with the fertility clinic in cases where the clinic, whether intentionally or mistakenly, did not disclose the abnormality to the parents that resulted in other physical abnormalities in the embryo due to a genetic disorder.

Nevertheless, the right to revocation cannot be given to the genetic parents at least after embryo ensoulment. However the contract has the financial burden and can be subject to the rules governing the termination of transactions. On the other hand, it is associated with human life. Other authors have discussed causes of malformed and pros and cons of patient fetus abortion (29). If a deformity or congenital defect in the fetus in the surrogate's uterus is one of the indications listed in the Therapeutic Abortion Act approved 30 of May 2005, then the genetic parents can terminate the contract with the carrier and abort the embryo. However, if the deformity or congenital defect is not listed in the Act or the parents are informed after the sixteenth week, by citing the terminate authority, the contract can no longer be terminated and the embryo aborted.

Under such conditions, the genetic parents, fertility clinic, and surrogate should fulfill their contractual obligations towards the embryo. The genetic parents should accept their own deformed or abnormal child after birth. This custodial right cannot be ignored, as it is a natural-genetic right whereby the parents should take care of the child once their mutual relationship occurs (dependent on both parents and the child) (30). However,

since taking care of a deformed child is financially more difficult than taking care of a normal child; therefore, the fertility clinic should compensate for this financial loss for not fulfilling its obligation to the issues of the surrogacy contract. The compensation rule exists, whether or not the fertility clinic had the obligation to create a healthy child. Based on common understanding, the contract relates to the creation of a healthy child unless the fertility clinic denies this responsibility (i.e., creating a healthy, not deformed child) in the contract.

Discussion

Use of surrogacy is a new phenomenon which, like any other method, has raised questions that are mostly beyond the medical scope. These questions are legal and jurisprudent. One important question that relates to surrogacy is whether each of the parties involved in the contract have the right to terminate the surrogacy contract. The current article attempts to find an answer to this question and in case of cancellation by any member, what decision should be made regarding continuation of the pregnancy and the resultant baby. Each of the effective parties involved in the surrogacy contract can annul the contract prior to embryo formation without the consent of the other sides. The permission for surrogacy contract revocation by each of the effective parties involved in the contract after egg cell formation and prior to its implantation in the surrogate's uterus is subject to not considering personhood for the embryo. The validity of embryo placement in the uterus does not permit its abortion. The permission for surrogacy contract revocation by each of the effective parties involved in the contract after embryo implantation in the surrogate's uterus prior to ensoulment is subject to not considering personhood for the embryo. Surrogacy contract revocation by each of the effective contractual parties after embryo implantation in the surrogate's uterus and its ensoulment is not permitted due to its inconsistency with the embryo's right to live. Surrogacy contract revocation after embryo (egg cell) implantation in the uterus due to violation of one of the parties from the terms of the contract is not permitted. The violator is obligated to fulfill the condition; otherwise, he/she should compensate for the loss(es) imposed on the other parties.

In case of the clinic's obligation to create a child with specific physical attributes which is not fulfilled, the surrogacy contract cannot be annulled based on a violation from shart e sefat. In this case, contract revocation by the genetic parents will result in an abortion or embryo abandonment (the burden of which would fall on the surrogate). If the fertility clinic is aware of a genetic disorder of the embryo prior to signing the surrogacy contract and does not, intentionally or mistakenly, tell the genetic parents about this defect, then the surrogacy contract can be annulled only before ensoulment (sixteenth week) based on option of deficiency (khiar al-ayb). Nevertheless, contract revocation after ensoulment is not permitted and the genetic parents should take custody of their abnormal/deformed child; however, the fertility clinic should compensate for the loss that resulted from not fulfilling its (clinic) obligations.

Conclusion

According to the result of this paper there is a direct relationship between legitimacy of surrogacy contract revocation and moral status of the embryo. Surrogacy contract revocation either by the infertile couple, surrogate, or the clinic is allowed by Muslim Jurists only when the embryo lacks personhood. Based on Islamic teachings, termination of a surrogacy contract in and after the sixteenth week of pregnancy, when the embryo acquires human soul (ensoulment), is not allowed. However, religious teachings emphasize that the moral status of the fetus before 16 weeks that leads to optional termination of the surrogacy contract becomes is not permissible when the fetus becomes a human being.

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References

1. Barry V. Bioethics in a cultural context: philosophy, religion, history, politics. 1st ed. Wadsworth: Cengage Learning; 2011; 71.
2. Modern family surrogacy center. The history of surrogacy. Available from: http://www.modernfamilysurrogacy.com/page/surrogacy_history. (1 Jul 2016).
3. Brinsden P. Clinical aspects of IVF surrogacy in Britain. In: Cook R, Day Sclater S, Kaganas F, editors. Surrogate mother-hood: international perspectives. Hart Publishing: Oxford- Portland Oregon; 2003; 99-100.
4. UK Parliament. Human fertilisation and embryology act 2008. Available from: http://www.legislation.gov.uk/ukpga/2008/22/contents. (12 Oct 2016).

5. Sadeghi Moghadam MH. Jurisprudence and law necessary for embryo donation to infertile couples. J Name Mofid. 2005; (46): 31-60.
6. Brinsden PR. Gestational surrogacy. Hum Reprod Update. 2003; 9(5): 483-491.
7. Fritz MA, Speroff L. Clinical gynecologic endocrinology and infertility. 8th ed. Philadelphia: Lippincott Williams & Wilkins; 2011; 1379.
8. Akhoundi MM, Behjati Ardakani Z. Surrogate uterus. 1st ed. Tehran: Samt Publication; 2009; 6.
9. Gorji A, Safai H, Araghi E, Emami A, Qasemzadeh M, Sadeghi M, et al. A comparative study of family law. 1st ed. Tehran: Tehran University; 2005; 435-436.
10. Nazari Tavakoli S. Introduction to legal principles infertility. Islamic Studies. 2007; 75: 175-223.
11. Ansari M. Kitab al-Makasib. 1st ed. Qum: Sheikh Azam Ansari World Congress; 1994; 53, 63.
12. Sabzevari A. Mohazab al ahkam fi bayan alhalal va alharam. 4th ed. Qum: Almenar Publication; 1993; 243.
13. Shahidi M. Decline Oobligation. 9th ed. Tehran: Majd Publication; 2010; 112.
14. Nazari Tavakoli S, Javanmard Farkhani E. The implantation uterus and its effect on the fetus associated rulings of Islamic Jurisprudence and law. J Medical Figh. 2012; 3-4(9-10): 37-63.
15. Quran. 23: 12-14.
16. Quran. 6: 16.
17. Nazari Tavakoli S. Masail Mustahdathe pizeshki (2). 2nd ed. Qum: Bustane Kitab; 2002; 75.
18. Quran. 16: 4.
19. Quran. 76: 2.
20. Quran. 23: 13.
21. Qurtubi Ansari MbA. Al-Jami li-Ahkam al-Quran. 1st ed. Riyaz: Dar Alam al-Kutub; 2002; 9.
22. Ibn Babuwayh Qummi MA. Man la Yahduruhu al-Faqih. Ali Akbar Ghaffari, editor. Qum: Muassisa-yi Nashr-i Islami; 1992; 171.
23. Irvani AA. Hashiyat al-Makasib. 1st ed. Tehran: Minister of Culture and Islamic Guidance; 1986; 72.
24. Nazari Tavakkoli S. Organ transplants in Islamic Jurisprudence. 2nd ed. Mashhad: Islamic Research Foundation of Astan Qods Razavi; 2008; 116-119.
25. Khui AQ, Tabrizi J. Figh al-Adar al-shariaya. 1st ed. Qum: Dar al-Sddiqah al-Shahida; 2007; 146.
26. Gulpaygani SMR. Irshad al- Sail. 2nd ed. Birut: Dar al-safvah; 1992; 173.
27. Rashti MH. Figh A-Imamyah (Ghesm Al- Khiyarat). 1st ed. Qum: Davari Book Store; 1987; 246.
28. Araki MA. Al-khiyarat. 1st ed. Qum: Rahe Hagh Institution; 1993; 494.
29. Shahrudi SM. Mosua al-figh al-Islami. 1st ed. Qum: Muassisat Dairat al-maaref al-figh al-Islami; 2002; 396-397.
30. Nazari Tavakoli S. Custody of children in Islamic Jurisprudence. 1st ed. Tehran: Samt Publication; 2007; 157-171.

Fertility Preservation in Iranian Cancer Patients: A Continuing Neglect

Gholamreza Toogeh, M.D.[1, 2*], Mohammadreza Razzaghof, M.D.[2], Fariba Zarrabi, M.Sc.[2]

1. Department of Internal Medicine, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
2. Thrombosis Hemostasis Research Center, Tehran University of Medical Sciences, Tehran, Iran

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It is not to be denied that one of the greatest breakthroughs of modern medicine is the day-to-day improvement in the diagnosis and treatment of cancer. Global statistics show a declining rate of mortality from cancer and rising rate of survival from this ominous disease (1). Mortality data over the past quarter-century is quite promising as it shows a decreasing mortality rate from all cancers combined by 1.5% per year since 1993 in men and by 0.8% per year since 1992 in women (2). It is most fortunate which all of the most common cancers in men (lung, colorectal, and prostate) and women (breast and colorectal) show this decreasing trend. Even lung cancer mortality in women has finally leveled off after several decades of increase. Despite such improvements in the survival rate of cancers, the incidence rate in Iran shows an increasing trend (3-5).

Iran, as a developing country, is undergoing an epidemiologic transition from communicable to non-communicable diseases (6). Breast cancer, the most common malignancy in women, has shown an increasing incidence in Iran in recent decades, especially in women of reproductive age (7, 8). The largest age group of Iranian women with breast cancer is among those 40-49 years of age. Although worldwide, breast cancer is uncommon in women less than 40 years of age, 23% of female breast cancer cases in Iran are under the age of 40 years (8). Thus, compared with the global average, the incidence of breast cancer in Iran is nearly one decade behind (9). A total of 42% of cervical cancer cases are diagnosed in women less than 45 years of age (10). In colorectal cancer, 42.9% of patients are younger than 50 years (11). Therefore, it appears that a considerable group of our cancer patients are or will be of reproductive or pre-pubertal age in the future.

Detrimental effects of cancer on fertility and mental health

It should be noted that cancer does not bequeath a valuable heritage to its survivors; rather, there are considerable prolonged physical and mental complications. One of the most important is the detrimental effect of cancer on fertility and reproduction in survivors. Fertility in patients with cancer can be impaired in one of two ways, either as a sequel of the cancer itself or an adverse effect of the treatment protocol in use such as radio-chemotherapy regimens or bone marrow transplantations (12). In Iran, the increasing incidence of cancer, improving trend in survival rates, and significant proportion of young patients with cancer attach considerable importance to this issue. Delaying childbearing for social and financial reasons causes even more women to endure fertility threats because of early-stage cancer diagnoses (13). Infertility that results from cancer or its treatment jeopardizes self-esteem, personal identity, sexuality, and self-image of cancer patients. It also causes feelings of emptiness and defeat, and a negative effect on families and marriages (14).

Available fertility preservation options

Fertility preservation options in male patients include sperm collection either by masturbation, electroejaculation, or testicular biopsy followed by cryopreservation of semen and testis tissue cryopreservation (15). In women, due to the non-replenishable number of ovarian follicles, fertility preservation is more complex and depends

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*Corresponding Address: P.O.Box: 1419733141, Thrombosis Hemostasis Research Center, Imam Khomeini Hospital Complex, Qarib Street, Keshavarz Blvd, Tehran, Iran
Email: toogeh.gh@gmail.com

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on patient age, urgency of the treatment, and the regimen and treatment dosages. These techniques include immature and mature oocyte cryopreservation, ovarian tissue cryopreservation, ovarian suppression with a gonadotropin-releasing hormone (GnRH) agonist, ovarian transposition, embryo cryopreservation, gonadal shielding, and conservative gynecologic surgery (16, 17). According to American Society of Clinical Oncology (ASCO) and European Society for Medical Oncology (ESMO) guidelines on fertility preservation for cancer patients, established, highly recommended fertility preservation methods include sperm cryopreservation in males and embryo and oocyte cryopreservation in females. Patients should also be informed that other methods (i.e., testicular or ovarian tissue cryopreservation) are experimental. Hormone therapy to preserve fertility should not be recommended in males or females, as there is insufficient evidence of its effectiveness (18, 19). Figure 1 summarizes the fertility preservation options for male and female patients. Of note, these fertility preservation methods are available, in various forms, in Iran (20-22).

Lack of knowledge: physicians versus patients

It is a fact that women with cancers report great emotional distress and misgiving from unmet information about fertility preservation options besides cancer treatment (16, 23, 24). Ghorbani et al. (25) studied Iranian oncologists' attitudes on fertility preservation. Only 46% of oncologists expressed awareness of fertility preservation techniques. Although the oncologists believed that radio-chemotherapy had a 30% damage rate on reproductive organs, 67% of them believed that fertility preservation should be offered to all patients. Howeveronly 40% offered fertility preservation. Of note, only 15% of oncologists delayed treatment to refer patients to fertility preservation

<table>
<tr><td rowspan="15">Fertility risk assessment (includes intrinsic and extrinsic factors)</td><td colspan="3" rowspan="3">Male</td><td>Patient assessment</td><td colspan="3">Intervention</td><td>Storage</td></tr>
<tr><td>Pre-pubertal</td><td colspan="3">Testis biopsy</td><td rowspan="2">Testis tissue cryopreservation</td></tr>
<tr><td>Pubertal</td><td rowspan="2">Able to produce a suitable semen sample</td><td>No</td><td>Testis biopsy/ gamete extraction</td><td rowspan="2">Sperm cryopreservation</td></tr>
<tr><td>Post-pubertal</td><td colspan="2">Yes</td></tr>
<tr><td rowspan="12">Female</td><td rowspan="3">Pre-pubertal</td><td rowspan="12">Treatment: Radiation to pelvis without any systemic chemotherapy</td><td rowspan="3">Yes</td><td colspan="3">Ovarian tissue biopsy</td><td>Ovarian tissue cryopreservation</td></tr>
<tr><td colspan="4">Ovarian transposition</td></tr>
<tr><td colspan="4">Ovarian shielding</td></tr>
<tr><td>No</td><td colspan="3">Ovarian tissue biopsy</td><td>Ovarian tissue cryopreservation</td></tr>
<tr><td rowspan="9">Post-pubertal</td><td rowspan="6">Yes</td><td colspan="4">Ovarian transposition</td></tr>
<tr><td colspan="4">Ovarian shielding</td></tr>
<tr><td rowspan="2">Ovarian stimulation</td><td colspan="2">Partner donor sperm</td><td>Embryo cryopreservation</td></tr>
<tr><td colspan="3">Oocyte cryopreservation</td></tr>
<tr><td rowspan="3">No</td><td rowspan="2">Ovarian stimulation</td><td colspan="2">Partner donor sperm</td><td>Embryo cryopreservation</td></tr>
<tr><td colspan="3">Oocyte cryopreservation</td></tr>
</table>

Fig.1: Fertility preservation options for both male and female with cancer.

centers. The most important reason why parents of children with cancer did not think of fertility preservation before cancer treatment was the lack of knowledge. Sadri-Ardekani et al. (26) studied on parental attitudes toward fertility preservation in 456 boys with cancer. They reported that parents of boys with cancer had limited knowledge about the risks of infertility due to cancer treatment. However, the majority desired some sort of fertility preservation once informed about these risks. More than one-third of parents wanted some sort of fertility preservation even if the chance of infertility was less than 20%.

In sum, the results of these studies highlight the fact that knowledge of both oncologists and patients about the necessity and importance of fertility preservation in Iran is inadequate. The increasing incidence of cancer, improving trend in survival rates, and significant proportion of young patients with cancer in Iran emphasize that this important issue, termed "oncofertility" by Dr. Teresa Woodruff in 2006 as new interdisciplinary field of obstetrics and gynecology (16), should be brought to the fore front in the health system policies of Iran. In order to achieve this, we make the following recommendations:

1. Ministry of Health and Medical Education organized and supervised educational programs, panels, and seminars should be held with the contribution of all related medical subspecialties including adult and pediatric oncologists, gynecologists, surgical oncologists, urologists, radiotherapists, and embryologists.
2. Regulations should be established by deputies of treatment in medical universities to oblige fertility counseling before the start of cancer treatment, in the same manner as routine laboratory tests and cardiology counseling.
3. National clinical guidelines should be developed for proper case selection and referral, and the choice of an appropriate fertility preservation technique. These guidelines should be developed by a committee of relevant specialist groups and supervised by the Treatment Deputy of the Ministry of Health and Medical Education.
4. Standard institutes specialized in the preparation and preservation of reproductive tissues that include sperm, ovule, fetus, and testis and ovary tissues should be endorsed, equipped and expanded under the supervision of the Ministry of Health and Medical Education.
5. Appropriate insurance and financial support should be provided for adequate coverage of costs, guaranteeing the integrity of tissues and compensation for probable damage.

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References

1. DeSantis CE, Siegel RL, Sauer AG, Miller KD, Fedewa SA, Alcaraz KI, et al. Cancer statistics for African Americans, 2016: Progress and opportunities in reducing racial disparities. CA Cancer J Clin. 2016; 66(4): 290-308.
2. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, et al. Cancer statistics, 2005. CA Cancer J Clin. 2005; 55(1): 10-30.
3. Mohagheghi MA, Mosavi-Jarrahi A, Malekzadeh R, Parkin M. Cancer incidence in tehran metropolis: the first report from the tehran population-based cancer registry,1998-2001. Arch Iran Med. 2009; 12(1): 15-23.
4. Mousavi SM, Gouya MM, Ramazani R, Davanlou M, Hajsadeghi N, Seddighi Z, et al. Cancer incidence and mortality in Iran. Ann Oncol. 2009; 20(3): 556-563.
5. Rafiemanesh H, Rajaei-Behbahani N, Khani Y, Hosseini S, Pournamdar Z, Mohammadian-Hafshejani A, et al. Incidence trend and epidemiology of common cancers in the center of Iran. Glob J Health Sci. 2015; 8(3): 146-155.
6. Kolahdoozan S, Sadjadi A, Radmard AR, Khademi H. Five common cancers in Iran. Arch Iran Med. 2010; 13(2): 143-146.
7. Keramatinia A, Mousavi-Jarrahi SH, Hiteh M, Mosavi-Jarrahi A. Trends in incidence of breast cancer among women under 40 in Asia. Asian Pac J Cancer Prev. 2014; 15(3): 1387-1390.
8. Harirchi I, Karbakhsh M, Kashefi A, Momtahen AJ. Breast cancer in Iran: results of a multi-center study. Asian Pac J Cancer Prev. 2004; 5(1): 24-27.
9. Sadjadi A, Nouraie M, Ghorbani A, Alimohammadian M, Malekzadeh R. Epidemiology of breast cancer in the Islamic Republic of Iran: first results from a population-based cancer registry. East Mediterr Health J. 2009; 15(6): 1426-1431.
10. Karimi-Zarchi M, Mousavi A, Gilani MM, Barooti E, Miratashi-Yazdi A, Dehghani A. Conservative treatment in early cervical cancer. Int J Biomed Sci. 2013; 9(3): 123-128.
11. Azadeh S, Moghimi-Dehkordi B, Fatem SR, Pourhoseingholi MA, Ghiasi S, Zali MR. Colorectal cancer in Iran: an epidemiological study. Asian Pac J Cancer Prev. 2008; 9(1): 123-126.
12. Lambertini M, Del Mastro L, Pescio MC, Andersen CY, Azim HA Jr, Peccatori FA, et al. Cancer and fertility preservation: international recommendations from an expert meeting. BMC Med. 2016; 14: 1.
13. Maltaris T, Koelbl H, Seufert R, Kiesewetter F, Beckmann MW, Mueller A, et al. Gonadal damage and options for fertility preservation in female and male cancer survivors. Asian J Androl. 2006; 8(5): 515-533.
14. Tschudin S, Bitzer J. Psychological aspects of fertility preservation in men and women affected by cancer and other life-threatening diseases. Hum Reprod Update. 2009; 15(5): 587-597.
15. Trost LW, Brannigan RE. Oncofertility and the male cancer

patient. Curr Treat Options Oncol, 2012; 13(2): 146-160.
16. Harp D, Chowdhury I. Oncofertility: an emerging branch of women's health. Int J Cancer Res Prev. 2015; 8(2): 291.
17. Hormozi M, Akbarpour M, Hosseini Gohari L, Heidari M, Salehkhou Sh, Jedi Tehrani M, et al. Common techniques for preserving fertility in girls and young women undergoing cancer treatment. J Reprod Infertil. 2011; 12(2): 85-92.
18. Loren AW, Mangu PB, Beck LN, Brennan L, Magdalinski AJ, Partridge AH, et al. Fertility preservation for patients with cancer: American Society of Clinical Oncology clinical practice guideline update. J Clin Oncol. 2013; 31(19): 2500-2510.
19. Peccatori FA, Azim HA Jr, Orecchia R, Hoekstra HJ, Pavlidis N, Kesic V, et al. Cancer, pregnancy and fertility: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2013; 24 Suppl 6: vi160-170.
20. Karimi Zarchi M, Mousavi A, Gilani MM, Barooti E, Amini Rad O, Ghaemmaghami F. Fertility sparing treatments in young patients with gynecological cancers: iranian experience and literature review. Asian Pac J Cancer Prev. 2011; 12(8): 1887-1892.
21. Eftekhar M, Pourmasumi S, Karimi-Zarchi M. Preservation of ovarian function during chemotherapy and radiotherapy in young women with malignancies. Iran J Reprod Med. 2014; 12(6): 377-382.
22. Amirjannati N, Sadeghi M, Hosseini Jadda SH, Ranjbar F, Kamali K, Akhondi MA. Evaluation of semen quality in patients with malignancies referred for sperm banking before cancer treatment. Andrologia. 2011; 43(5): 317-320.
23. Canada AL, Schover LR. The psychosocial impact of interrupted childbearing in long-term female cancer survivors. Psychooncology. 2012; 21(2): 134-143.
24. Crawshaw MA, Sloper P. 'Swimming against the tide'-the influence of fertility matters on the transition to adulthood or survivorship following adolescent cancer. Eur J Cancer Care (Engl). 2010; 19(5): 610-620.
25. Ghorbani B, Madahi P, Shirazi E, Ardekani HS, Kamali K. Iranian oncologists' attitude towards fertility preservation in a sample group. J Reprod Infertil. 2011. 12(1): 33-36.
26. Sadri-Ardekani H, Akhondi MM, Vossough P, Maleki H, Sedighnejad S, Kamali K, et al. Parental attitudes toward fertility preservation in boys with cancer: context of different risk levels of infertility and success rates of fertility restoration. Fertil Steril. 2013; 99(3): 796-802.

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