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Revisiting The Relationship between The Ejaculatory Abstinence Period and Semen Characteristics

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Abstract

Variation in the ejaculatory abstinence period suggested by different guidance bodies have resulted in a growing concern among researchers and clinicians over what the precise period of ejaculatory abstinence ought to be for an optimal semen sample. Several studies have thus been undertaken to examine the association between the length of sexual abstinence and semen characteristics. Not all studies, however, have arrived at the same conclusions. This study aims to review all existing literature published during the past few decades pertaining to the influence of ejaculatory abstinence on semen quality. For the purpose of this systematic review, all data related to sexual abstinence duration and seminal parameters were re-analysed to homogenize the current data. Thorough PubMed, MEDLINE and Google Scholar, a literature search was conducted using the keywords "sexual abstinence", "ejaculatory abstinence", "semen", "spermatozoa", "semen analysis", "sperm parameters", "motility", "reactive oxygen species (ROS)" and "DNA fragmentation". After carefully reviewing all the literature, 30 relevant papers, both written in English and published between January 1979 and December 2016, were included in this review. The weight of the evidence suggests that the decline in semen volume and sperm concentration with shorter abstinence periods is accompanied by a substantial improvement in sperm motility characteristics, especially progressive motility and velocity. Nevertheless, available data are insufficient to support definitive conclusions regarding the influence of the ejaculatory abstinence period on advanced semen parameters (ROS, DNA fragmentation and seminal plasma antioxidant capacity) and pregnancy rates. In conclusion, taking all data into account, shortening of the abstinence period may be beneficial to sperm quality. Furthermore, we recommend that the current guidelines regarding the prescribed abstinence period should be revisited.

Keywords: DNA Fragmentation, Semen Analysis, Sexual Abstinence, Spermatozoa, Sperm Motility

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Introduction

Infertility is the "failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" and is a condition estimated to affect about 15% of all couples of reproductive age. Male factor infertility has been found to be the sole contributor in approximately 20% of all infertility cases and is partially implicated in another 30-40% (1). When the attributable causes of female infertility have been eliminated and/or semen analysis results fail to meet the World Health Organization (WHO) criteria, male infertility is taken into consideration as the likely etiological factor. Therefore, semen analysis still remains the established cornerstone of the laboratory assessment of male infertility.

A considerable amount of variability has been shown to exist in various semen characteristics within and among individuals (2). These variations have been largely attributed to several modifiable intrinsic and extrinsic factors. These factors include the length of sexual abstinence, ejaculation frequency and method of collection. Other factors that have the potential to influence semen quality are general health and lifestyle, infection, dysfunction of male sex glands, urogenital surgery as well as therapeutic and environmental exposures (3).

The WHO manuals for examining and processing human semen provide a practical guide for standardizing semen analysis. These manuals have been periodically published and actively developed since its first edition in 1980. The WHO criteria for semen analysis have been adopted by most human andrology and fertility laboratories around the world for more than thirty years. The most recent guidelines of WHO recommend that the minimum period of ejaculatory abstinence prior to semen collection should not be less than 2 days and more than 7 days (4). The Nordic Association for Andrology (NAFA) and the European Society of Human Reproduction and Embryology (ESHRE) (5), however, outline a narrower range of 3-4 days of abstinence. The basis for these recommendations is nevertheless not supported by sufficient scientific evidence and requires further clarification.

In light of the differing ejaculatory abstinence periods suggested by various regulatory bodies, a growing concern has resulted over what the precise period of ejaculatory

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abstinence ought to be for an optimal semen sample. This has prompted several studies to examine the influence of abstinence periods on various semen parameters. The results are, however, not conclusive. Interestingly, some studies have even challenged the recommended guidelines in favour of extremely shorter periods (i.e. <1 hour to 4 hours) due to their advantageous effects on semen characteristics (6-9). Studies on the association of abstinence length with semen quality have examined a wide range of abstinence intervals. Although numerous related articles have been published to this date, a systematic review has not been undertaken. This study therefore aims to review the existing scientific literature over the past few decades pertaining to this association in humans to evaluate the weight of evidence for the optimal time period of ejaculatory abstinence.

Materials and Methods

For the purpose of this systematic review, all data related to sexual abstinence duration and seminal parameters were re-analysed to homogenize the current data. An extensive review of the existing literature was performed in various electronic databases, namely MEDLINE, PubMed and Google Scholar by using the keywords "sexual abstinence", "ejaculatory abstinence", "semen", "spermatozoa", "semen analysis", "sperm parameters", "motility", "reactive oxygen species (ROS)" and "DNA fragmentation". A total of 34 relevant articles were obtained, all of which were written in English and published between January 1979 and December 2016. Four of these were excluded due to a lack of numerical data. After careful review of the abstracts, these 30 studies were included in the current review, of which 25 were prospective and five were retrospective. The majority of the included studies had used donors recruited from the general population, while twelve studies selected patients from infertility clinics or assisted reproduction units. The seminal parameters examined were semen pH, semen volume, sperm concentration, sperm motility, sperm morphology, sperm intracellular ROS and DNA fragmentation, and seminal plasma antioxidant capacity. Here, ejaculatory abstinence was classified into the time periods of ≤1 day, 2-3 days, 4-5 days, 6-7 days and >7 days.

Ejaculatory abstinence and conventional semen parameters

The majority of the studies investigating the influence of ejaculatory abstinence on semen quality (Table 1) had assessed the most conventional semen parameters (e.g. volume, count and concentration, motility and morphology) as described by WHO (4) with the latest version reporting a reference range based on men with proven fertility.

Seminal pH

A slightly alkaline seminal fluid is necessary to neutralize the acidic environment of the vagina, which can negatively impact sperm function (10). A substantial reduction in sperm motility was reported in patients with semen pH less than the WHO lower-bound threshold value of 7.2 (11), however, the correlation between semen pH and sperm motility was not statistically significant (12). Only three studies considered seminal pH as a parameter when investigating the relationship between the abstinence period and semen quality (13-15). Blackwell and Zaneveld (13) analysed semen samples from ten men with abstinence periods of 1, 2, 3, 4, 5 and 10 days, and found that seminal pH remained essentially unchanged. In addition, De Jonge et al. (14) examined ejaculates from 11 men who had abstained for 1, 3, 5 and 8 days, and reported no significant changes in seminal pH across the four abstinence periods. Similar results were also reported by Agarwal et al. (15) who collected semen samples from seven men each abstaining sequentially for 1, 2, 5, 7, 9 and 11 days, and observed that semen pH remained relatively stable but declined significantly after 11 days of abstinence. The scarcity of studies examining seminal pH indicates that the significance of this semen marker has been underestimated.

Semen volume

According to the latest WHO guidelines, the lower-bound reference value for semen volume is 1.5 ml. Accurate measurement of the ejaculate volume is important as the concentration of spermatozoa and non-sperm cells in the ejaculate are based on the initial volume. Semen volume-after the recommended standard period of abstinence-has consequently been suggested to be an early indicator of low semen quality even before identifying any abnormality in concentration, motility and morphology of spermatozoa. Semen volume has also been suggested to be a reliable indicator of the secretory functions of the accessory glands, particularly the seminal vesicles (4).

The relationship between the abstinence period and semen volume was reported in twenty-four studies (Table 1). In all but one, there is robust and consistent evidence for the significant increase in semen volume with increase in abstinence period (6, 8, 9, 13-32). In a retrospective longitudinal study (22), the greatest overall mean of daily increase in semen volume was observed at 11.9% per day during the first 4 days of abstinence. However, only one study (33) failed to show any significant change in semen volume in both normozoospermic and asthenozoospermic populations which is likely to be due to the small sample size studied and the protracted period of the short abstinence.

Short abstinence-associated decreases in the ejaculate volume may be attributed to insufficiency of the accessory sex glands to make an adequate contribution to the ejaculate volume, particularly the seminal vesicles and the prostate gland, which are the major contributors. The epithelial tissues of these organs are targeted by androgen, which is thought to regulate their mRNA production as well as the synthesis of rough endoplasmic reticulum, thereby enhancing the production of seminal plasma proteins (34). Improved secretory capacity of the seminal vesicles and the prostate gland has been associated with higher endogenous serum testosterone levels in rats (35) and men (36). In addition, higher testosterone serum levels have been reported following a prolonged abstinence period compared with a shorter

abstinence (37). Therefore, the potential stimulating effect of testosterone on the major accessory glands associated with long abstinence periods may contribute to the increased semen volume after prolonged abstinence periods.

Sperm concentration and total count

Concentration of spermatozoa in semen, expressed as millions per millilitre, is a critical indicator of semen quality and a prognostic factor for fertility potential (38). However, it is not recommended as an accurate measure of spermatogenesis because it is influenced by the volume of secretions of the accessory sex glands in which the concentrated epididymal spermatozoa are diluted in during ejaculation (4). The total number of spermatozoa in the ejaculate, expressed as millions per total ejaculate and obtained by multiplying the sperm concentration by the semen volume, is suggested to be a better parameter for the evaluation of spermatogenic statuses (39). The lower-bound threshold values of sperm concentration and total count recommended by the WHO are 15×10^6 spermatozoa/mL and 39×10^6 spermatozoa/ejaculate respectively (4).

The influence of the abstinence period on sperm concentration was assessed in twenty-two of the studies listed in Table 1. Of these, twenty (91%) reported a linear increase in sperm concentration with increased abstinence periods (8, 9, 13-16, 19, 21-26, 28-30, 32, 33, 40, 41). The highest rise in the overall mean of sperm concentration (14×10^6/mL) occurred when the abstinence period increased from 2-3 days to 4-5 days (Table 2). Two studies found a non-significant mild increase in sperm concentration after long abstinence compared with short abstinence periods (18, 20). Eighteen studies (6, 9, 13, 15, 16, 18, 19, 21-24, 29-33, 41, 42) reported a significant association between long abstinence periods and increased total sperm count in the ejaculate. The largest increase in the overall mean of total sperm count was recorded when the abstinence period extended from 6-7 days to >7 days.

During sexual inactivity an estimated 400 million spermatozoa are reserved within the epididymis with the majority stored in the cauda epididymis and lesser in the caput and corpora with an average of 90 million in each of these sections. The paired vas deferens with its ampulla is estimated to contain about 75 million spermatozoa (39). During the arousal phase, but prior to the emission phase, the population of spermatozoa in the paired ampulla increases dramatically as they move distally towards the urethra (43). After particularly long periods of abstinence, the bulk of the sperm population in the first ejaculate mainly comprise spermatozoa stored in the ampulla and vas deference, and partly in the cauda epididymis. Consequent ejaculates in quick successions are typically characterized by a lower total count of spermatozoa as the residual spermatozoa are flushed from the proximal cauda and corpus, and thereafter from the caput (6), all of which contain much lower sperm reserves (39). Despite these findings, Bahadur et al. (8) interestingly suggested that "combining the initial and consecutive ejaculates allows for a potential shift of severe and oligozoospermia patients towards the normospermia range". This approach may lead to a change in the treatment strategies by possibly avoiding testicular biopsies.

The observed consistent positive correlation of sperm concentration and total count with increasing abstinence durations can be ascribed to daily sperm production, which is determined to be approximately $130\text{-}270\times10^6$ per day (39). The regulation of testicular functions and spermatogenesis necessitates a complex combination of endocrine and paracrine signals. Relatively higher levels of testosterone are essential for the maintenance and proceeding of spermatogenesis. Serum testosterone levels were shown to fluctuate mainly from the second to the fifth day of abstinence, reaching a peak (about 145% of the baseline) after the seventh day of abstinence and remaining relatively constant even when the abstinence period was prolonged (37).

Sperm motility and kinematics

Assessment of motility characteristics of ejaculated spermatozoa has been shown to have the utmost importance for the diagnosis of male fertility potential since it provides vital information on the functional competence of the spermatozoon. The percentage of motile spermatozoa in the ejaculate provides an indication of epididymal sperm maturation (44). However, progressive motility is required for the spermatozoa to migrate through the harsh environment of the female genital tract to reach the ovum. Motility is not only necessary for sperm transit, but changes in flagellar motion also play an essential role at the site of fertilization. The mechanical driving force generated by motility help the sperm to propel through the outer layers of the cumulus-oocyte complex (45). The lower-bound WHO threshold values for the percentages of total motility and progressive motility are 40 and 32% respectively (4).

Twelve studies examined the relationship between the abstinence period and the total motile sperm (TMS) count in the ejaculate. Eight of these (15, 23, 24, 26, 29, 40-42) reported an increase in TMS count with increase in the abstinence period, while the other four did not find any significant effect of abstinence period on TMS (9, 22, 28, 33). The overall mean of TMS increased substantially as the abstinence period increased from ≤ 1 to 3 days (Table 2). The mean TMS remained relatively stable between the fourth and the seventh day, increased on the subsequent days (>7) and declined gradually after day 9 to 10 of abstinence (17, 19). The influence of abstinence length on the percentage of motile spermatozoa was investigated in seventeen studies (6, 9, 14, 15, 17, 19, 21, 23, 24, 26-30, 32, 41, 42) (Table 1). We found little consensus among the results of these studies. A slight or lack of association between abstinence period and motile sperm percentage was reported in eleven studies (9, 14, 15, 17, 21, 27, 28, 30, 32, 41, 42). In contrast, six studies (6, 19, 23, 24, 26, 29) reported a substantial decrease in the percentage of motile spermatozoa with increasing abstinence; the highest overall mean sperm motility percentage was observed after ≤ 1 day of abstinence (Table 2).

Table 1: Abstinence periods and semen characteristics

Type of study	Abstinence periods	Subjects	Number of subjects/samples	Volume (mL)	Concentration (10^6/mL)	TSC (10^6/ejaculate)	TMS (10^6/ejaculate)	Motility (%)	Progressive motility (%)	Viability (%)	Normal morphology (%)	DNA fragmentation (%)	ROS
Prospective	4 hours and 3-5 days	Volunteers	11	↑	--	↑	--	↓	↓	--	--	--	--
Prospective	3 hours and 96 hours	Normozoospermic	21	--	--	--	--	--	--	--	--	↔	--
Prospective	40 minutes and 2-7 days	Oligozoospermic	73	↑	↑	--	--	--	↓	--	↓	--	--
Prospective	2 hours and 3-4days	Healthy	3	↑	↑	↑	↔	↔	↔	↔	--	↓	↔
Prospective	1, 2, 3, 4, 5 and 10 days	Volunteers	10	↑	↑	↑	--	--	--	↔	↑	--	--
Prospective	1, 3, 5, and 8 days	Volunteers	11	↑	↑	--	--	↔	--	↔	↔	↔	--
Prospective	1, 2, 5, 7, 9 and 11 days	Normozoospermic	7	↑	↑	↑	↑	↔	--	↓	--	↑	↔
Prospective	1, 2, 3, 4, 5, 6 and 7 days	Normal	36	↑	↑	↑	--	--	--	--	--	--	--
Retrospective	≤1, 2, 3, 4, 5, 6 and 7 days	Suspected infertile	1801	↑	--	--	--	↔	--	↔	↔	--	--
Prospective	8 hours and 3 days	Volunteers	7	↑	↔	↑	--	--	--	--	↔	--	--
Prospective	12 hours and 7 days	Volunteers	10	↑	↑	↑	--	↓	--	--	↔	--	--
Prospective	2-4, 5-7 and >7 days	Healthy men	195	↑	↔	--	--	--	--	--	--	--	--
Prospective	2, 4, 7, 10, 15 and 18 days	Volunteers	6	↑	↑	↑	--	↔	--	--	↔	--	--
Prospective	<4, 4-6 and >6 days	Healthy	27	↑	↑	↑	↔	--	--	--	↔	--	--
Prospective	2-3 and 4-7 days	Non-azoospermic	422	↑	↑	↑	↑	↓	↓	--	↓	--	--
Retrospective	1, 2, 3, 4, 5, 6, 7, 8-10 and 11-14 days	Oligozoospermic	3506 samples	↑	↑	↑	↑	↓	--	--	↓	--	--
Retrospective	1, 2, 3, 4, 5, 6, 7, 8-10 and 11-14 days	Normozoospermic	5983 samples	↑	↑	↑	↑	↓	--	--	↔	--	--
Prospective	2, 3, 4 and 5 days	Fertile	500	↑	↑	--	--	--	↓	--	↔	--	--
Retrospective	≤2 and 3-7 days	Undergoing IUI	372	↑	↑	--	↑	↓	--	↓	--	--	--
Prospective	1 and 4 days	Undergoing ICSI	40	↑	--	--	--	↔	--	--	--	↑	--
Prospective	18-30 hours and 3-5 days	Healthy	57	↑	↑	--	↔	↔	--	--	↑	↑	--
Prospective	1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days	Normozoospermic	100 samples	↑	↑	↑	↑	↓	--	↔	↔	--	--
Prospective	1 and 4 days	Planning IUI	40	↑	↑	↑	--	↔	--	--	↔	--	--
Retrospective	2-3, 4-5 and 6-7 days	Attending Infertility Unit	730	↑	--	↑	--	--	↓	↓	↔	--	--
Prospective	1 and 3-4 days	Healthy	6	↑	↑	↑	--	↔	↔	↔	↔	↔	↔
Prospective	3, 6 and 10 days	Normozoospermic	7	↔	↑	↑	↔	--	↓	↔	↔	--	--
Prospective	3, 6 and 10 days	Asthenozoospermic	7	↔	↑	↑	↔	--	↔	↔	↔	--	--
Retrospective	≤3, 4-10 and >10 days	Undergoing IUI	929	--	↑	--	↑	--	↔	--	--	--	--
Prospective	4 and 14 days	Nonobstructive azoospermic	50	--	↑	↑	↑	↔	--	--	--	--	--
Prospective	1, 2, 4, 7, 10 and 14 days	Healthy	4	--	--	↑	↑	↔	↔	↔	↔	--	--
Prospective	1 and 3-4 days	With high DNA fragmentation levels	35	--	--	--	--	--	--	--	--	↑	--
Prospective	1-10 days	Healthy	36 samples	--	--	--	--	--	--	--	--	--	↔

TSC; Total sperm count/ejaculate, TMS; Total motile sperm/ejaculate, ROS; Reactive oxygen species, ICSI; Intracytoplasmic sperm injection, IUI; Intrauterine insemination, ↑; Increase significantly with increasing abstinence period (P≤0.05), ↓; Decrease significantly with increasing abstinence period (P≤0.05), ↔; Not significantly different, and --; Not investigated.

Table 2: The overall mean values of basic semen parameters in relation to different abstinence periods calculated from values reported in relevant studies referred to in Table 1

Semen parameter	Day				
	≤1	2-3	4 -5	6-7	>7
Semen volume (mL)	2.198 n=15	2.72 n=13	3.251 n=18	3.773 n=13	4.229 n=14
Concentration (10^6/mL)	54.363 n=16	52.038 n=11	66.849 n=16	64.623 n=11	70.474 n=13
Total sperm count (10^6/ejaculate)	99.911 n=9	114.306 n=9	172.591 n=10	225.792 n=10	288.642 n=12
Total motile sperm (10^6/ejaculate)	36.56 n=8	49.618 n= 8	81.114 n=9	78.517 n=8	94.612 n=9
Motility (%)	56.03 n=15	44.813 n=11	52.044 n=15	41.277 n=12	43.325 n=13
Progressive motility (%)	57.083 n=6	54.533 n=3	53.887 n=6	49.15 n=2	- -
Viability (%)	66.29 n=4	72.37 n=5	73.622 n=5	68.4 n=5	66.41 n=6
Normal morphology (%)	8.453 n=1	9.644 n=14	10.16 n=15	8.45 n=14	8.590 n=13

The average reported in each study contributed equally to the overall mean. Only studies reporting absolute values were included. All studies were included in the calculations (e.g. normozoospermic, oligozoospermic, volunteers, patients, etc.).

Ten studies (6, 8, 9, 23, 25, 31-33, 40, 42) investigated the relationship between ejaculatory abstinence and progressive motility (Table 1). Five studies (6, 8, 23, 25, 31) reported a significantly higher percentage of progressively motile spermatozoa with shorter abstinence periods, with the overall mean peak of progressive motility observed after ≤1 day of abstinence (Table 2). Interestingly, shortening the abstinence interval to about 30 minutes resulted in a significant increase in the percentage of fast progressive (type A) spermatozoa (8). The results of Magnus et al. (33) were consistent with those of the abovementioned studies where the progressive motility of a normozoospermic population was found to increase with decreasing abstinence time. However, they analysed an asthenozoospermic population and found no such association, corroborating the findings of the other relevant studies (9, 32, 40, 42).

Motility assessment in the majority of the studies was performed manually using a light microscope and only five studies (23, 27-29, 42) used computer-aided sperm analysis (CASA). Manual assessment of sperm motility is subjective and is strongly associated with inter- and intra-laboratory variation (46). The potential counting and interpretation errors associated with the subjective visual assessment of sperm motility have made automated semen analyses an absolute necessity. CASA, in contrast to subjective motility estimation, is certainly a powerful approach for the objective assessment of sperm motion. The most recent WHO guidelines on semen analysis nevertheless indicate that the assessment of sperm motility percentage using CASA may be unreliable due to the potential misidentification of particulate debris as immotile spermatozoa (4). This issue has recently been addressed as modern CASA systems such as the sperm class analyser (SCA6) are now equipped with intelligent filters to accurately identify the spermatozoa and eliminate the debris and other cells. The automatic analysis of sperm motility by CASA instruments enables the objective estimation of various parameters which translate into certain kinematic measures of sperm movement (47). The only study investigating the impact of ejaculatory abstinence on sperm kinematics, among other determinants of semen quality, had been conducted by Elzanaty et al. (23). In this study, semen samples collected from patients with a wide age range, undergoing infertility assessment, were grouped into three categories based on the abstinence period (i.e. 2-3 days, 4-5 days and 6-7 days). Significantly higher straight-line velocity (VSL) and linearity (LIN) were found in the group with the shortest abstinence period, while average path velocity (VAP) and curvilinear velocity (VCL) were not significantly different among the three abstinence groups.

Variation in semen characteristics among individuals may enhance the potential for observation bias (48) since other factors besides ejaculatory abstinence may account for the effects observed. However, collecting replicate semen samples from the same individual is likely to be an effective approach to controlling confounding factors. The increase in semen volume and sperm concentration with prolonged abstinence periods was almost consistently accompanied by substantial deterioration in sperm motility characteristics, especially progressive motility and velocity. Although the exact mechanism as to how ejaculatory abstinence may affect changes in semen quality is unknown, a number of possibilities have been suggested. For instance, reduction in the storage period within the epididymis may minimize the exposure of unejaculated spermatozoa to motility inhibitory factors and enzymes released from the degenerating cells within the same microenvironment (6). Furthermore, the sperm

reservoir capacity of the cauda epididymis is limited (49), thus the substantial increase in sperm concentration during prolonged ejaculatory abstinence may result in the depletion of energy reserves and allow for senescent spermatozoa to accumulate in the epididymis. The relative contribution of these senescent spermatozoa to the subsequent ejaculate impairs semen quality (27, 50). Extending the abstinence time may also enhance susceptibility of unejaculated spermatozoa to recurrent genital heat exposure, causing detrimental changes to the membrane phospholipid architecture of epididymal spermatozoa (51) and the functional properties of the motor apparatus of the sperm flagellum (52). Therefore, reducing the abstinence period may minimize the frequency and time span of heat exposure, thereby leading to improved motility.

Sperm viability

Sperm viability is one of the parameters that is routinely assessed in basic semen analysis, and is especially recommended in samples where the percentage of motile spermatozoa is less than about 40% (4). The viability status of spermatozoa selected for intracytoplasmic sperm injection (ICSI) has to be precisely examined since the injection of a live spermatozoon is vital to the success of the ICSI outcome (53). Furthermore, a direct correlation has recently been identified between sperm viability and the level of DNA fragmentation, showing that the viability status may be a potential indicator of DNA integrity of the ejaculated spermatozoa (54). The lower-bound reference limit for sperm viability is estimated to be 58% (4). The influence of abstinence duration on sperm viability was examined in eleven studies (9, 13-15, 17, 26, 29, 31-33, 42). This was done by using various techniques including a dye exclusion assay (14, 15, 29, 33), the hypo-osmotic swelling test (13, 31, 42) and flow cytometry (9, 32). Most of these studies reported slight or no statistically significant negative association between sperm viability and abstinence period. The overall mean percentage of viable spermatozoa peaked and remained relatively unchanged between the second and the fifth day of abstinence, and declined thereafter (Table 2).

Sperm morphology

To be considered morphologically normal, the whole spermatozoon and its three distinct areas, the head, midpiece and the tail, must fit with stringent criteria in terms of their size and shape. The 5th centile lower-bound reference limit for normal forms is 4% (4). It has also been reported that morphologically abnormal spermatozoa, with a special focus on the acrosomal region, have a lower chance to bind to the zona pellucida (55). A correlation has also been observed between sperm head abnormalities and DNA integrity. Therefore, analysis of sperm morphology, which may provide crucial evidence about semen quality, is assessed by fairly simple and inexpensive methods compared with expensive and elaborate assays such as DNA fragmentation (56) and acrosome reaction (57). The relationship between the abstinence duration and sperm morphology was investigated in eighteen studies (8, 13, 14, 17-19, 21-25, 28-33, 42). All had assessed sperm morphology manually via visual assessment except one (29) which had used CASA.

Most of the studies (14 out of 18) reported no significant association between sperm morphology and the period of abstinence. In contrast, one study reported significantly higher percentages of spermatozoa with tail defects when the abstinence period was extended from 2-3 days to 6-7 days. However, the overall proportion of normal morphology did not differ between the two abstinence groups (23). Furthermore, Levitas et al. (24) reported that among mild to moderate oligozoospermic samples, the highest percentage of normal morphology was reported at $\leq$2 days of abstinence but this association was not observed in a normozoospermic population. Bahadur et al. (8) recently reported that an extremely short abstinence period of 30 minutes could significantly improve sperm morphology among oligozoospermic men, all candidates for intrauterine insemination (IUI) treatment. By contrast, shortening the abstinence duration in normal individuals from 3-5 days to only 18-30 hours resulted in a considerably lower percentage of morphologically normal spermatozoa (28). It may therefore be advantageous for patients with oligozoospermia to abstain for shorter periods before sperm collection in the process of fertility treatment. However, it must be re-iterated that manual assessment of sperm morphology is a subjective analysis with inter- and intra-laboratory variation. This variability may be attributed to several factors including the use of different fixation and staining techniques (58), differences in interpretation (59) and technician expertise (60). Another important factor that needs to be taken into consideration is that the WHO guidelines and reference ranges have changed over the years and may thus lead to differences in interpretation (4).

Ejaculatory abstinence and advanced semen parameters

Conventional semen parameters provide the essential information on which clinicians base their preliminary diagnosis (61). Approximately 25-40% of idiopathic infertile males have been reported to have normal semen profiles (62). Therefore, a range of advanced sperm quality parameters have been developed to circumvent the limitations of the conventional semen analysis (63).

DNA fragmentation

Assessment of sperm DNA integrity, in addition to routine semen analysis, provides further valuable information about sperm quality as well as pregnancy outcomes (64, 65). It has been shown that high proportions of spermatozoa with DNA fragmentation above 20% increase the risk of infertility regardless of having normal basic semen parameters (61). Eight studies (7, 9, 14, 15, 27, 28, 32, 66) had investigated the relationship between the abstinence period and sperm DNA fragmentation. Three studies (7, 14, 32) did not find any effect while half of the studies (15, 27,

28, 66) showed an increase in sperm DNA fragmentation rates with prolonged abstinence. Interestingly, the report by Mayorga-Torres et al. (9) was to the contrary, showing considerable increase in DNA fragmentation levels after an extremely short abstinence periods of 2 hours compared with the initial ejaculate that was collected after 3-4 days of abstinence. The latter finding could be purely a result of the extremely small and underrepresented sample size (n=3) but still merits further investigation.

Reactive oxygen species production

Normal physiological levels of ROS are crucial for maintaining various vital functions in spermatogenesis at different maturational stages. These highly reactive species can also act as essential mediators for signal transduction involved in sperm capacitation, hyperactivation and acrosome reaction (67). However, ROS levels must be maintained within physiological ranges since ROS overproduction or insufficient antioxidant defense can result in a state of oxidative stress (68).

Three studies (9, 15, 32) examined the relationship between the abstinence period and sperm intracellular ROS production, while only one study examined the relationship in terms of seminal ROS concentration (69). These studies consistently reported no association of abstinence duration with either intracellular ROS production or seminal ROS levels. However, among the relevant studies a general trend of reduction, albeit non-significant, was observed in intracellular ROS levels after short abstinence in comparison with long abstinence. Interestingly, when four repeated ejaculates were collected on the same day at 2 hour intervals, a significant reduction in intracellular ROS production was observed in the fourth ejaculate compared with the initial one obtained after 3 to 4 days of abstinence (9). During their maturation and storage, spermatozoa are continuously susceptible to oxidative damage induced by intracellular and extracellular reactive species. Spermatozoa are highly sensitive to ROS damage by lipid peroxidation due to their membranes being highly rich in polyunsaturated fatty acids (67). Therefore, the release of spermatozoa through more frequent ejaculations may possibly minimize their adverse effects on sperm quality (9).

Seminal plasma antioxidants

Spermatozoa have limited intracellular enzymatic defense against oxidative stress, partly due to cytoplasmic extrusion during spermatogenesis. This deficient capacity is effectively compensated for by a group of cellular detoxifying enzymes with powerful antioxidant properties including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidises found within the seminal plasma (68). Surprisingly, only one study had examined the influence of ejaculatory abstinence period on seminal plasma antioxidants and lipid peroxidation of the sperm membrane (30). By analysing ejaculates of forty men undergoing IUI, Marshburn et al. (30) observed a significant improvement in the total antioxidant capacity of seminal plasma after one day of abstinence compared to four days. Lipid peroxidation of the sperm membrane remained unchanged between the two abstinence periods. They therefore suggested that short abstinence-related increase of total antioxidant capacity in seminal plasma could defend spermatozoa against oxidative stress through a mechanism that is independent of lipid peroxidation.
Hitherto, there are no available data on the effect of the abstinence period on acrosome reaction or any individual antioxidants. With respect to the detoxyifying enzymes, however, we have observed in our laboratory that a short abstinence period of four hours led to a significant increase in SOD activity but did not change the activity of catalase in seminal plasma (unpublished data).

Pregnancy rate

The conventional parameters of semen analysis provide fundamental information for the initial diagnosis of male infertility, but none is reliable enough to predict pregnancy (38). Few studies had examined the influence of ejaculatory abstinence period on pregnancy rate, all of which had recruited patients from infertility and assisted conception clinics. For instance, among infertile couples undergoing ovulation induction followed by IUI, the highest pregnancy rate was observed for those with an abstinence period of ≤3 days, while a sharp decline in pregnancy rate was observed for those with ≥10 days of abstinence. Interestingly, the relationship between ejaculatory abstinence period and pregnancy rate was independent of the variation in conventional semen parameters (40). Another study, examining a more general infertile population, revealed that the highest IUI pregnancy rates were associated with ≤2 days of abstinence (26). Sánchez-Martín et al. (27) reported that serial ejaculation every 24 hours for four days with an ultimate abstinence of 12 hours, along with sperm selection by density gradient centrifugation, could significantly improve pregnancy rate with ICSI. More recently, Bahadur et al. (70) showed in a pilot study that recurrent ejaculates successfully improved IUI pregnancy rates. These findings can be supported by the fact that fertilization rates are directly related to sperm progressive motility and inversely related to DNA fragmentation in vitro (71) with both parameters generally found to be improved with shorter abstinence periods. However, importantly, large prospective randomized controlled trials are required to validate that short abstinence periods improve pregnancy and live birth rates, and may thus be recommended for infertility treatments.

Conclusion

We conclude that in spite of the varied quality of existing studies, the weight of evidence suggests that reducing the ejaculatory abstinence period may positively influence semen quality based on a consistent trend towards an increase in the percentage of motile, progressively motile and rapid spermatozoa with shorter abstinence periods. However, the small number of studies examining ROS production, DNA fragmentation and seminal plasma antioxidant capacity

limit any definitive conclusion regarding its effect on advanced semen parameters. Further clinical trials with sufficient number of subjects, and controlling for potential confounders, may shed further light on this association. We recommend that future studies incorporate CASA as a more accurate and objective measurement tool as well as utilize more sensitive measures of sperm function such as sperm hyperactivity, sperm-zona binding ability, acrosome reaction, and total and individual seminal plasma antioxidants. It is, however, worth mentioning that even after short abstinence periods of ≤1 day, the overall mean values of the conventional semen parameters were always above the lower-bound reference limits recommended by WHO (fifth version). Therefore, shortening the abstinence period may be a potential strategy to improve sperm quality. It is thus recommended that the current guidelines regarding the prescribed abstinence period are revisited.

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Author's Contributions

B.M.A.; Helped design the study, searched the data base, analysed the results and wrote the manuscript. G.V.d.H.; Helped with the study design and reviewed the final version of the manuscript. S.S.D.P.; Helped with the study design and assisted with the writing of the manuscript.

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Original Article

Gene Polymorphism of Matrix Metalloproteinase 9 in Asthenozoospermic Male Subjects

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Abstract

Background: Matrix metalloproteinase (MMPs) play important roles in the structural and functional properties of reproductive organs. The aim of this study is to determine the prevalence of C-1562T *MMP-9* (rs3918242) gene polymorphism in fertile and infertile men. In addition, we aim to determine the association between C-1562T *MMP-9* and G-1575A *MMP-2* gene polymorphisms.

Materials and Methods: A total of 400 subjects, including 200 fertile and 200 infertile men, were recruited for this case-control study. The allele frequencies and genotype distributions of single nucleotide polymorphism in the promoter regions of *MMP-9* (C-1562T) were determined using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis. The chi-square (χ^2) test was used to assess the distribution of genotype frequencies.

Results: There were no significant differences found in the genotype distributions or allele frequencies between fertile and infertile men for the C-1562T *MMP-9* gene polymorphism. The percent of immotile sperm in infertile men with the CC and CT genotypes of C-1562T *MMP-9* gene polymorphism significantly differed compared with that of subjects with the TT genotype. The frequency of CC/GA-combined genotypes of C-1562T *MMP-9* and G-1575A *MMP-2* gene polymorphisms significantly differed in fertile and infertile men (P=0.031).

Conclusion: Our results suggest that genetic polymorphisms in MMP may impact male fertility.

Keywords: Infertility, Matrix Metalloproteinase, Polymorphism, Semen, Single Nucleotide

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Introduction

Infertility is a clinical problem that affects married couples. Nearly 20% of couples experience infertility and 50% of these cases are related to male reproductive disorder (1). Male infertility is influenced by many environmental and genetic factors (2, 3). Reproductive organs undergo major alterations in their structural and functional properties throughout adult life. These changes involve remodeling of connective tissues. Matrix metalloproteinases (MMPs), due to their specific features, play important roles in these modifications (4). Mammals have 28 types of MMP (5). These enzymes require zinc for their activities and are involved in the degradation of both the extracellular matrix and basement membrane. MMPs and their tissue inhibitors (TIMPs) participate in a number of physiological processes, such as ovulation and fertilization. These enzymes have been frequently studied in the female reproductive system, but not extensively examined in terms of male fertility. One study showed that the MMP profile between normal and abnormal sperm samples differed and suggested that sperm abnormality might occur because of the presence or absence of a specific MMP (6). Accumulating evidence has shown that MMPs impact male fertility for three reasons. First, these enzymes play an important role in the development of reproductive organs (7). Second, MMPs are required for spermatogenesis (1). Third, the breakdown of physical barriers between egg and sperm and the contact of sperm with the egg surface, along with certain specific features of MMPs, suggest that they may be involved in fertilization (8, 9).

Gelatinases, a subtype of MMPs (including MMP-2 and -9), are usually involved in the degradation of collagen type IV and gelatine (10, 11). MMP-2 (col-

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lagenase A with a molecular weight of 72 kDa) and MMP-9 (collagenase B with molecular weight of 92 kDa) present in the acrosome and tail of the sperm, respectively (12). Single-nucleotide polymorphisms (SNPs) in the promoter region of *MMP* genes may influence their expression and thus alter their enzyme activities. Several polymorphisms, such as G-1575A of *MMP-2* and C-1562T of *MMP-9*, have been identified in the promoter region of *MMP-2* (ID 4313) and *MMP-9* (ID 4318) genes, which affect their expression (13). In our previous report, we have determined the G-1575A genetic polymorphism of *MMP-2* in fertile and infertile men. Our result showed that the frequencies of GA genotype in fertile and infertile men significantly differed (14). The C-1562T gene polymorphism in the *MMP-9* gene is located at position -1562 of the promoter site, where a transition occurs between C and T. This site is relative to the transcription start site. The C allele results in lower promoter activity than the T allele due to its lower affinity to a nuclear protein (13).

Considering the role of *MMP-9* in both remodeling and destruction of the extracellular matrix, genetic variations may affect the transcription of the gene and activity of this enzyme, resulting in a propensity towards male infertility. To the best of our knowledge, the prevalence of C-1562T *MMP-9* gene polymorphisms as well as the association of C-1562T *MMP-9* and G-1575A of *MMP-2* gene polymorphisms has not been previously investigated in male fertility. Therefore, the aim of this study was to determine the prevalence of C-1562T *MMP-9* (rs3918242) gene polymorphism in fertile and infertile men. In addition, the association between C-1562T of *MMP-9* and G-1575A of *MMP-2* gene polymorphism in fertile and infertile men was determined.

Materials and Methods

A total of 400 subjects (200 fertile and 200 infertile men) participated in this study. A literature review showed no previously published study on the C-1562T *MMP-9* gene polymorphism in male fertility; therefore, we determined the sample size based on the predominant genotype frequency of the C-1562T *MMP-9* polymorphism in the population. Based on the average frequency of the predominant genotype in the study population (85%), the minimum difference in dominant genotype frequency between two groups was set at 10%, with a type I error set at α=0.05 and type 2 error considered to be β=20%.

The fertile men were staff members of the Hamadan University of Medical Sciences who voluntarily participated in this study. Fertile individuals had no specific diseases. Infertile men were randomly selected from patients admitted to the Fatemieh Fertility Clinic at Hamadan University of Medical Sciences and all had abnormal spermogram results according to World Health Organization laboratory guidelines (2010). In this case-control study, the fertile men had children born within the past 5 years. Patients had idiopathic infertility (failure to achieve a clinical pregnancy after 12 months or more of regular unprotected intercourse). We excluded all cases with specific reasons for their disease, such as varicoceles and abnormal karyotype (15) from the study. Subjects from two fertile and infertile groups were matched for age (29-42 years) to achieve no significant difference between the two groups. All participants provided written informed consent and the Research Ethics Committee of Hamadan University of Medical Sciences approved this study.

Semen analysis

We collected and examined semen samples from 200 infertile men according to the World Health Organization (2010) laboratory guidelines (16). The ejaculates were collected into sterile containers during masturbation after at least 72 hours of sexual abstinence and allowed to liquefy at room temperature. Semen suspensions were analyzed for sperm concentration, linear progressive movement (motility), and morphology. The infertile individuals were divided into asthenozoospermic and teratoastheozoospermic subjects. Criteria for asthenozoospermia was defined by progressive motility<32%, sperm concentration $\geq 20\times10^6$/ml, and normal morphology ≥15%. Criteria for teratoastheozoospermia was defined by progressive motility <32%, sperm concentration $\geq 20\times10^6$/ml, and normal morphology<14%.

DNA extraction

We collected 3 ml whole blood into EDTA coated tubes. Genomic DNA was extracted using the ethanol-chloroform extraction method (17). DNA concentration was determined by spectrophotometry at 260 nm. The *MMP-9* nucleotide polymorphism at position -1562 was determined by polymerase chain reaction (PCR) combined with the restriction fragment length polymorphism (RFLP) method using forward, (5′-GCCTGGCACATAGTAGGCCC-3′) and reverse, (5′-CTTCCTAGCCAGCCGGCATC-3′) primers (18). For PCR of each sample, a premix PCR kit (Bioneer, Korea) was used. For the PCR cycle, after DNA denaturation at 94°C for 5 minutes, the reaction mixture was subjected to 30 cycles at 94°C for 45 seconds, 55°C for 45 seconds, and 72°C for 45 seconds with a final extension time of 5 minutes. Electrophoresis was performed with Syber Safe staining on 2% agarose gel to confirm the 435 bp size of the PCR product.

Genotyping

To analyze the presence of polymorphism, PCR products were digested using the sphI restriction enzyme. Amplified PCR products (10 µl) were digested in a 30 µl final reaction volume that consisted of 2 µl of 10x Reaction Buffer and 5 units of sphI restriction endonuclease (Fermentas, USA), incubated at 37°C overnight. The enzyme digested products for *MMP-9* gene were

analyzed on a 2% agarose gel. Gels pre-stained with Syber Safe (for visualization under a UV light) were run at 96 mV in 1X tris-borate-EDTA (TBE) buffer for 45 minutes. The homozygote CC genotype yielded a 435 bp product, whereas we observed two fragments (247 bp and 188 bp) for the TT homozygote subjects. Heterozygotes with the CT genotype, on the other hand, yielded three PCR product fragments of 435 bp, 247 bp, and 188 bp after sphI digestion (Fig.1). DNA sequencing confirmed the genotypes identified by the RFLP method.

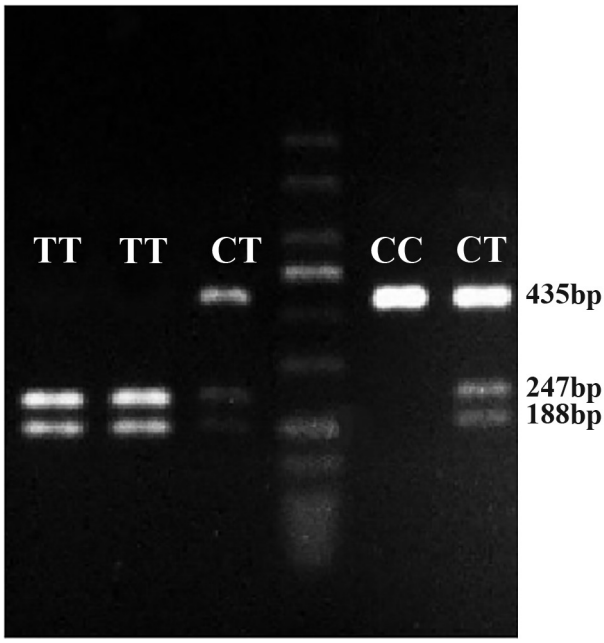

Fig.1: The polymerase chain reaction-based SphI restriction fragment length polymorphism (PCR-RFLP) products for C-1562T *MMP-9* gene polymorphism on agarose 2%. The PCR products were digested by sphI restriction endonuclease. The CC genotype produced a 435 bp,TT genotype 247bp and 188 bp, and CT genotype 435 bp, 247 bp and 188 bp PCR product.

Statistical analysis

All the statistical analyses were carried out using the Statistical Program of Social Sciences (SPSS) version 16.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± standard deviation and statistical significance was defined as $P<0.05$. The chi-square (χ^2) test was used to assess the distribution of genotype frequencies for deviation from the Hardy-Weinberg equilibrium. We calculated the allele frequencies from the observed numbers of genotypes. Odds ratios (OR) with 95% confidence intervals (CI) were calculated as estimates of relative risk for infertility.

Results

We examined the association between the C-1562T polymorphism in the *MMP-9* gene and the risk of male infertility. The homozygous CC genotype was detected in 59% of the fertile men compared to 66% of the infertile men. The homozygous TT genotype was detected in 4.5% of the fertile men and 3.5% of the infertile men. The heterozygous CT genotype was detected in 36.5% of fertile men and 30.5% of infertile men. The frequencies of CT and TT genotypes did not show significant differences between fertile (P=0.174) and infertile (P=0.482) men. We determined allele frequencies of the C-1562T *MMP-9* gene polymorphism (Table 1). There was no association found between the allele frequencies of the C-1562T *MMP-9* gene polymorphism and the risk of infertility. Table 1 shows the genotype and allele OR of the C-1562T *MMP-9* gene polymorphism in fertile and infertile men. Our results indicated that the variant genotype -1562 CC in the *MMP9* gene did not have a significant association with an increased risk of infertility (adjusted OR=1.34, 95% CI: 0.88-2.02 and OR=1.44, 95% CI: 0.52-3.98).

Table 1: The frequency of C-1562T *MMP-9* genotypes, alleles, and odds ratios in fertile and infertile males

C-1562T *MMP-9*	Infertile men n=200	Fertile men n=200	OR (95% CI)
C/C[a]	132 (66%)	118 (59%)	1.00
C/T	61 (30.5%)	73 (36.5%)	1.34 (0.88-2)
T/T	7 (3.5%)	9 (4.5%)	1.44 (0.52-3.98)
Alleles			
C[a]	325 (81.25%)	309 (77.25%)	1.00
T	75 (18.75%)	91 (22.75%)	1.27 (0.9-1.8)

[a]; Reference group, OR; Odds ratios, and CI; Confidence interval.

The semen profiles of infertile subjects with reference to the *MMP-9* genotypes are described (Table 2). The percent of immotile sperm in infertile men with the CC and CT genotypes of C-1562T *MMP-9* gene polymorphism showed a significant difference compared to subjects with the TT genotype. According to the spermiogram, we divided the infertile men into two groups: asthenozoospermia (n=97) and terato-asthenozoospermia (n=103). We determined the genotype distribution of C-1562T *MMP-9* gene polymorphism in the three groups of fertile, asthenozoospermia, and terato-asthenozoospermia (Table 3). We found no statistically significant difference in genotype distribution of the C-1562T polymorphism in these three groups. Since we previously reported the genotype and allele frequencies of the G-1575A *MMP-2* gene polymorphisms in these subjects (14), in the current study, we analyzed the association of the G-1575A *MMP-2* and C-1562T *MMP-9* gene polymorphisms. The results demonstrated that the frequencies of CC/GA-combined genotypes of C-1562T *MMP-9* and G-1575A *MMP-2* gene polymorphisms had a significant difference between fertile and infertile men (P=0.032, χ^2=4.6, df=1) (Table 4). We also analyzed the synergistic effect of alleles C-1562T *MMP-9* and G-1575A *MMP-2* gene polymorphisms on male infertility (Table 5). Synergistic analysis of the allele frequencies of G-1575A and C-1562T gene polymorphisms showed that the frequency of individuals with negative T allele (MMP-9) and positive A allele (MMP-2) between fertile and infertile men significantly differed (P=0.038).

Table 2: Semen profiles of infertile men regarding genotype of C-1562T *MMP-9* gene polymorphism

C-1562T *MMP-9*	Motility (%) (mean ± SD)			Sperm count (million/ml)	Normal morphology (%)
	Progressive	Non progressive	Immotile		
C/C (n= 132)	7.24 ± 7.95	17.97 ± 13.05	57.4 ± 25.2	44.17 ± 26.77	19.37 ± 11.05
C/T (n=61)	8.65 ± 8.05	16.32 ± 10.3	55.43 ± 27.39	40.69 ± 31.01	20.53 ± 14.8
T/T (n=7)	7.15 ± 8.72	16.73 ± 23.74	20.85 ± 27.27	35.5 ± 41.49	14.16 ± 13.93
P value	0.312	0.734	0.004	0.630	0.481

Table 3: The genotype distribution of C-1562T *MMP-9* gene polymorphism in three groups of fertile, teratoasthenozoospermia and asthenozoospermia infertile men

C-1562T *MMP-9*	Fertile men n=200	Infertile men n=200		P value
		Teratoasthenozoospermia n=103	Asthenozoospermia n=97	
C/C[a]	118 (59%)	68 (66.02%)	64 (65.98%)	
C/T	73 (36.5%)	31 (30.09%)	29 (29.89%)	0.358 (χ^2=2, df=2)
T/T	9 (4.5%)	4 (3.89%)	4 (4.13%)	0.891 (χ^2=0.22, df=2)

[a]; Reference group, x^2; Chi-square, and df; Degree of freedom.

Table 4: The combined genotype frequencies of G-1575A *MMP-2* and C-1562T *MMP-9* gene polymorphisms in fertile and infertile men

MMP-9 -1562/*MMP-2*-1575	Infertile men	Fertile men	OR (95% CI)	P value
CC/GG[a]	81 (40.5%)	57 (28.5%)	1.00	
CC/GA	48 (24%)	59 (29.5%)	1.74 (1-2.9)	0.032 (χ^2=4.6, df=1)
CC/AA	4 (2%)	2 (1%)	0.7 (0.12-4)	0.702 (χ^2=0.151, df=1)
CT/GG	37 (18.5%)	45 (22.5%)	1.72 (0.99-3)	0.051 (χ^2=3.81, df=1)
CT/GA	22 (11%)	27 (13.5%)	1.74 (0.9-3.36)	0.093 (χ^2=2.78, df=1)
CT/AA	1 (0.5%)	1 (0.5%)	1.42 (0.09-23.2)	0.814 (χ^2=0.061, df=1)
TT/GG	6 (3%)	5 (2.5%)	1.18 (0.35-4)	0.794 (χ^2=0.072, df=1)
TT/GA	1 (0.5%)	4 (2%)	5.6 (0.6-52)	0.093 (χ^2=2.95, df=1)
TT/AA	0	0	NA	NA

[a]; Reference group, NA; Not applicable, OR; Odds ratios, CI; Confidence interval, x^2; Chi-square, and df; Degree of freedom.

Table 5: Synergism of alleles of G-1575A *MMP-2* and C-1562T *MMP-9* gene polymorphisms in fertile and infertile men

MMP-9 -1562/*MMP-2*-1575	Infertile men	Fertile men	OR (95% CI)	P value
Both negative MMP9 T allele and MMP-2 A allele[a] (CC+GG)	81 (40.5%)	57 (28.5%)	1.00	
Negative MMP9 T allele and positive MMP-2 A allele (CC+AA+GA)	51 (25.5%)	61 (30.5%)	1.7 (1-2.8)	0.038 (χ^2=4.29, df=1)
Positive MMP9 T allele and negative MMP-2 A allele (TT+CT+GG)	44 (22%)	50 (25%)	1.29 (1-1.7)	0.071 (χ^2=3.18, df=1)
Both positive MMP9 T allele and MMP-2 A allele (TT+CT+AA+GA)	24 (12%)	32 (16%)	1.9 (1-3.5)	0.045 (χ2=4, df=1)

[a]; Reference group, OR; Odds ratios, CI; Confidence interval, x^2; Chi-square, and df; Degree of freedom.

Discussion

In this study we determine the prevalence of the C-1562T *MMP-9* gene polymorphism and its association with the G-1575A of *MMP-2* gene polymorphism in fertile and infertile men. The study indicated that in the C-1562T *MMP-9* gene polymorphism, the frequencies of CT and TT genotypes did not significantly differ in fertile and infertile men. The risk of infertility in individuals with the CC genotype was 1.3-times more than individuals who carried the CT genotype and 1.4-times more than those with the TT genotype; however, these associations did not reach statistical significance. Based on logistic regression analysis, the T allele could

have a protective effect and possibly decrease the risk of male infertility by approximately 1.27 times, however the P value was not statistically significant. The number of participants with the T allele in the fertile and infertile groups was most likely not adequate to reach a statistically significant conclusion. It was reported that the T allele carriers of C-1562T *MMP-9* gene polymorphism had a higher enzyme activity and protein level compared to C allele carriers (19). Since nuclear proteins have a lower affinity to the C allele compared to the T allele, individuals with the C allele have a lower promoter function, and consequently, a lower MMP-9 enzyme activity (13). Therefore, most likely, the T allele increases the expression of *MMP-9* and has a positive effect on male fertility.

Our results were consistent with the results of a study conducted by Patricia et al. which showed that the TT genotype in the C-1562T *MMP-9* gene polymorphism had a protective effect against the development of lung cancer compared to the reference genotype (20). Here we confirmed that the TT genotype in the C-1562T *MMP-9* gene polymorphism had a protective effect on sperm motility and might indirectly improve male fertility. On the other hand, Wang and Shi (21) showed that East Asian T allele carriers (TT+TC) compared with C allele carriers had a significantly higher risk of coronary artery diseases. These results suggested that this polymorphism could have different effects in different diseases.

In this study, we divided the infertile men into asthenozoospermic and terato-asthenozoospermic groups according to their semen profiles, and determined the frequencies of the genotype C-1562T *MMP-9* gene polymorphisms. We found that genotype distribution of C-1562T polymorphism in asthenozoospermic, terato-asthenozoospermic, and fertile men did not statistically differ. These results suggested that the C-1562T *MMP-9* gene polymorphisms had no significant effect on the morphology of spermatozoa. In accordance with these observations, semen analysis showed that the genotype frequencies of the C-1562T *MMP-9* gene polymorphisms were not significantly different in terms of morphology. On the other hand, we found a lower percentage of immotile sperm in men who carried the TT genotype compared to the CC and CT genotype carriers of the C-1562T *MMP-9* gene polymorphism.

Ferrer et al. (12) identified MMP-9 activities in human spermatozoa that were mainly in the mid-piece of the sperm tail. According to previous reports, T allele carriers of the C-1562T *MMP-9* gene polymorphism had higher MMP-9 activity and protein level compared to C allele carriers (19). We could explain the lower percentage of immotile sperm from the TT genotype compared to the CT and CC genotypes. On the other hand, higher MMP-9 activity in the sperm tail of males with the TT genotype might improve sperm cell motility. However, we did not determine the MMP-9 enzyme activity in participants enrolled in this study. In addition, due to ethical issues, we did not have access to the semen of fertile men t*o* strengthen our conclusion.

MMP-9 gene polymorphisms could be associated with other *MMP* polymorphisms in the genome. We analyzed the synergism of genotypes and alleles of G-1575A *MMP-2* and C-1562T *MMP-9* gene polymorphisms on male infertility. In our previous report on G-1575A *MMP-2* polymorphism, we showed that the frequencies of GA genotype of fertile and infertile men were significantly different; however, the frequency of AA and GG genotypes didn't show any significant differences. In G-1575A *MMP-2* polymorphism, the risk of infertility in individuals with AA genotype was 2.14-fold more than individuals carrying GA genotype (14). The synergistic analysis of genotypes of C-1562T *MMP-9* and G-1575A *MMP-2* gene polymorphisms showed that the frequency CC/GA-combined genotype was significantly different between fertile and infertile men. It demonstrated a protective effect which could increase male fertility about 1.7 times.

The synergistic effects of different MMP polymorphisms on male infertility is very complex, and further investigations with larger sample size are needed to clearly delineate the impact of *MMP* polymorphisms on male infertility. On the other hand, synergistic analysis of alleles G-1575A *MMP-2* and C-1562T *MMP-9* gene polymorphisms showed that the frequency of individuals with negative MMP-9 T allele and positive MMP-2 A allele between fertile and infertile men was significantly different. We also found that the frequency of individuals with both positive MMP-9 T allele and MMP-2 A allele was significantly different in the fertile group compare with the infertile individuals.

A previous study has shown that the systemic lupus erythematous (SLE) patients with A allele (GA+AA genotypes) of G-1575A MMP-2 gene polymorphism have higher levels of MMP-2 activity than the control subjects (22). Two polymorphisms that were examined in this study (G-1575A *MMP-2* and C-1562T *MMP-9*) are located in the promoter regions of their corresponding genes. Promoter regions of *MMP-2* and *MMP-9* contain regulatory elements which are affected by transcription factors. The substitution of T with C in position of -1562 from *MMP-9* gene is associated with up-regulation of promoter activity (23). In addition, it has been shown that C-1562T MMP-9 polymorphism influence on gene expression of *MMP-9* (13). These results suggest that the synergism of genotypes and alleles of G-1575A *MMP-2* and C-1562T *MMP-9* gene polymorphisms can have an impact on male fertility.

There are some limitations in this study. The relatively wider range of CI observed in TT genotypes of C-1562T *MMP-9* gene polymorphism (0.52-3.98) was probably due to the lower sample size requited in this study. Further studies should be performed with larger sample size

together with the determination of MMP-9 and MMP-2 activities to provide more information about the impact of these polymorphisms on male infertility.

Conclusion

Our results suggest that the T allele carriers of C-1562T *MMP-9* gene polymorphism have lower immotile sperm number. In addition, a relationship was observed between combined *MMP-2* and *MMP-9* variant genotypes and male infertility. Therefore, it can be concluded that genetic polymorphisms in matrix metalloproteinases may impact on male fertility.

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Author's Contributions

H.T.; Contributed to the conception and design and was responsible for overall supervision. S.M.; Contributed to all experimental work and the acquisition of data. M.K.; Contributed to the statistical analysis, and interpretation of data. I.A; Performed sample collection. S.M.; Drafted the manuscript, which was revised by H.T. and I.K. All authors read and approved the final manuscript.

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Original Article

Multiplex-Polymerase Chain Reaction for Detecting Microdeletions in The Azoospermia Factor Region of Y Chromosome in Iranian Couples with Non-Obstructive Infertility and Recurrent Pregnancy Loss

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Abstract

Background: Approximately 15% of couples are infertile with the male factor explaining approximately 50% of the cases. One of the main genetic factors playing a role in male infertility is Y chromosomal microdeletions within the proximal long arm of the Y chromosome (Yq11), named the azoospermia factor (*AZF*) region. Recent studies have shown there is a potential connection between deletions of the *AZF* region and recurrent pregnancy loss (RPL). The aim of this study is to examine this association by characterizing AZF microdeletions in two infertile groups: in men with non-obstructive infertility and in men with wives displaying RPL.

Materials and Methods: In this is a case-control study, genomic DNA was extracted from 80 male samples including 40 non-obstructive infertile men, 20 males from couples with RPL and 20 fertile males as controls. Multiplex polymerase chain reaction was used to amplify 19 sequence tagged sites (STS) to detect AZF microdeletions. Differences between the case and control groups were evaluated by two-tailed unpaired t test. $P<0.05$ were considered statistically significant.

Results: Only one subject was detected to have Y chromosome microdeletions in *SY254*, *SY157* and *SY255* among the 40 men with non-obstructive infertility. No microdeletion was detected in the males with wives displaying RPL and in 20 control males. Y chromosome microdeletion was neither significantly associated with non-obstructive infertility ($P=0.48$) nor with recurrent pregnancy loss.

Conclusion: Performing Testing for Y chromosome microdeletions in men with non-obstructive infertility and couples with RPL remains inconclusive in this study.

Keywords: Infertility, Multiplex Polymerase Chain Reaction, Y Chromosome

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Introduction

Y chromosome is the shortest chromosome in the human genome. It has the least number of genes among all human chromosomes (1). The human Y chromosome is necessary for human sex determination, and male germ cell development and maintenance (2). Of the 60 Mb length of the Y chromosome, 3 Mb belongs to pseudoautosomal regions (PAR1 and PAR2 on the Yp and Yq respectively) and 57 Mb to a nonrecombining region (NRY). The NRY region can be classified to heterochromatic and euchromatic regions. The euchromatin contains all of the known genes in the Y chromosome. The euchromatic regions on the Y are about 23 Mb consisting of 8 Mb on the short arm and 14.5 Mb on the long arm (1, 3). Genes located on the euchromatic region of the proximal long arm of the Y chromosome (Yq11), named *azoospermia factor (AZF)* region, plays an essential role in spermatogenesis (4, 5). Recent studies have suggested that the *AZF* region cause male infertility and also recurrent pregnancy loss when it is disrupted (6, 7).

Y Chromosome and male infertility

Approximately 15 percent of couples are infertile with the male factor being responsible for approximately 50% of the

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cases. It is defined as a multifactorial syndrome encompassing a wide variety of disorders (8). In about 50-60% of male infertility cases, the etiology can be identified, however, when the cause is unknown, it is referred to as idiopathic infertility (9). A significant proportion of idiopathic male infertility is associated with azoosperima or severe oligozoospermia, which may be due to genetic alterations. Nevertheless, the underlying etiology is still poorly understood (10).

Recent studies have shown that both genetic and environmental factors are involved in the reduction of reproductivity in males. The main genetic factors in male infertility are Y chromosomal microdeletions within the Yq11 region and somatic chromosomal abnormalities. After Klinefelter syndrome, Y chromosomal microdeletion is the most frequent cause of male infertility (11) and the second most frequent genetic cause of spermatogenic failure (12). The microdeletions in the *AZF* region occur in infertile men (13). Studies have shown that the *AZF* region is deleted in about 13% of men with non-obstructive azoospermia and in 7 to 10% of men with oligozoospermia (14). Testicular tissue sections of azoospermic men with these Yq11 aberrations showed intense spermatogenesis disruption. This suggested that there is an essential function of *AZF* for differentiation and proliferation of human male germ cells (15). The *AZF* region consists of four sub-regions, namely *AZFa*, *AZFb*, *AZFc* and *AZFd* (14, 16, 17). Each of these regions are associated with a particular testicular histology, and a number of candidate genes have been found within these regions (13). Deletions in the *AZF* region occur as six classical types of Yq deletions consist of *AZFa*, *AZFb*, *AZFc*, *AZFbc*, *AZFabc* and partial *AZFc* (3) as described in Table 1.

Table 1: Genotype-phenotype correlation of *AZF* regions (3, 18)

Deletion	Deletions are known to correspond to:
AZFa deletion	Complete *AZFa* deletions: severe testicular phenotype, SCOS and spermatogenic arrest
	Partial *AZFa* deletions: extremely rare
AZFb deletion	Complete *AZFb* deletions: spermatogenic arrest
	Partial AZFb deletions: variable phenotypes from hypospermatogenesis to SCOS extremely rare
AZFc deletion	Complete *AZFc* deletions: variable phenotype which may range from mild oligospermia to azoospermia and SCOS
Partial *AZFc* deletion	Variable phenotypes from hypospermatogenesis to the SCOS
AZFbc deletion	SCOS/spermatogenic arrest
AZFabc deletion	SCOS

***AZF; Azoospermia factor* and SCOS; Sertoli cell-only syndrome.**

Y Chromosome and recurrent pregnancy loss

Recurrent pregnancy loss (RPL), recurrent miscarriage or habitual abortion is the occurrence of three or more consecutive pregnancies that terminate through miscarriage before fetus viability (for instance, 24 weeks of gestation). About 1% of couples trying to have children are affected by recurrent miscarriage (19). RPL is a multifactorial condition with several etiologic factors including genetic abnormalities of the parents, endocrinologic, anatomic, hematologic and immunologic abnormalities along with nutritional, infectious and environmental factors (20, 21). The most commonly accepted etiology of RPL is maternal, however, most cases are classified as idiopathic, with no identifiable cause in either partner (20, 22). The repetitive pregnancy loss in some couples plus the high percentage of idiopathic RPL indicate that the underlying causes of RPL needs to be investigated (23). Mutations including small deletions, duplications and substitutions cannot be detected by cytogenetic analysis. These genetic abnormalities may thus account for a large number of miscarriages with unknown causes (24).

New evidence indicates that male factors may play a major role in RPL (25). Sperm integrity is required for fertilization, sperm-egg interactions and early embryonic development. Sperm quality affects the ability of the embryo to reach the blastocyst phase and develop into implantation. Paternally expressed genes control the proliferation and invasiveness of trophoblast cells, and also placental proliferation (7). The cause of pregnancy loss in approximately 50% of women with RPL remains unexplained despite many investigations (26). Recent studies have shown there is a potential connection between deletions of the *AZF* region and RPL (7, 26, 27). In a study by Dewan et al. (7), analysis of male partners in couples with RPL showed 82% of the men had at least one *AZF* microdeletion. Studying Y microdeletion is thus crucial in understanding and predicting the outcome of future pregnancies, and making informed decisions regarding treatment such as assisted reproductive technology (ART). Therefore, the aim of this study was to detect Y chromosomal microdeletions in men with non-obstructive infertility and in men having spouses with RPL by using a multiplex PCR design.

Materials and Methods

This was a case-control study. It consisted of three groups. The first group comprised 40 infertile men (azoospermic and severe oligozoospermic) aged 20-53 years old, who were referred to the Ghaem General Hospital and Novin Infertility Clinic in Mashhad, Iran, between September 2012 and September 2013. All patients in this group had primary infertility with normal karyotype and absence of obstructive azoospermia. The second group consisted of 20 men aged 17-42 years from couples with history of three or more consecutive idiopathic miscarriages, all of whom were referred from the High Education Center of Jahad Daneshgahi, Mashhad, Iran, from 2011 to 2013. In this group all men and their spouses had a normal karyotype. Other causes of pregnancy loss including infectious disease, and psychological, uterine anatomic and endocrine disorders along with immunologic and haemostatic changes were excluded. A group of 20 healthy men aged 25-42 years from couples with at least one live birth and no history of miscarriage was considered as the control group (third group).

Statistical analysis

Differences in microdeletion frequency were examined

by two-tailed unpaired t test. A P<0.05 was considered statistically significant. Demographic data of the patient and control groups were also analyzed. All the above were computed using the SPSS-V11 software. Our study was approved by the Ethics Committee of Mashhad University of Medical Sciences. An informed consent was obtained from each individual participating in this study.

Y microdeletion multiplex polymerase chain reaction detection assay

Genomic DNA was extracted from 3 ml of peripheral blood lymphocyte samples using a standard salting-out method. Isolated DNA was stored at -20°C. Following DNA extraction, *AZF* microdeletions were screened by multiplex polymerase chain reaction (PCR). Nineteen sequence-tagged sites (STSs) within the long arm of the Y chromosome were selected to cover *AZFa, b, c* and proximal *AZFc (AZFd)* regions. For each participant, *18 STS* in *AZFa* (*sY81*, *sY86*, *sY182*), *AZFb (sY121*, *sY124*, *sY127*, *sY128*, *sY130*, *sY133*, *sY134*, *SYPR3*), *AZFc* (*sY157*, *sY208*, *sY242*, *sY254*, *sY255*) and *AZFd* (*sY145*, *sY152*) sub-region were typed. The primers were combined into five sets for multiplex PCR for the purpose of determining the presence of all 19 sequence-tagged sites by performing five parallel PCR amplifications from multiplex A to E.

Multiplex reaction A amplified *SY81, SY130, SY157, SY182, SY254,* B amplified *SYPR3, SY127, SY208, SY242,* C amplified *SY121, SY128, SY145, SY255,* D amplified *SY124, SY133, SY152, SMCX* and E amplified *SY14(SRY) SY86, SY134 ZFX/Y.* Multiplex D contained a control primer pair amplifying a fragment of the X-linked SMCX locus and multiplex E contained a control primer pair amplifying a unique region present on both the Y and X chromosomes (*Zinc Finger Protein* of *Y* and *X chromosomes ZFX/ZFY*). These control primer pairs were used as internal controls to check amplification of DNA and also the integrity of the genomic DNA sample used. Finally, the multiplex E reaction included a primer pair amplifying a region of SRY (*sex-determining region of the Y*). The presence of the short arm of the Y chromosome (Yp) was tested with *STS SY14*, located within *SRY*. The *SRY* was examined to confirm the sex of the sample donor.

PCR was carried out in a total volume of 15 µl containing 150 ng of genomic DNA, 1X PCR buffer, 2 mM of $MgCl_2$, 1 unit of Taq DNA polymerase (Genet Bio, South Korea), 0.2 mM of dNTP mix and 4 pmol of each primer. The cycling conditions were an initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds at 59°C in multiplex A, at 57°C in multiplex B and at 56°C in multiplex reactions C, D, E, and extension at 72°C for 35 seconds, followed by a last extension at 72°C for 5 minutes and a cooling step at 4°C. The PCR products were separated on a 3.5% agarose gel using 1X TAE. PCR bands were visualized using DNA Green Viewer and under ultraviolet light.

Results

No microdeletion in the *AZFa, AZFb, AZFc* and *AZFd* sub-regions was observed in male partners of women with RPL (Fig.1) and men in the control group (Fig.2). Among the 40 infertile men, only one subject (2.5%) had microdeletions in multiplex reactions A and C, indicative of a microdeletion in the *AZFc* region (Fig.3A, B). *AZF* microdeletion was neither significantly associated with non-obstructive infertility (P=0.48) nor with RPL.

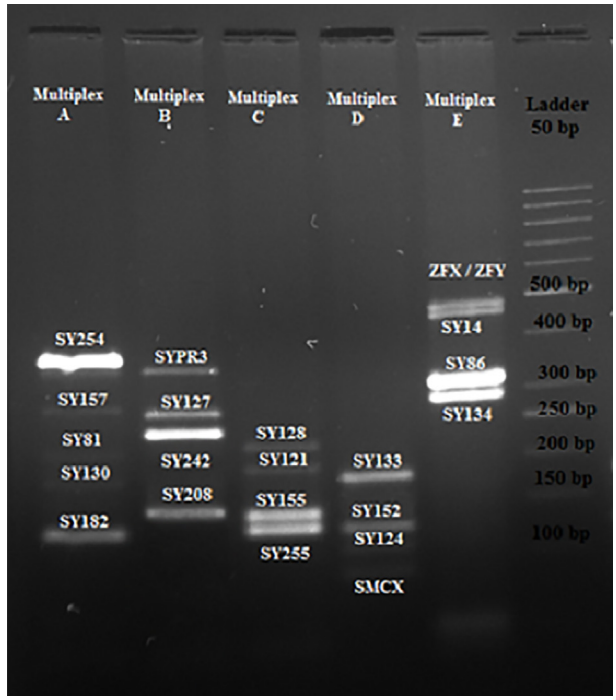

Fig.1: Results of the multiplex reactions A, B, C, D and E in male partners of women with recurrent pregnancy loss (RPL). Polymerase chain reaction (PCR) fragments were separated on a 3.5% agarose gel. Lane 1; Multiplex A, Lane 2; Multiplex B, Lane 3; Multiplex C, Lane 4; Multiplex D, and Lane 5; Multiplex E. Molecular weight marker (50 bp ladder).

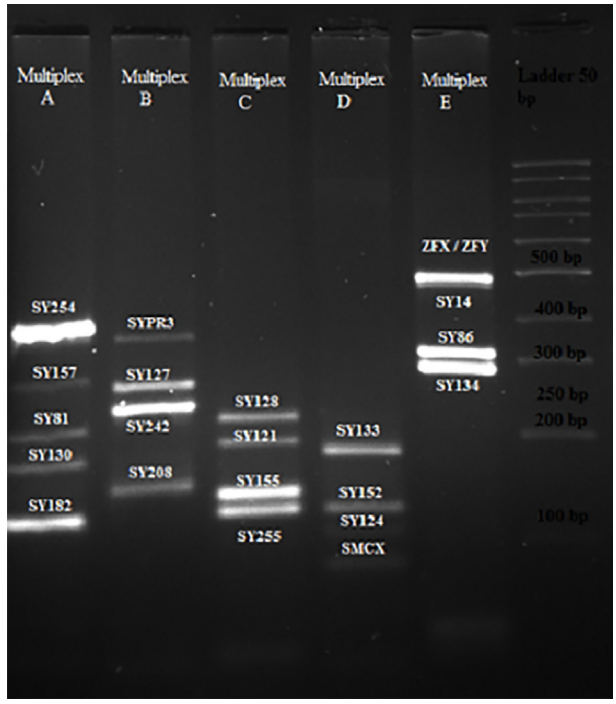

Fig.2: Results of the multiplex A, B, C, D and E in control group. Polymerase chain reaction (PCR) fragments were separated on 3.5% agarose gel. Lane 1; Multiplex A, Lane 2; Multiplex B, Lane 3; Multiplex C, Lane 4; Multiplex D, and Lane 5; Multiplex E. Molecular weight marker (50 bp ladder).

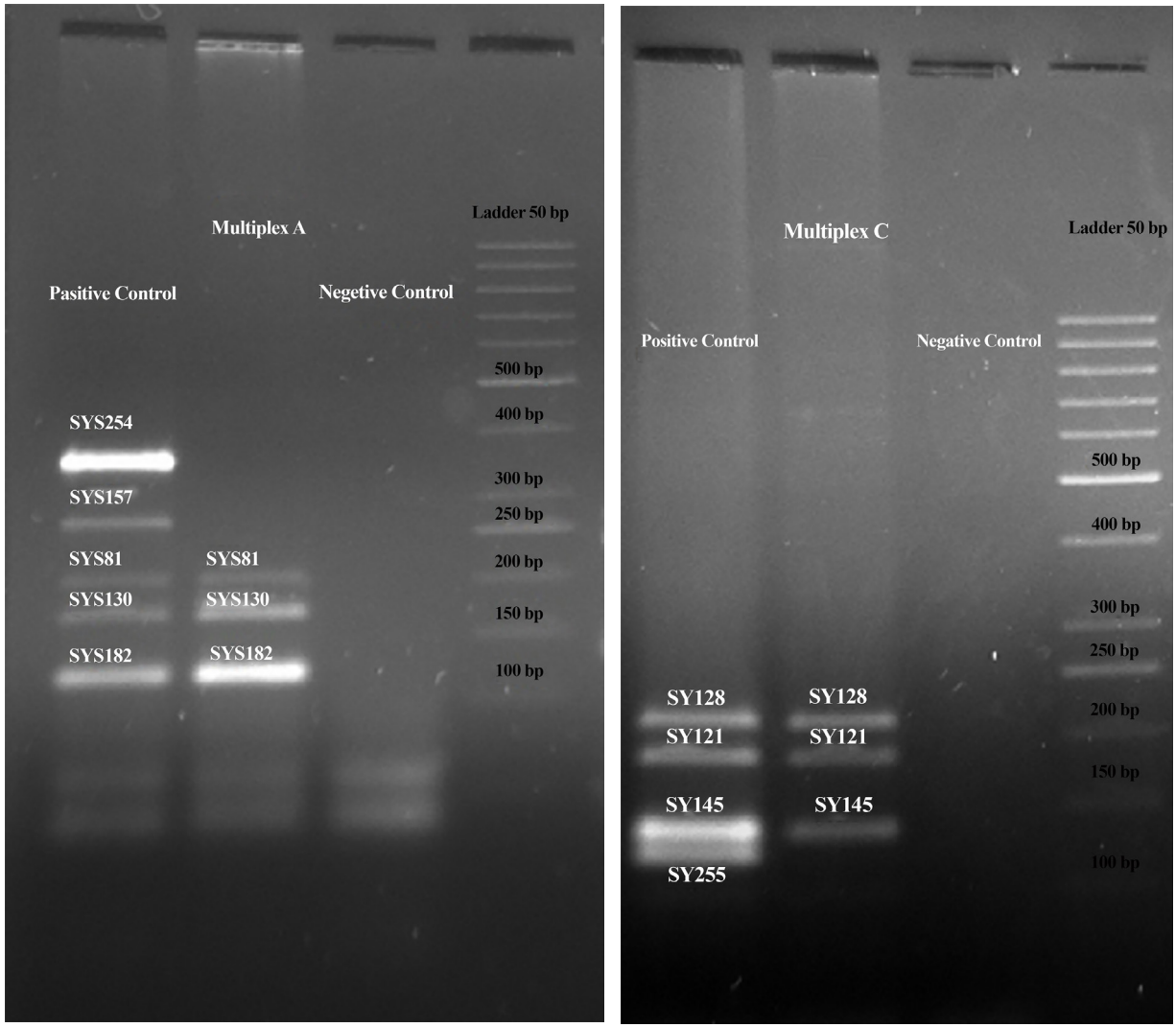

Fig.3: Detection of partial *AZFc* deletion in an infertile male patient. **A.** The microdeletions observed were in Multiplex A (*SY254* and *SY157*) and **B.** In Multiplex C (*SY255*). Polymerase chain reaction (PCR) fragments were separated on a 3.5% agarose gel. Lane 1; Fertile control, Lane 2; Infertile man, and Lane 3; Negative control. Multiplex A reaction contained *SY81* (209 bp), *SY130* (173 bp), *SY157* (290 bp), *SY182* (125 bp) and *SY254* (380 bp), and multiplex C reaction contained *SY121* (190 bp), *SY128* (228 bp), *SY145* (143 bp) and *SY255* (124 bp). Molecular weight marker (50 bp ladder).

Discussion

The AZF region was originally identified by Tiepolo and Zuffardi (28). These microdeletions are thought to be pathogenetically involved in some cases of male infertility who have azoospermia or severe oligozoospermia (4). Although chromosomal abnormalities in sperms of infertile men may lead to RPL (29), microdeletions in the AZFc region of the Y chromosome may have an important function in embryo "competency" or in maintaining gestation. This has led to Y-chromosome AZFc microdeletion testing in RPL cases when no other explanation for RPL is known (7).

The Y chromosome is extremely rich in repetitive sequences, organized in amplicons forming eight palindromes. Most of the genes deleted in infertile men are located in the palindromic regions of the Yq and are exclusively expressed in the testes (3, 13). Since AZF microdeletions usually include more than one gene, the role of a single AZF gene cannot be specified and thus unclear. Gene-specific deletions removing a single gene has been only reported in the AZFa region (30). In our study, a single infertile man (2.5%) had microdeletion in the AZFc region (partial AZFc deletions), which displays a lower frequency of AZF microdeletions than other reports in Iran (5, 31-33).

Y chromosome microdeletions were neither found in the male partners of women experiencing RPL nor in the control group. Although this finding is in agreement with the results obtained by Ghorbian et al. (24), it does not support the results of Soleimanian et al. (27) who detected Y chromosome microdeletions in male partners of women with RPL. This discrepancy could be explained by the small sample size, which is a limitation of the current study. In addition, differences in genetic background of the population studied here and the typing of different sets of STS used in different studies may explain the differences in the frequency of AZF microdeletions. Adjusted sample size and use of identical sets of STS could lessen the variation in results.

Conclusion

We showed Y chromosome microdeletions were not associated with non-obstructive infertility and recurrent pregnancy loss in our population study. Thus, this study is not supporting to test for AZF microdeletions in these two groups.

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Author's Contributions

H.A., M.M.T., M.A.K.; Contributed to conception and design. A.M.S.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. M.A.K., A.T.; Were responsible for overall supervision. A.M.S.; Drafted the manuscript, which was revised by H.A. and M.A.K. All authors read and approved the final manuscript.

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Original Article

Body Mass Index Effects Kruger's Criteria in Infertile Men

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Abstract

Background: The aim of this study is to evaluate the relationship between sperm parameters and body mass index (BMI) in the male spouses with infertility complaints, who had reffered to our clinic.

Materials and Methods: The male spouses from 159 couples reffering to our clinic because of infertility, during a six-month period, were included in the study. In this prospective case control study, the included men were categorized as non-obese (BMI<25 kg/m^2), overweight (BMI 25-29 kg/m^2) and obese (BMI ≥30 kg/m^2) according to their BMIs. The assessed sperm parameters consisted of; sperm concentration, Kruger morphology, progressive motility level, and volume pH levels. The statistical significant level was set as less than 0.05.

Results: The assessed group consisted of 159 patients applying to our clinic with infertility symptoms. Fifty-three non-obese, 53 overweight and 53 obese men were eligible for the study. There was statistically significant differences in sperm volume (P<0.001), progressive motility (P<0.001), postwash sperm count (P<0.001) and Kruger (P<0.001) morphology among the patient groups grouping according to the BMI levels.

Conclusion: In this study, increased BMI was associated with decreased semen quality, affecting volume, concentration, and motility. further studies with a wider range of prospective cases need to be conducted in order to investigate the effects on male fertility in more detail.

Keywords: Body Mass Index, Male Infertility, Obesity

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Introduction

The definition of obesity given by the World Health Organization (WHO) is; a body mass index (BMI) of over 30 kg/m^2 (1). The epidemic global obesity displays a parallel relation with the decrease in semen quality. The connection between the sperm parameters and obesity is debatable. Obesity reduced semen quality and the sperm mitochondrial activity. Also itincreases sperm DNA damage and miscarriage, induces seminal oxidative stress, impairs blastocyst development, or reduces pregnancy outcome following assisted reproduction (2). Furthermore; there are other outcomes such as impaired erectile function, and other physical problems, such as increased scrotal temperatures and sleep apnea (3, 4). The male reproductive function can be significantly affected by the hormonal changes related to obesity. A reduction in the binding capacity of sex hormone-binding globulin with increased serum oestradiol reduced luteinizing hormone (LH) pulse amplitude. Decreased levels of serum gonadotropin and testosterone may be among these changes (5). Although there are conflicting reports in terms of obesity and sperm parameters; insulin-suppressed sex hormone binding globulin (SHBG) in obese men increases the androgen availability required by the adipose aromatase for the production of estrogen which may result in a reduced gonadotropin secretion (2).

In the subfertile couples, the increased abdominal adiposity in men is related to decreased concentration, motility, and sperm count (6) but it is also associated in some studies (7, 8) not all (9, 10) with an increased incidence of asthenozoospermia and oligozoospermia. In this present prospective study, the aim was to look into various semen parameters (sperm concentration, semen volume, morphology) in infertile obese-men and also to evaluate their association with BMI.

Materials and Methods

This study was conducted at the Zekai Tahir Burak Women's Health Education and Research Hospital, Ankara from February 2016 to June 2016. The study protocol was in conformity with the principles of the Declaration of Helsinki, and approval of the institutional review board obtained. Each patient had his informed consent. In this prospective case-control study, the sample group consisted of 159 infertile Turkish men from

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central Anatolia between the ages of 22-54 years. Initially, all men were admitted to our infertility outpatient clinics with their spouses. Medical and infertility history of each patient was recorded. Also patients were excluded if any chronic diseases (chronic liver and kidney problem, asthma, epilepsy, steroid users, chronic inflammatory bowel disease, history of thromboembolism and cerebrovascular event, hypotroidea) were evident. They measured each patients' weight and height with a professional, calibrated device. BMI was calculated by dividing the weight to height squared. Patients were stratified according to their BMI into three groups. The evaluation process included a total of 159 patients with infertility symptoms. Fifty-three non-obese (BMI<25 kg/m^2), 53 overweight (25-29), 53 (BMI≥30 kg/m^2) obese men were eligible for the study.

They asked the patients to refrain from sexual activity for a period of 3 days. They collected semen samples from the patients by masturbation in a private room nearby the laboratory. The collected semen specimens were assessed by using a computerized semen analyzer for conventional semen parameters including sperm motility and sperm concentration (pre-washed for half an hour at room temperature) after liquefaction. Standard swim-up method with a sperm preparation media (Ferticult Flushing medium TM, FertiProNV, Beernem, Belgium) was used to process the rest of the semen. A computer-assisted semen analyzer was used for the post-wash analyses. In relation to the quality control program; the same andrology laboratory technician performed the sperm analyses. The World Health Organization guidelines (11) were used to assess the sperm analyses. For each man sperm count, pH, semen volume, percentages of motility, sperm concentration, and normal sperm morphology were recorded.

Statistical analysis

All statistical analyses were performed by using SPSS for Windows 11.5 software program (SPSS Inc., Chicago, IL). Except descriptive statistics, one-way ANOVA and Kruskal Wallis were used to approximate statistical disparity on the collected data between BMI groups. A statistically significant difference was found between the group while evaluating the quantitative data of more than two groups. Also Post Hoc analyses were performed with Tukey HSD Test for the normally distributed data and paired Mann Whitney-U Tests conducted for abnormally distributed data and finally Bonferroni correction was applied. The statistical significance level was set as less than 0.05. The significance limit in the Bonferroni correction was determined to be 0.05/k with the evaluation number of the paired Mann Whitney-U being "k". The statistical significance level was set as less than 0.05.

Results

The evaluated group consisted of 159 patients applying to our clinic with infertility symptoms. A total of 159 patients were grouped as non-obese (BMI<25 kg/m^2) (53 men), overweight (BMI 25-29 kg/m^2) (53 men) and obese (BMI ≥30 kg/m^2) (53 men). The demographic and clinical characteristics of the patients has been shown (Table 1).

Table 1: Comparison of demographic and clinical characteristics of the patients

Variable	Obese group BMI≥30 kg/m^2 n=53		Overweight group BMI 25-29 kg/m^2 n=53		Non obese group BMI<25 kg/m^2 n=53		P value
	Mean ± SD	Median (Minimum-Maximum)	Mean ± SD	Median (Minimum-Maximum)	Mean ± SD	Median (Minimum-Maximum)	
Age (Y)	33.32 ± 6.64	32.00 (23.00-54.00)	32.93 ± 4.92	32.00 (22.00-45.00)	32.21 ± 5.82	32.00 (24.00-52.00)	0.610
Sperm volume (cc)	1.97 ± 1.61	2.00 (0.00-6.00)	3.36 ± 1.43	3.00 (1.00-8.00)	2.94 ± 1.20	3.00 (1.00-6.00)	<0.001*
pH	6.30 ± 3.27	8.00 (0.00-8.00)	7.83 ± 0.24	8.00 (7.50-8.00)	7.88 ± 0.43	8.00 (6.00-8.00)	0.033*
Concentration (m/mL)	23.07 ± 30.38	12.00 (0.00-150.00)	44.12 ± 47.13	25.00 (0.00-170.00)	45.39 ± 45.36	33.00 (0.00-180.00)	0.014*
Progressive motility (%)	21.49 ± 21.44	11.00 (0.00-60.00)	40.60 ± 11.21	39.00 (19.00-75.00)	41.02 ± 11.89	44.00 (4.00-57.00)	<0.001*
Postwash sperm count (m/mL)	8.40 ± 11.05	5.00 (0.00-43.00)	26.10 ± 19.62	21.00 (2.00-65.00)	29.66 ± 19.39	22.00 (2.00-65.00)	<0.001*
Postwash progressively motility (%)	38.63 ± 42.66	19.00 (0.00-100.00)	27.75 ± 13.91	27.00 (10.00-70.00)	29.60 ± 12.46	33.00 (2.00-48.00)	0.446*
Morphology (%)	3.15 ± 2.93	3.00 (0.00-10.00)	5.90 ± 2.09	6.00 (3.00-11.00)	6.09 ± 2.28	7.00 (2.00-13.00)	<0.001*
Concentration <15 m/cc n (%)	1.00 ± 0.00	1.00 (1.00-1.00)	1.00 ± 0.00	1.00 (1.00-1.00)	1.00 ± 0.00	1.00 (1.00-1.00)	1.000*
Azoospermia n (%)	1.00 ± 0.00	1.00 (1.00-1.00)	1.00 ± 0.00	1.00 (1.00-1.00)	1.00 ± 0.00	1.00 (1.00-1.00)	1.000*

*; Kruskal Wallis Test analysis results and BMI; Body mass index.

Statistically significant differences were found in sperm concentration among the three groups (P=0.014). The statistically significant difference was because of the difference between the obese and non-obese groups (P=0.019).

There were also statistically significant differences in sperm volume (P<0.001) among three groups. The statistically significant difference was because of the difference observed between the obese and non-obese groups (P=0.010) and also the obese and overweight groups (P<0.001). Also There were statistically significant differences in pH among three groups (P=0.03). The statistically significant difference was because of the difference observed between the obese and non-obese groups (P=0.010) and also the obese and overweight groups (P=0.007).

There were also statistically significant differences in progressive motility among three groups (P<0.001). The statistically significant difference was due to the difference between the obese and non-obese groups (P<0.001) and the obese and overweight groups (P=0.001). There were also statistically significant differences in postwash sperm count among three groups (P<0.001). The statistically significant difference was due to the difference observed between the obese and non-obese groups (P=0.008) and the obese and overweight groups (P=0.013). There were also statistically significant differences in Kruger morphology among three groups (P<0.001). The statistically significant difference was due to the difference observed between the obese and non-obese groups (P<0.001) and the obese and overweight groups (P<0.001).

Discussion

The principal finding of this study is that; in terms of semen parameters there are statistically significant differences among the non-obese, overweight and the obese infertile patients. The effect of obesity on sperm quality is still under discussion. Nearly 40% of all infertility cases have a male origin (12). The reasons for male infertility may be numerous. Infertility may be induced in males by a number of reasons; the presence of antibodies against sperm, sperm production disorders, hormonal disorders, blockage in the sperm ducts, testicular trauma, varicocele, anatomical problems, certain medications and infections can be named among them. Hence, for eliminating factors that could play a role in changing the semen parameters alongside BMI, we excluded factors such as smoking, varicocele, hormonal changes, and other fertility-affecting factors in our study. Based on the recent reports by the WHO, obesity is a major concern; as in 2008; globally more than 1.4 billion were overweight and 500 million were obese (13). Nevertheless, theeffect of obesity on sperm parameters is conflicting. Although the semen parameters established by the WHO were used in various studies, the results obtained on the impact of obesity on sperm parameters were dissimilar.

The sperm concentration of infertile men in this study displayed a statistically meaningful relation with BMI. According to the recent WHO surveillance study; conducted on male partners of pregnant women; determined that the total sperm count of the obese-men were significantly lower than the non-obese men (mean 231×10^6 vs. 324×10^6, respectively), even though other sperm parameters did not reveal any signs of being affected (14). Hofny et al. (15) demonstrated decreased sperm count in obese normozoospermic men compared with nonobese fertile subjects. Bounartzi et al. (16) showed that overweight and obese men had reduced sperm volume and the BMI was correlated with the percentage of degenerated spermatozoa and increased sperm DNA fragmentation. Belloc et al. (17) revealed that increased BMI was associated with decreased semen quality which affects volume, concentration, and motility.

Our results are contradictory with those published by the analysis of a database of 2139 patients, which displayed no significant change in obese men. The effect of it on sperm count may be negligible (mean total sperm count for BMI 20-25 kg/m^2: 231×10^6; BMI.30: 265×10^6) (5). According to Martini et al. (18), no relationship between BMI and sperm concentration was noted. Similar findings were mentioned in other studies (19, 20). Still, conflicting with these studies; a study, including healthy volunteers showed that overweight men, in comparison to men with normal BMI, had significantly lower total sperm count and sperm concentration (21). Our results display a significant association between the semen volume and BMI. Wang et al. (22) revealed lower sperm quality (total sperm count, sperm concentration, motile sperm, relative amounts of type A motility, and progressive motility sperm [A + B]) in overweight and obese participants than in those with normal body mass index. In a more recent study by Rosenblatt et al. (23) it was shown that among the obese men the sexual quality of life was diminished, and increased BMI correlated with reduced sexual activity. A large clinical study conducted by Belloc et al. (24) on >10,000 samples; displayed that there was a distinct link between obesity and sperm production (total sperm count, volume and, concentration), whereas no other statistically significant relationship was found in terms of other semen parameters, especially the sperm morphology.

Evaluation of the sperm morphology may be disputable since it continues to be affected by influences of the subjectivity of the observer and because of the lack of objective measurement standards. A statistically significant association between the sperm morphology and BMI was observed by us. A study with a small sample size demonstrated that weight loss led to improvement in semen quality including sperm count and normal sperm morphology (25). The results of a study declared that there was a significant positive correlation between abnormal sperm morphology and BMI (15). In order to have a better idea about the effects of excess weight or obesity on reproduction; men should be included in the studies notonly for the BMI butalso for the factors related to excess weight which could also affect fertility; such as fat distribution, lifestyle habits, and associated pathologies.

As an example, Hammiche et al. (6), noted that not only the BMI, but also another factor such as waist circumference of 102 cm, which is a measure of central adiposity, was inversely associated with total motile sperm count and sperm concentration. Håkonsen et al. (26), came to the conclusion that; weight loss could improve the semen quality in a study with a relatively low sample size. In addition Anifandis et al. (27) the sperm DNA integrity, as a potential clinical marker of semen quality SDF only correlated with sperm characteristics.

On the other hand, while BMI of men affects fecundity, it is ambiguous that BMI has really an empact on sperm parameters. A retrospective study embracing in 301 subjects was carried out by Anifandis et al. (28) who unveils that there is an affiliation between male BMI and embryo quality; while male BMI does not correspond to sperm parameters which is no effect on the *in vitro* fertilization (IVF) outcome as well. Besides, Thomsen et al. (29) conducted on 612 infertile couples submitted to ART at a Danish fertility center. Semen parameters (sperm concentration, total sperm count, seminal volume and motility) were statistically insignificant influenced by male BMI. Furthermore, fertilization rate, number of good quality embryo, implantation and pregnancy as the outcomes of ART were not depended upon by the escalation of male BMI. One prime limitation of our study was that due to the low number of patients, it could not be a population-based study. Also there were no records of whether the same patients had multiple sperm analyses during the study period and how they were conducted. There was also another limitation due to the heterogeneity of the populations (both general population and infertile couples) under focus. On the other hand, one of the important sperm characteristics, sperm DNA fragmentation (SDF) data were not included in our study due to insufficient funds.

Conclusion

The effect of obesity on the conventional semen parameters in infertile men was evaluated in the study. In this study, we established statistically significant differences between the BMI and sperm concentration, motility, semen volume, and normal sperm morphology. Large prospective randomized clinical studies, including men with no infertility problems should be conducted in order to support these findings and investigate the effects on male fertility in more detail. Further studies at the level of advanced semen parameters such as the sperm DNA integrity are needed.

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Author's Contributions

Y.E.U, N.Y., N.A.; Contributed to conception and design. A.D.T, B.B.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. Y.E.U, N.A., A.A.; Were responsible for overall supervision. N.A.; Drafted the manuscript, which was revised by Y.E.U. All authors read and approved the final manuscript.

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Original Article

Royal Jelly Promotes Ovarian Follicles Growth and Increases Steroid Hormones in Immature Rats

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Abstract

Background: Royal jelly (RJ) is a complementary diet widely prescribed by traditional medicine specialists for treatment of infertility. The aim of present study was to evaluate the effects of RJ on a set of reproductive parameters in immature female rats.

Materials and Methods: In this experimental study, thirty two immature female rats (30-35 g) were divided into four groups (n=8/group): three experimental groups and one control. The experimental groups received 100, 200 and 400 mg/kg/body weight doses of RJ daily for 14 days, and the control group received 0.5 ml distilled water interaperitonealy (i.p). The treated rats were sacrificed and their ovaries were dissected for histological examination. The serum levels of ovarian hormones, nitric oxide (NO) and ferric reducing antioxidant power (FRAP) were evaluated, and the ratios of the ovarian and uterine weight to body weight were calculated. One-way ANOVA was used for data analysis.

Results: The body weights were significantly different (P=0.002) among the rat groups, with an increase in all RJ treated animals. Uterine and ovarian weights and the serum levels of progesterone (P=0.013) and estradiol (P=0.004) were significantly increased in experimental groups compared to the control group. In addition, a significant increase in the number of mature follicles and corpora lutea (P=0.007) was seen in RJ recipients compared to the controls. A significant increase in the serum levels of FRAP (P=0.009) and a significant decrease in NO level (P=0.013) were also observed.

Conclusion: RJ promotes folliculogensis and increases ovarian hormones. This product can be considered as a natural growth stimulator for immature female animals.

Keywords: Fertility, Immature Rats, Ovary, Royal Jelly

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Introduction

Puberty is the underlying reproductive process, in which several maturation changes occur in the body to promote adult phenotype. The onset of puberty is sensitive to the amount of energy reserves of the organism and nutritional status and it is associated with the dynamic dialogue between the environment and genes (1). In females, estrogens have a key role in the differentiation, growth and function of the reproductive organs. They are identified as the principal hormones in regulation of the female reproductive system (2). Royal jelly (RJ) is the most important apiary product, secreted by 5 to 15 day old worker honeybees (3). It possesses several health promoting properties such as antitumor effect, antioxidant activity, and improvement of menopausal symptoms and infertility (4). There are four unsaturated fatty acid compounds in RJ (10H2DA, 10HDA, 2DEA and 24MET) that showed estrogen receptors' (ERs') β-binding activity. These compounds exhibited estrogenic effects mediated through interactions with ERs, leading to alterations in gene expression and cell proliferation (5). Nonetheless, RJ has been shown to inhibit the harmful effects of exogenous estrogen on male reproductive tract (6).

Previous study showed that RJ treatment increased plasma progesterone levels in sheep (7). RJ could also be effective in ameliorating pregnancy rate and the lambing rate in Awassi ewes (8). These data prompted us to study the effect of RJ on puberty and fertility parameters in immature female rats. To our knowledge, the mechanism of action of RJ on reproductive function is still unknown. It may exert its effects via changing hormonal secretions or by containing hormone-like compounds. Other studies have shown that treatment of ovine oocytes with 10 mg/mL of RJ during *in vitro* maturation (IVM) increases oocyte and nuclear maturation rate, fertilization rate and blastocyst formation, which might be due to increased activity of antioxidant enzymes in both oocyte and cumulus cells (9). It has been reported that therapy with bee honey plus RJ may be an effective approach to treat infertility due to asthenozoospermia (10).

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It has been suggested that over-nutrition before puberty, as well as excessive weight gain might affect the maturation of reproductive system and induce an early onset of puberty (11). It should be noted that during the final oocyte maturation stage, alterations at levels of proteolytic enzymes, cytokines, prostaglandins and nitric oxide (NO) lead to an increase in the level of reactive oxygen species (ROS), thereby inducing ovarian blood flow and helping follicular rupture (12). It has also been suggested that excessive amount of follicular ROS may disrupt the antioxidant defense system, leading to oocyte injury (13). Interestingly, abnormal ROS levels might facilitate various pathological processes, specifically in the ovary and uterus, causing decreased pregnancy maintenance hormones and luteal regression (14, 15). The antioxidant effect of RJ has been widely examined and confirmed on the male reproductive system (15), but there is no data regarding the effect of RJ on immature female reproductive tract. The present study is designed to investigate the effects of RJ on ovarian maturation as well as changes in related parameters in immature female rats.

Materials and Methods

In this experimental study, 32 immature female Wistar rats aged about 25 days (30-35 g) were used for our study. They were maintained under standard environmental conditions of light (12 hour cycle), temperature (24 ± 2°C) and humidity (30-70%), and were fed with a standard laboratory diet and water ad libitum. The protocol of this study was approved by Animal Care and Use Committee at Kermanshah University of Medical Sciences. In addition, all animal procedures were in agreement with the guidelines of the Ethical Committee for research on laboratory animals at Kermanshah University of Medical Sciences (KUMS.REC.1395.168).

RJ was gathered from six colonies of Iranian honeybees (Apis mellifera) at the apiary of Urmia region, North-West of Iran. RJ samples were harvested in sterile bottles at 68-72 hours after bee larvae were transferred into the queen cell. Different doses of RJ were dissolved in 0.5 ml distilled water; this mixture was prepared freshly every day.

Experimental design

The rats were randomly classified into 4 groups (n=8): three groups received 100, 200 and 400 mg/kg/day doses of RJ interaperitonealy (i.p). These doses were chosen according to previous studies (16, 17).The fourth group was our control, which received distilled water for 14 days (i.p) (18). On day 14, the rats were weighted and euthanatized by chloroform inhalation in a closed chamber. Blood was collected by cardiac puncture and centrifuged at 2500 rpm for 15 minutes at 4ºC, and the collected serum was stored at -20ºC for the hormones assay (estradiol and progesterone), FRAP, and NO assay.

Hormonal assay

The levels of estradiol and progesterone in the collected sera were determined by ELISA kit (DRG International, GmbH, USA), and the reagents to perform the assays were purchased from GBC (General Biological Corporation, Hsin Chu, 30 077, Taiwan, R.O.C). The concentrations of these hormones were assayed via absorbance reading to Microtiter (well reader LabSystems Multiskan RC, 351, FIN-0 0881, Helsinki, Finland) at 450 nm.

Ferric Reducing Antioxidant Power assay

The total antioxidant capacity in the collected sera was evaluated by Ferric Reducing Antioxidant Power assay (FRAP) method. Briefly, 150 µl serum was mixed with 1.5 ml of fresh FRAP reagent (10 mM 2, 4, 6- Tripyridyl-s-Triazine, 20 mM $Fecl_3$, $6H_2O$ solution and 300 mM acetate buffer pH= 3.6), and incubated at 37˚C for 10 minutes. Absorbance at 593 nm was then measured using a spectrophotometer (Pharmacia, Novaspec II, Biochrom, England) and was compared with a standard curve constructed with known concentrations of $FeSO_4$ $7H_2O$. Results were expressed in µM (19).

Nitric oxide assay

The serum levels of NO were determined colorimetrically by Griess method, which includes the conversion of nitrate to nitrite. Griess reagent facilitates the conversion of nitrite to a deep pink azo substance (20). Briefly, equal volumes of Griess reagent and serum samples were mixed and incubated at room temperature for 30-45 minutes. Next, the absorbance rate was determined at 540 and 630 nm using ELISA reader (STAT Fax 100, USA).

Ovarian histology evaluation

The ovaries and uteri were dissected and cleaned from fatty tissues and were weighed afterwards. The ovaries were fixed in 10% formalin, sectioned at 6 µm thickness and stained with hematoxylin and eosin (H and E) for histological analysis. The five largest sections of each ovary were used for follicular and corpora lutea investigation. Histopathological examination was performed to measure the effects of RJ on the mean number of primary, secondary, graffian follicles and corpora lute. Samples were evaluated for histological changes under an optical microscope and images were captured for further analysis using an image processing software (Motic 2000) (21).

Statistical analysis

All data were analyzed by SPSS software (version 18) and presented as mean ± SE. There were no outliers, and the data were normally distributed for each group, as evaluated by box plot and Shapiro-wilk test, respectively. The assumption of homogeneity of variance was also evaluated (P value for Levene's test was not significant). Comparison of data between the groups was performed by one-way ANOVA followed by post-hoc Tukey test with significance level of $P<0.05$.

Results

Whole body, ovarian and uterine weight changes

Administration of different RJ doses to immature female rats for 14 days resulted in a significant increase

(P=0.002) in body weights compared to the control rats. On the 1st day, there was no significant difference (P=0.9) between all groups in the mean body weight (Fig.1). All three doeses of RJ significantly increased the ovarian (P=0.002) and uterine (P=0.007) weights in comparison to controls (Fig.2A, B).

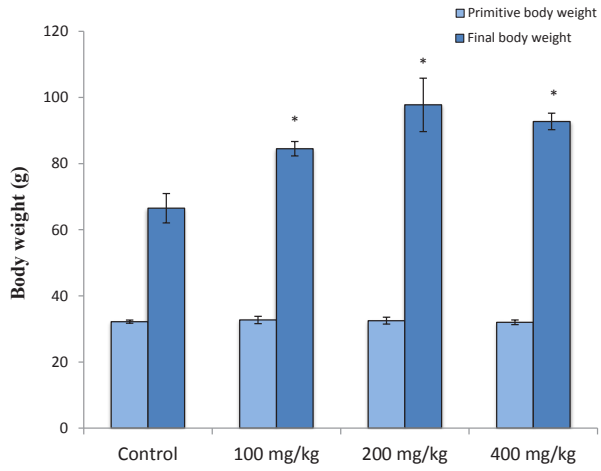

Fig.1: Effect of royal jelly (RJ) on body weight (first and final weight). Values represent mean ± SE for 8 rats in each group.
*; P< 0.05 shows significant difference from control group.

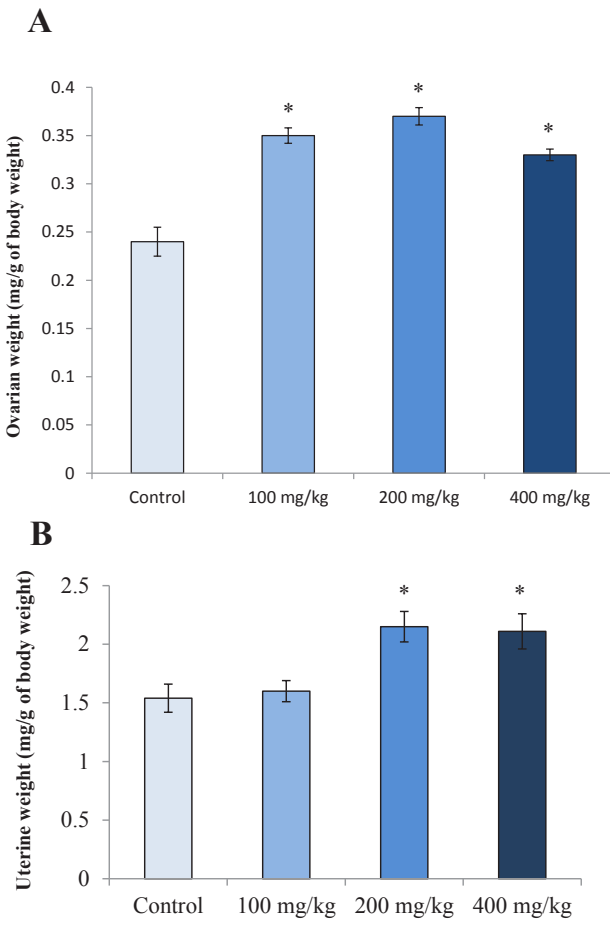

Fig.2: Royal jelly (RJ) effect on the ovarian and uterine weights. **A.** Histogram of ovarian weights and **B.** Histogram of uterine weights.
*; Values significantly different at P<0.05 from control group (ANOVA). Each histogram represents the mean ± SE of the values for 8 rats.

Ovarian hormones

The effect of RJ on production of ovarian hormones was a significant rise in the serum estradiol levels in the groups treated with 200 and 400 mg/kg doses of RJ (P=0.004) compared to the control group (Fig.3A). Serum estradiol values for doses 100, 200 and 400 mg/kg of RJ were 198.7 ± 8.3, 273.0 ± 32.6 and 252.7 ± 39.29 (pg/ml), respectively, whereas it was 150.0 ± 9.28 (pg/ml) for the control group. Similarly, RJ treatment led to a significant increase (P=0.013) in serum progesterone levels. The progesterone concentration was measured to be 12.73 ± 7.8, 17.62 ± 7.82, 41.00 ± 1.44 and 21.45 ± 3.64 (pg/ ml) in control, 100, 200 and 400 mg/kg doses of RJ, respectively (Fig.3B).

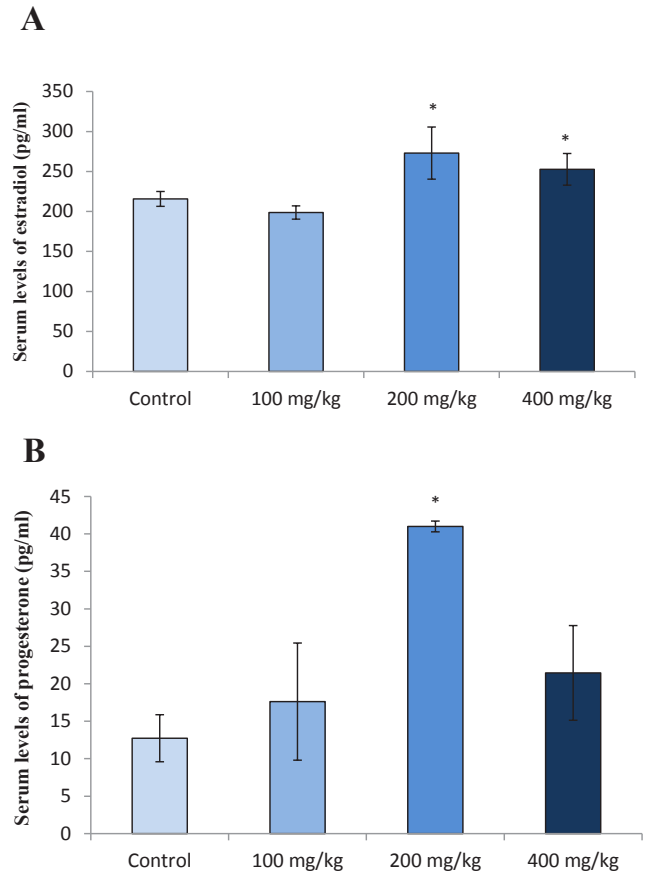

Fig.3: Effect of royal jelly (RJ) on the serum levels of estradiol and progesterone. Values significantly different respectively at P<0.05 from control group (ANOVA). **A.** Histogram of serum levels of estradiol and **B.** Histogram of serum levels of progesterone. Each histogram represents the mean ± SE of the values for 8 animals.
*; P<0.05 represents significant difference from control group.

Nitric oxide and ferric reducing antioxidant power levels

NO concentration was measured in the rat sera and the results showed that RJ decreased NO levels in immature rats significantly (P=0.013) (Fig.4A). Also, significant increase (P=0.009) in serum levels of FRAP was observed in normal rats treated with doses 100 and 200 of RJ compare to control group (Fig.4B)

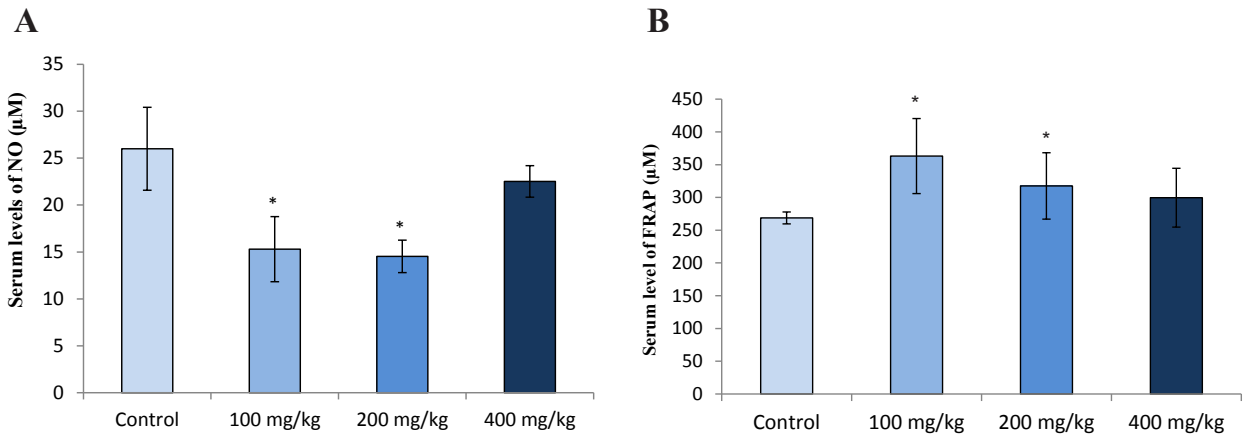

Fig.4: Effect of royal jelly (RJ) on serum NO and FRAP in immature female rats. **A.** Histogram of serum levels of NO and **B.** Histogram of serum levels of FRAP. Data represent the mean ± SE (n=8 for each group).
NO; Nitric oxide, FRAP; Ferric reducing antioxidant power, and *; P<0.05 indicates significant difference from control group.

Ovarian structure

Microscopic observations of the treated rat ovaries showed an increase in the mean numbers of secondary, antral and graffian follicles when 100 and 200 mg/kg doses of RJ were administered. There was also a significant increase (P=0.007) in the number of corpora lutea of the rats treated with 100 and 200 mg/kg doses of RJ compared to the control rats (Figs.5, 6).

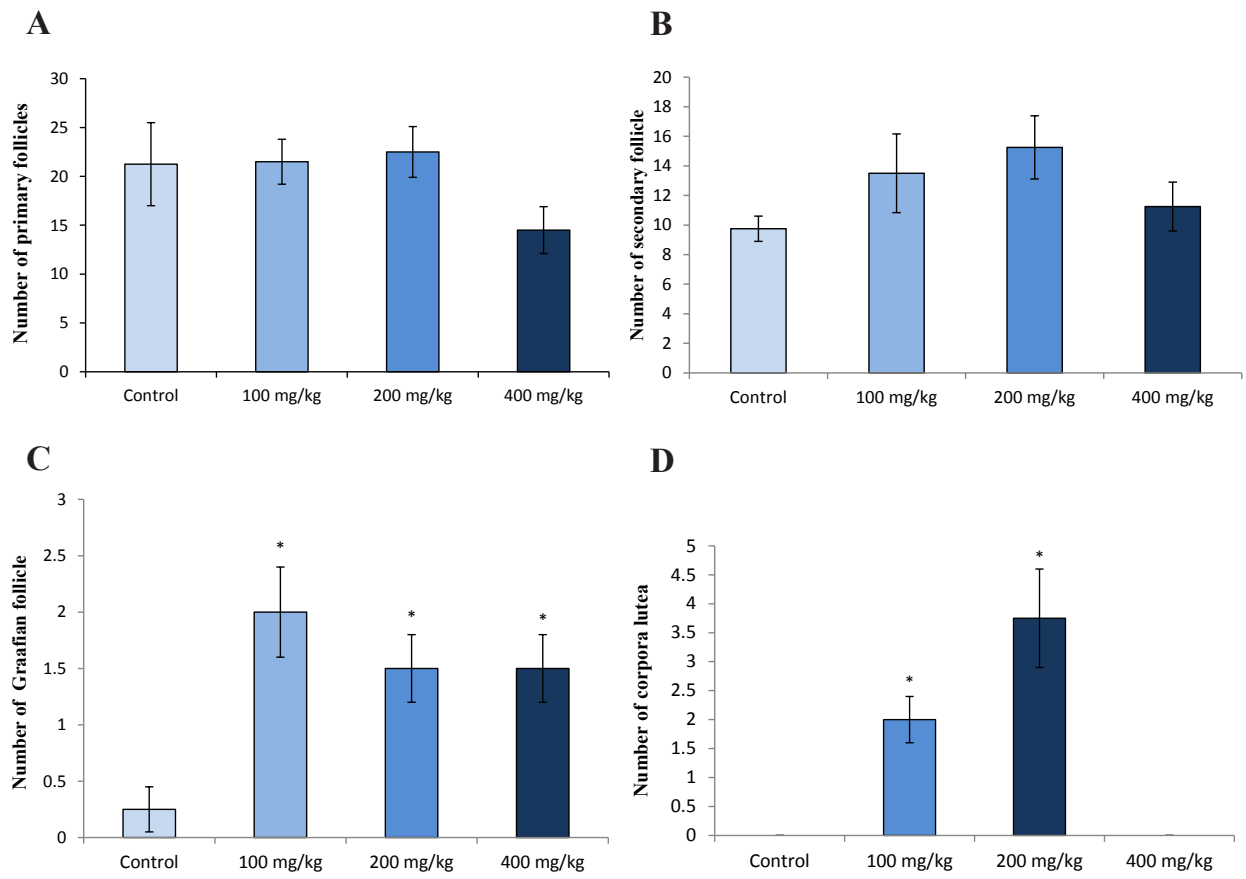

Fig.5: Effect of royal jelly (RJ) on the number of follicles and corpora lutea in ovarian cortex. **A.** Histogram of the number of primary follicle, **B.** Histogram of the number of secondary follicles, **C.** Histogram of the number of Graffian follicles, and **D.** Histogram of the number of corpora lutea.
*; P<0.05 shows statistically significant difference from control group.

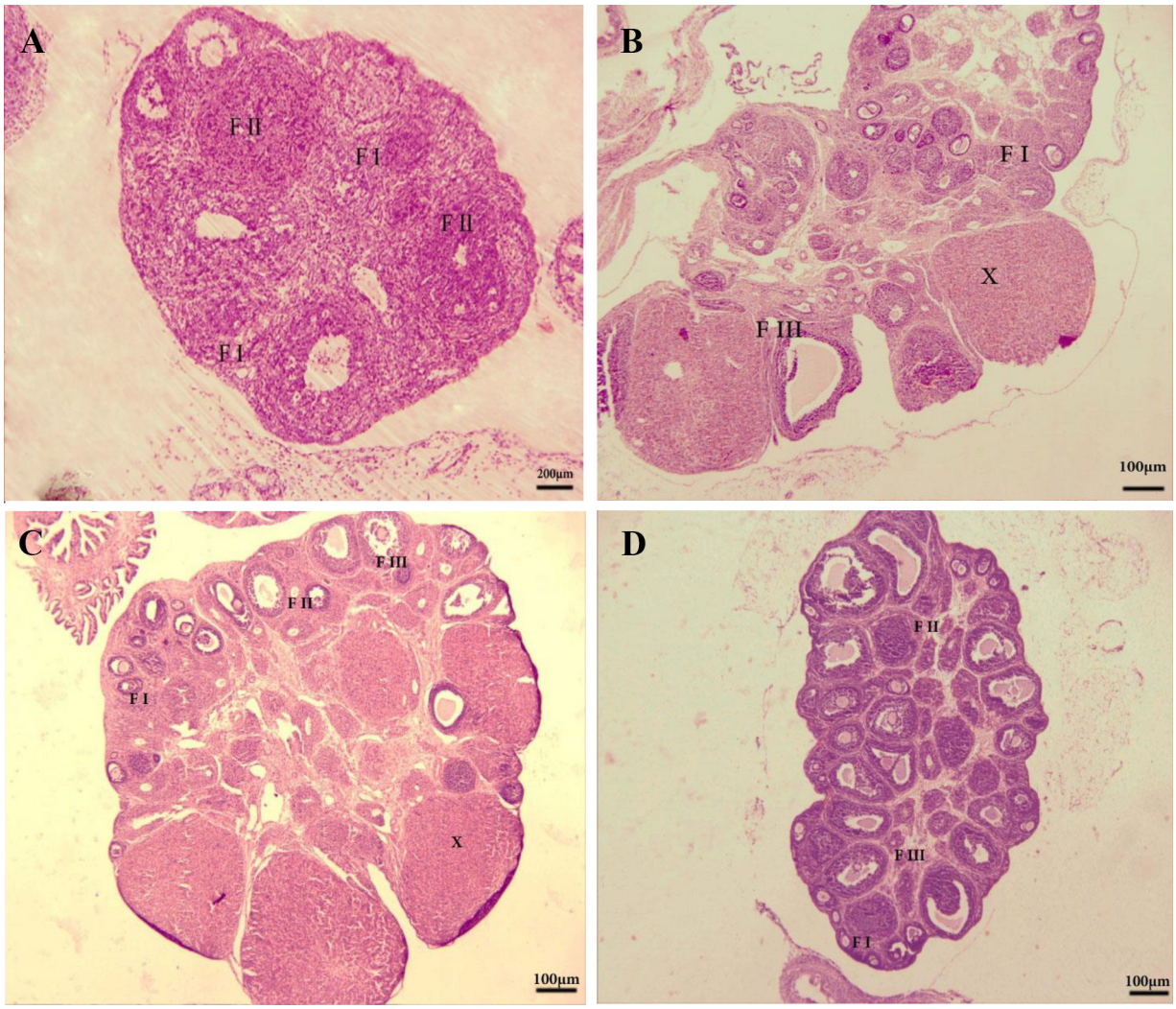

Fig.6: Representation of histological study on immature female rat ovaries. **A.** Ovaries from the control (magnification: ×10, scale bar=200 µm), **B.** Treated with 100 mg/kg, **C.** 200 mg/kg royal jelly (RJ), and **D.** 400 mg/kg of RJ. The figures show primary, secondary and graffian follicles and also observed the corpora lutea (magnification: ×4, scale bar=100µm). Primary follicles (F I), the secondary follicles (F II), the antral follicles (F III) and Corpora lutea (X).

Discussion

In this study, administration of different doses of RJ to immature female rats led to remarkable increase in the serum steroid hormones, and in body, uterine and ovarian weights. These results suggest that, as a consequence of its estrogenic effects, RJ may have beneficial reproductive influence on both ovarian and uterine structures (22). Pervious studied have shown that consumption of dietary estrogenic compounds in immature rodents promotes female sexual maturation (23).

Ovarian steroids have pivotal roles in fertility, maturation and growth of reproductive organ. It seems that RJ components exert their effects by binding to estrogen receptors. Previous reports have shown that four main isolated components from RJ bind preferentially to estrogen receptor β rather than estrogen receptor α in immature rats (5). In addition, different bioactive compounds in RJ have been identified that promote cell growth, cell survival and cell differentiation in insects (24). Also, a RJ protein (350 kDa), which seems to be a stimulator of reproductive system development has been reported (25), which might be the growth promoting factor leading to the weight changes that we observed in the rat ovaries as a result of RJ treatment.

The weight gains that were noticed following RJ administration in our study may be related to high amino acid and protein contents of RJ, which might be involved in metabolic pathways for tissue synthesis and body growth. On the other hand, royalactin, a 57-kDa protein in RJ, induces the development of honey bee larvae to queens via epidermal growth factor receptor-mediated signaling pathway, as well as elevating body size and ovarian development (26). Additionally, it has been shown that treatment with RJ may beneficially impact ovulation rate, which might be associated with an increase in progesterone levels in luteal phase (7).

Kridli et al. (27) have reported a minor decrease in interval to the onset of estrus, as the effect of RJ administration which can be associated with the stimulating properties of RJ on follicular development and growth. Similar to our findings, other studies suggest that RJ increases the development and growth of follicle resulting in estradiol

secretion required to stimulate behavioral estrus, luteinizing hormone (LH) surge and ovulation (7). To assess the dose of RJ required for an optimal effect, we selected three different doses of RJ according to our previous experiments (15) and other related studies (16, 28, 29). Our data showed that 200 mg/kg of RJ is an efficient dose in our study and exerts significant differences in many studied parameters.

Since we were interested in studying the effects of RJ administration in immature rats, we chose 25 day old rats and treated them for a period of 14 days to complete our study before complete rat maturation. In terms of maturation, our data showed that treatment with 200 mg/kg RJ increased the mean number of secondary, antral and graffian follicles and corpus luteum, indicating that RJ exerts a stimulating effect on folliculogenesis and ovulation.

Recent studies have suggested that ovarian/oocyte NO production plays a key role in oocyte meiotic maturation and ovulation. Also, at the time of ovulation, NO may function as a signal for somatic cells of the follicle wall, which are necessary for ovulation (30). In the present study, however, the serum NO levels in immature female rats significantly decreased with administration of RJ. This reduction of serum NO might be the result of increasing serum levels of FRAP. These observations are consistent with the findings of other researchers, who have stated that paclitaxel administration increases NO level, while treatment with RJ (50, 100 and 150 mg/kg) is able to significantly protect this reduction of NO level (29).

In female animals changes in the physiological concentration of ROS possess a pivotal role in the reproductive functions, including oocyte maturation, ovarian steroidogenesis, folliculogenesis, luteolysis and ovulation. In addition, cumulus cells, follicular fluid and oocytes are endogenous sources of free radicals, such as NO, in female reproductive tissues (31). Although free radicals possess many physiological outcomes, higher production of such compounds (e.g. NO, superoxides and H_2O_2) may lead to an elevated risk of ovarian pathology that would probably intensify under decreased antioxidant defense system (32). The possible antioxidant property of RJ makes it a potential scavenger for free radicals. We thought that increasing FRAPS and reduced NO levels in our study might be related to these beneficial effects.

In addition, Ramadan and Al-Ghamdi (24) showed that 29 peptides with antioxidative activity were separated from RJ protein hydrolysate. Albeit, 12 of these antioxidative peptides with 2-4 amino acid residues had the highest free radical scavenging effects. In another study, Silici et al. (33) showed that administration of RJ (100mg/kg) to cisplatin-treated rats increased antioxidant enzyme activities (SOD), catalase (CAT) and glutathione-peroxidase, while decreasing malondialdehyde levels in their samples. Accordingly, the results of our study showed that RJ administration caused a significant increase in serum FRAP levels in immature female rats. This is in agreement with the findings of previous studies that reported a significant increase in FRAP and antioxidant enzyme activity levels in diabetic male rats treated with RJ (14). Consequently, our data emphasize the antioxidant effects of RJ. We suggest that appropriate administration of RJ may affect female fertility due to its antioxidant and estrogenic effects. Further research is required to evaluate the molecular mechanisms associated with the effects of RJ on female reproductive system, especially prior to using this compound as a treatment for human patients.

Conclusion

Administration of RJ to immature female rats promotes follicular growth and development in their ovaries. The mechanism of its action might be through its antioxidant and estrogenic effects on reproductive system to ameliorate the fertility parameters. RJ can potentially be considered as a treatment to promote fertility.

Acknowledgements

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Author's Contributions

E.G., M.R.K., M.K.; Participated in study design, carried out the experiment, data collection, and wrote the manuscript. M.R.K.; Wrote parts of the manuscript and acted as corresponding author. M.K.; Were responsible for overall supervision, drafting and statistical analysis, interpretation of data, editing and approving the final version of this paper for submission and collaboration in the preparation/revision of the submitted manuscript. V.N.; Preparing Royal Jelly.

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Original Article

N-Acetylcysteine Compared to Metformin, Improves The Expression Profile of Growth Differentiation Factor-9 and Receptor Tyrosine Kinase c-Kit in The Oocytes of Patients with Polycystic Ovarian Syndrome

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Abstract

Background: Paracrine disruption of growth factors in women with polycystic ovarian syndrome (PCOS) results in production of low quality oocyte, especially following ovulation induction. The aim of this study was to investigate the effects of metformin (MET), N-acetylcysteine (NAC) and their combination on the hormonal levels and expression profile of GDF-9, BMP-15 and c-kit, as hallmarks of oocyte quality, in PCOS patients.

Materials and Methods: This prospective randomized, double-blind, placebo controlled trial aims to study the effects of MET, NAC and their combination (MET+NAC) on expression of GDF-9, BMP-15 and c-kit mRNA in oocytes [10 at the germinal vesicle (GV) stage, 10 at the MI stage, and 10 at the MII stage from per group] derived following ovulation induction in PCOS. Treatment was carried out for six weeks, starting on the third day of previous cycle until oocyte aspiration. The expression of GDF9, BMP15 and c-kit were determined by quantitative real time polymerase chain reaction (RT-qPCR) and western blot analysis. Data were analyzed with one-way ANOVA.

Results: The follicular fluid (FF) level of c-kit protein significantly decreased in the NAC group compared to the other groups. Significant correlations were observed between the FF soluble c-kit protein with FF volume, androstenedione and estradiol. The GDF-9 expression in unfertilized mature oocytes were significantly higher in the NAC group compared to the other groups ($P<0.001$). Similar difference was not observed between the MET, NAC+MET and control groups. The c-kit expression in unfertilized mature oocytes were significantly lower in the NAC group compared to the other groups ($P<0.001$). Similar difference was not observed between the MET, NAC+MET and control groups (Registration number: IRCT201204159476N1).

Conclusion: : We concluded that NAC can improve the quality of oocytes in PCOS.

Keywords: Gene Expression, Metformin, N-acetylcysteine, Oocyte, Polycystic Ovarian Syndrome

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Introduction

Anovulation associated with polycystic ovary syndrome (PCOS), as a common metabolic disorder, is the major cause of female infertility (1, 2). The principal feature of PCOS is the large number of follicles arresting at early growth stage. The cytoplasmic and nuclear maturity of oocytes is reduced following ovarian stimulation and may account for embryo quality in these couples (3). Exclusive oocyte secreted factors (OSFs), such as growth differentiation factor-9 (GDF-9) and bone morphogenetic factor-15 (BMP-15), belonging to transforming growth factor-β superfamily, are essential for oocyte competence (3-5). The receptor tyrosine kinase c-kit is another OSFs which plays important role in oogenesis and folliculogenesis (6). Recent evidence suggest possible involvement of c-kit and its receptor, kit ligand (KL) in PCOS pathology (7). Indeed, it has been shown that the aberrant or low expression of these exclusive oocyte secreting factors (BMP-15 and GDF-9), lead to over expression of c-kit and its receptor (8-

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10). Therefore, drugs which can modulate the regulation of these intra-ovarian factors may play a role in the clinical management of PCOS.

To improve the quality of oocyte, various protocol for have been designed, tested and verified for ovulation induction along with insulin-sensitizing drugs in PCOS patients. But, the risks of poor response, ovarian hyperstimulation, production of low quality oocytes, reduced fertilization rates and poor embryo quality remains among concern to be dealt within PCOS women undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (11). Metformin, an insulin-lowering agent, has been extensively used for treatment of anovulation and infertility in PCOS patients. However, the efficacy of MET treatment is still disputed (12). In this regard, background studies indicate that MET does not improve the overall outcomes of assisted reproductive procedure in term of the aforementioned parameters (13, 14).

On the contrary, administration of N-acetylcysteine (NAC) has been shown to improve not only the number and also the quality of oocytes in these patients. This phenomenon has been mainly related to the strong antioxidant effect of NAC, which has been shown to reduce follicle atresia and improve the quality of oocyte (15). *In vitro*, NAC plays a key role in cell survival through the production of trophic factor and follicular preservation (16, 17). In line with these reports, Sacchinelli et al. (18) showed that co-administration of inositol and NAC improve ovarian function of PCOS patients. Therefore, considering the fact that oocyte secretory factors are hallmarks of oocyte quality, this study aims to evaluate the effects of NAC, MET and their co-administration on the expression of GDF-9, BMP-15 and c-kit in PCOS individuals undergoing ovarian stimulation in ICSI cycle.

Materials and Methods

Antibodies directed against c-kit and β-actin was obtained from Cell Signaling Technology (Beverly, MA, USA). BMP-15 antibody was obtained from Abcam Technology (Cambridge, MA, USA) and GDF-9 antibody from Santa Cruz Biotechnology (CA, USA). Other reagents used in this study were obtained from Sigma-Aldrich (St. Louis, MO, USA). Cell culture media and sera were obtained from Gibco BRL (Carlsbad, CA, USA).

Study design

This study was performed in continuation of our prospective randomized, double-blind, placebo controlled trial, in the IVF Unit of Infertility Research Center of the Academic Center for Education, Culture and Research (ACECR), Qom/Iran. 80 infertile PCOS women at the age of 25-35 years, in the interval between July 2012 and February 2013, who planned to undergo ICSI were included in this study (19). Individuals were diagnosed as PCOS according to the Rotterdam consensus workshop (20). Based on this consensus, each individual needed to have two out of three criteria: i. Biochemical or clinical hyperandrogenism, ii. Chronic oligo or anovulation and iii. Polycystic ovaries at ultrasound examination. Ethical consideration and further information on this clinical trial are provided in previous studies (19). This study was approved by the Ethics Committee (EC/91/1041) of Royan Institute, Tehran, Iran. The patients provided an informed consent and committed to avoid any changes in their normal physical activity, diet or starting a new medical regimen throughout the study.

Treatment design and ovulation induction

The female partner of ICSI candidates were examined and randomly divided into 4 groups (n=20): i. Placebo (PLA) receiving oral rehydration solution (ORS, Poursina, Iran), ii. MET receiving MET (Glucophage, Merck, West Drayton, UK, 500 mg), iii. NAC receiving NAC (Holzkrichen, Germany, batch no. 6N5483, 600 mg), and iv. MET+NAC group receiving the combination of MET and NAC at the aforementioned doses. Treatment was carried out three times daily for a period of six weeks. The dose and duration of NAC treatment was chosen according to recent studies (21-23).

Gonadotropin-releasing hormone (GnRH) agonist protocol (18) was used to induce ovulation. The female partner of ICSI candidates randomized to four groups received PLA, MET, NAC or MET+NAC from the third day of last menstrual period (LMP) of previous cycle until the day of oocyte aspiration. Oral contraceptive pills (OCPs) were also included in the regimen for 21 days starting simultaneously with placebo, MET, NAC or MET+NAC on the day 3 of menstrual cycle prior to the treatment cycle. For ovarian down-regulation, daily injections of Bucerelin acetate (1 mg, Suprefact, Aventis, Germany) were administered from the day 19 of the preceding, menstrual cycle until day 2 of the next cycle. On the second day on the next cycle if the endometrial thickness was less than 4 mm, the dose of Burcerelin acetate was reduced to 0.5 mg.

Ovulation induction was induced from the day two of the cycle with average daily injections of 2 ampoules of recombinant follicle stimulating hormone (rFSH, Gonal-f, Merck Serono S.A., Geneva, Switzerland). Vaginal ultrasound (Honda Electronics HS 4000-Japan) was also used to monitor the cycles. 10,000 IU human chorionic gonadotropin (hCG, Pregnyl, Organon, Netherlands) was administered to induce ovulation. 36 hours after the administration of hCG, when at least three follicles had reached the diameters of 16-18 mm, transvaginal oocyte aspiration was performed under ultrasound guidance and general anesthesia. This protocol of induction ovulation was used for all the individuals in the 4 group. During the treatment the par-

ticipants were asked to report any probable side effects such as abdominal discomfort, diarrhea and nausea. Due to these side effects, 20 couples (5 per group) were excluded from the study (Fig.1).

Preparation of oocytes, follicular fluid and blood samples

Based on our pervious study, oocytes and follicular fluid (FF) from multiple follicles, from each subject were pooled as explained (18). Following oocyte retrieval, their cumulus cells were removed by exposure to 20 IU/ml hyaluronidase (ART-4007A, SAGE BioPharma, USA) in HEPES-based medium for 30 seconds followed by mechanical pipetting in HEPES-buffered HTF containing 5 mg/ml human serum albumin (ART-3001, SAGE BioPharma, USA).

The nuclear status of each oocyte was determined under the stereo microscope (Olympus Co., Japan) and classified into three categories: i. Unfertilized mature oocyte [metaphase II (MII)] following ICSI, ii. Germinal vesicle (GV) stage, iii. Without first polar body called metaphase I (MI). For gene expression analysis, in each experimental group, 10 GV, 10 MI, and 10 MII oocytes were separately pooled and washed in phosphate-buffered saline (PBS) and transferred into RNase-free microcentrifuge tubes. 50 µl of RNAlater, RNA Stabilization Reagent (Qiagen, USA) were added to each tube and all samples were stored in a -80°C freezer until analysis. Only MII oocytes were used for ICSI.

The FF, from the first aspirated with no visible blood contamination was collect and immediately centrifuged at 3000 rpm for 10 minutes, and the supernatants were stored at -70°C for further analysis. Fasting blood sample were also collected from each participant once prior to the start of treatment (day 2 of pervious cycle) and once on the day of ovum pick up of ICSI cycle. The samples were immediately centrifuged for 10 minutes at 3000 rpm (Hettich, EBA20, UK) and the resulting serum were stored at -70°C for evaluation.

The levels of luteinizing hormone (LH, mIU/ml), FSH (mIU/ml), total testosterone (TT, ng/ml), Progesterone (ng/ml), estradiol (E2, pg/ml) and androstenedione (ng/ml) in the FF and serum were measured in all samples using the ELISA enzyme immunoassay (Demeditec Diagnostics GmbH, Germany) according to the manufacturer's protocol. The FF soluble protein level of c-Kit (pg/ml) was measured with the ELISA Kit (Abnova Corporation, Taiwan) by sandwich enzyme immunoassay technique, according to the manufacturer's protocol.

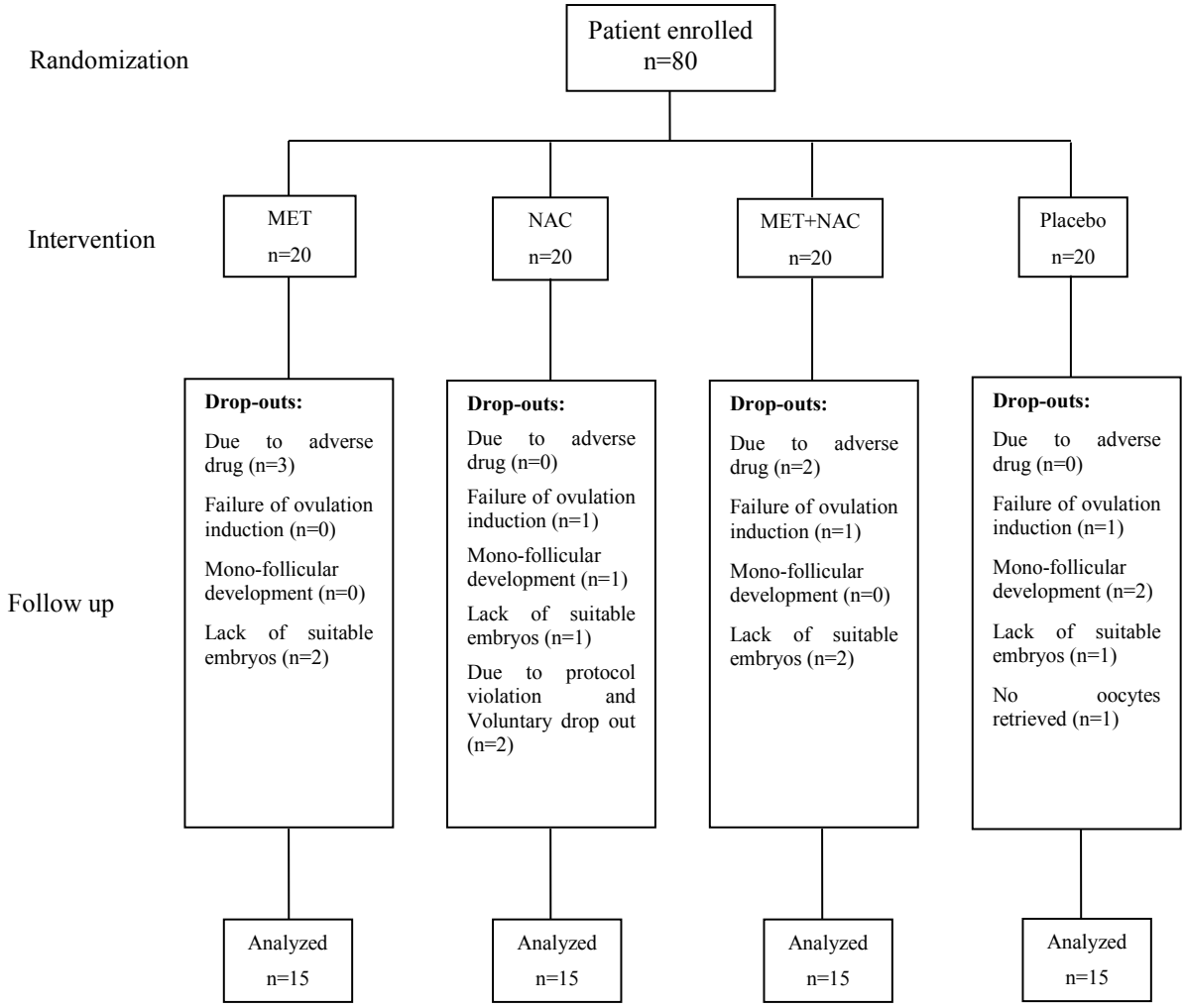

Fig.1: Flowchart of participants in this study.
MET; Metformin, NAC; N-acetylcysteine, and PLA; Placebo.

Gene expression analysis

Total RNA from the oocytes of each group were isolated using the EZ-10 total RNA mini-prep Kit (Bio Basic Inc., Canada), according to the manufacturer's protocol. All samples were stored at -80ºC till analysis. Complementary DNA (cDNA) was synthesized, using random hexamers [using the RevertAid First Strand cDNA synthesis Kit (Thermo scientific, USA)]. To determine the relative expression of target genes, quantitative real time polymerase chain reaction (RT-qPCR) was carried out using SYBR-Green/ROX qPCR master mix assay (Thermo scientific, USA) by gene-specific primers (Table 1). Relative gene expression was calculated as the abundance ratio of each target gene relative to *β-actin*. The ABI step one plus (ABI, USA) instrument was used for real time PCR experiments and the ΔΔCt method for data calculation.

Table 1: Identity and sequence details of polymerase chain reaction (PCR) primers used to analyses mRNA expression in oocytes

Gene	Sequence primer (5´-3´)
GDF-9	F: CCAATAGAAGTCACCTC R: GCGATCCAGGTTAAATAGCA
BMP-15	F: CAGTCCTCTATTGCCCTTCT R: AATGGTGCGGTTCTCTCTA
c-Kit	F: ACGAATGAGAATAAGCAGAATGAA R: GAGAGGACAGCGGACCAG
β-actin	F: GGACTTCGAGCAAGAGATGG R: AGCACTGTGTTGGCGTACAG

Total proteins from each pool of oocytes were extracted using RIPA lysis and extraction buffer Kit (Cat No: 89900, Thermo Scientific, USA), according to the manufacturer's instruction. Concentration of proteins was determined according to Bradford's method using bovine serum albumin (BSA) as a reference standard (Bradford, 1976). Total proteins were electrophoresed in 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, transferred to polyvinylidene fluoride membranes, and probed with specific antibodies. Membranes were developed using enhanced chemiluminescence reagents (Amersham Bioscience, USA) and the intensity of immunoreactive polypeptides was analyzed subsequent to visualization of the bands developed on a photographic film. Protein bands on photographic film were quantified by densitometry scanning after background subtraction. Integrated densities of bands were measured by Image J software.

Statistics

The normality of continuous variables was confirmed using the Kolmogrov-Smirnov test and data were reported as means ± SEM. Data analysis were performed using one-way ANOVA and Tukey's test for post-hoc. Means were considered significantly different at $P<0.05$. Pearson's correlation test defined the relation between variables. All data were analyzed with the statistical software SPSS (version16.0 for windows, Chicago, IL, USA).

Results

Patients characteristics including age, body mass index (BMI), the level of LH, FSH, E2 and TT were not significantly different among the groups PCOS prior to treatment.

Follicular fluid analysis

FF volume and FF level of androstenedione, E2 and progesterone were similar in all groups ($P>0.05$, Table 2), but the level of soluble c-kit protein in the FF significantly decreased in the NAC group compared to other groups ($P<0.01$). Our results also showed a significant correlation between the soluble c-kit protein in the FF of all the population with the FF volume ($r=0.508$, $P=0.02$), androstenedione ($r=0.682$, $P=0.01$), and E2 ($r=0.638$, $P=0.01$) (Fig.2).

Evaluation of oocyte and embryo quality

The number of immature oocytes (MI+GV) and abnormal mature oocytes significantly decreased in the NAC group ($P<0.01$) compared to the other groups. Similar reduction was also observed in MET and MET+NAC groups but the reduction was not significant compared to the placebo group ($P>0.05$). The fertilization rate of metaphase II oocytes were similar in all groups ($P>0.05$). The number of good embryo (grade I) on day 3 showed a significant increase in the NAC group ($P<0.02$) compared to placebo group. This improvement was also observed in the MET and MET+NAC groups when compared to the placebo ($P>0.05$), but remained insignificant (Fig.3). The percentage of top grad embryos was not different between the three NAC with MET and MET+NAC groups.

Table 2: Comparison of the biochemical parameters of follicular fluid in PCOS patients

Parameter	NAC	MET	NAC+MET	PlA
Follicular fluid Volume (ml)	5.4 ± 1.13[a]	5.7 ± 1.1[a]	4.96 ± 1.1[a]	4.9 ± 1.1[a]
Estradiol (pg/ml)	466.6 ± 34.8[a]	496.6 ± 44.8[a]	470.2 ± 48.4[a]	436 ± 40.16[a]
Progesterone (ng/ml)	3983.7 ± 353.9[a]	3916.9 ± 359.5[a]	3501.2 ± 326.9[a]	3255.3 ± 414.8[a]
Androstenedione (ng/ml)	426.2 ± 30.3[a]	435.2 ± 46.7[a]	487.6 ± 42.9[a]	548.3 ± 42.36[a]
Soluble c-Kit (pg/ml)	317.8 ± 27.5[b]	380.8 ± 30.3[a]	429.8 ± 28.7[a]	455.2 ± 28.75[a]

Data are shown as mean ± SEM. Analysis was performed by ANOVA and Tukey's test for multiple comparisons.
Means without a common letter are significantly different (P<0.05). PCOS; Polycystic ovarian syndrome, MET; Metformin, and NAC; N-acetylcysteine, and PLA; Placebo.

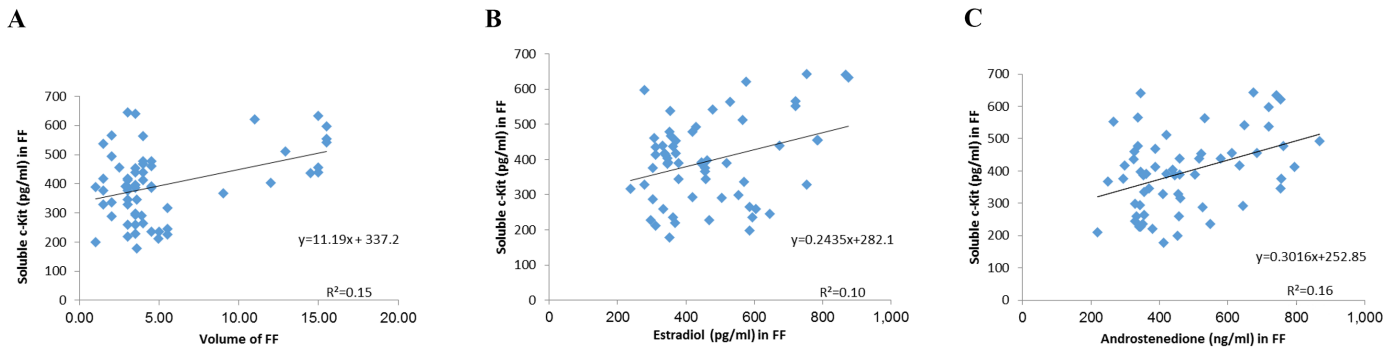

Fig.2: Correlation between parameters of follicular fluid (FF) in all the population. **A.** Soluble c-Kit with volume of FF, **B.** Estradiol (E2), and **C.** Androstenedione. R^2; Determination of coefficient.

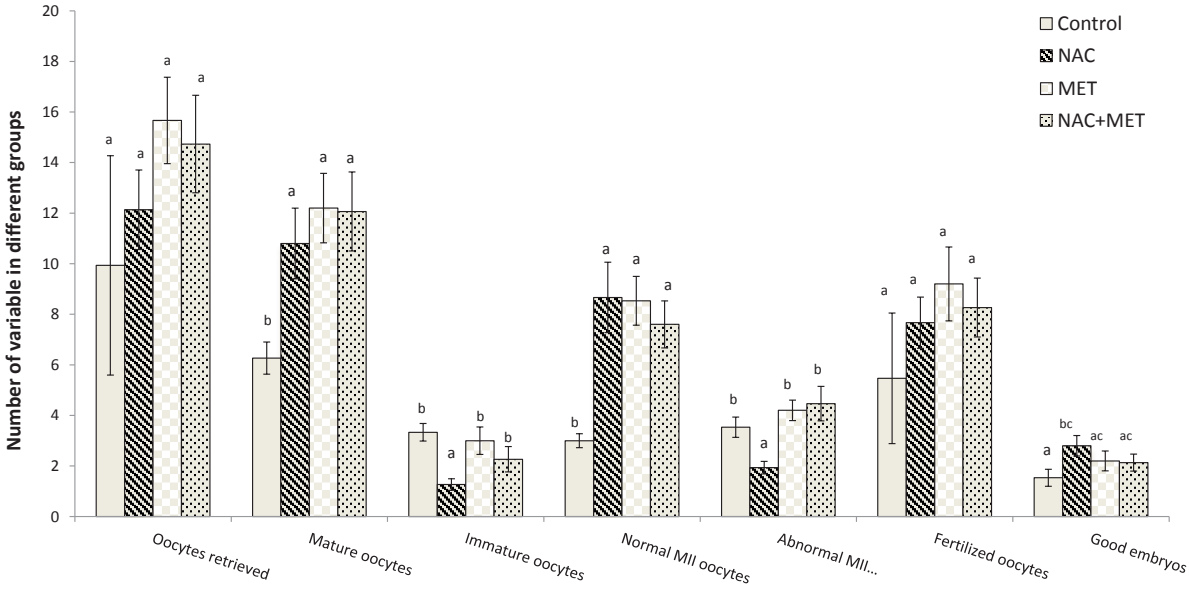

Fig.3: Distribution of oocytes retrieved, quality of oocytes and embryos in polycystic ovary syndrome patients undergoing treatment of N-acetylcysteine (NAC), metformin (MET), NAC+MET and Placebo (control).
Data are the mean ± SEM. Statistical analyses were performed by ANOVA followed by Tukey's test for multiple comparisons. Means without a common letter are significantly different (P<0.05).

Expression profile of GDF-9, BMP-15 and c-Kit in oocytes

The level of BMP-15 protein in the mature unfertilized oocytes and GV oocytes did not differ significantly among the groups (P>0.05, Figs.4, 5A, D, E). The expression level of GDF-9 in the GV oocytes significantly increased in all groups compared to the placebo (P<0.001) (Fig.4B, D, F), while for unfertilized mature oocytes, GDF-9 mRNA and protein levels only significantly increased in the NAC group (P<0.001, Fig.5B, D, F). The expression of c-kit in the GV oocytes significantly decreased in the NAC and MET groups compared to the placebo group (P<0.001), but not in NAC+MET group (Fig.4C, D, G). The c-kit expression in the unfertilized mature oocytes significantly decreased in the NAC group compared to the MET and other treatment groups (P<0.001), but no significant difference was found in the MET and NAC+MET groups when compared to the placebo group (P>0.05, Fig.5C, D, G). It is important to note that the results for MI oocytes were similar to GV oocyte; therefore, in this article only the results of GV were presented. This observation is in accordance with pervious literature (24).

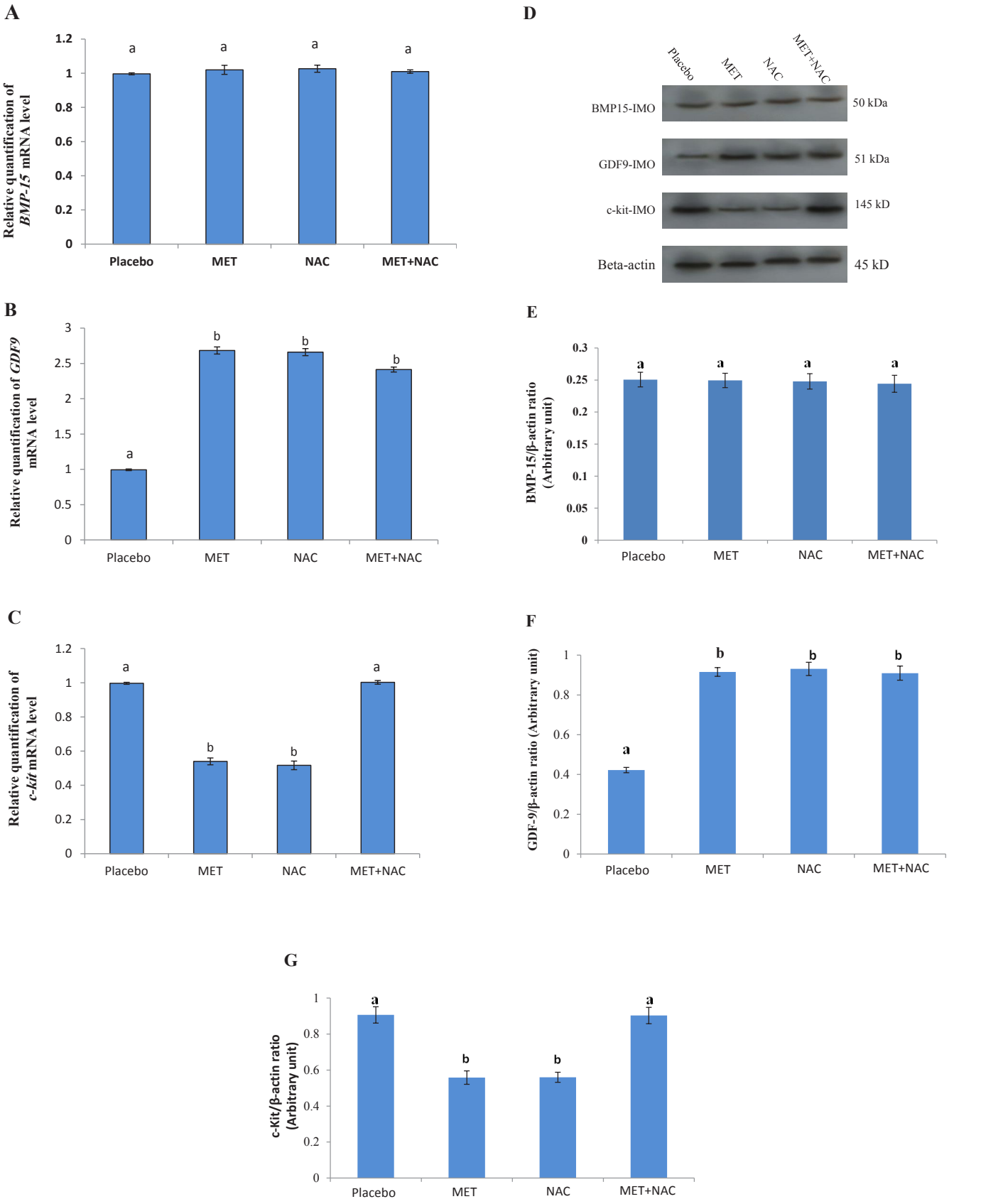

Fig.4: Effects of NAC and MET on *BMP-15*, *GDF-9* and *c-kit* mRNA and protein expression in immature oocytes (IMO, GV oocytes) of PCOS patients. Results of reverse transcriptase real-time polymerase chain reaction (PCR) for mRNAs of **A.** *BMP-15*, **B.** *GDF-9*, **C.** *c-kit* in GV oocytes, **D.** Immunoblots of BMP-15, GDF-9 and c-kit from oocyte cell lysates. Densities of **E.** BMP-15, **F.** GDF-9, and **G.** c-kit protein bands in the experimental groups are shown. Means without a common letter are significantly different (P<0.05).
NAC; N-acetylcysteine, MET; Metformin, GV; Germinal vesicle, and PCOS; Polycystic ovarian syndrome.

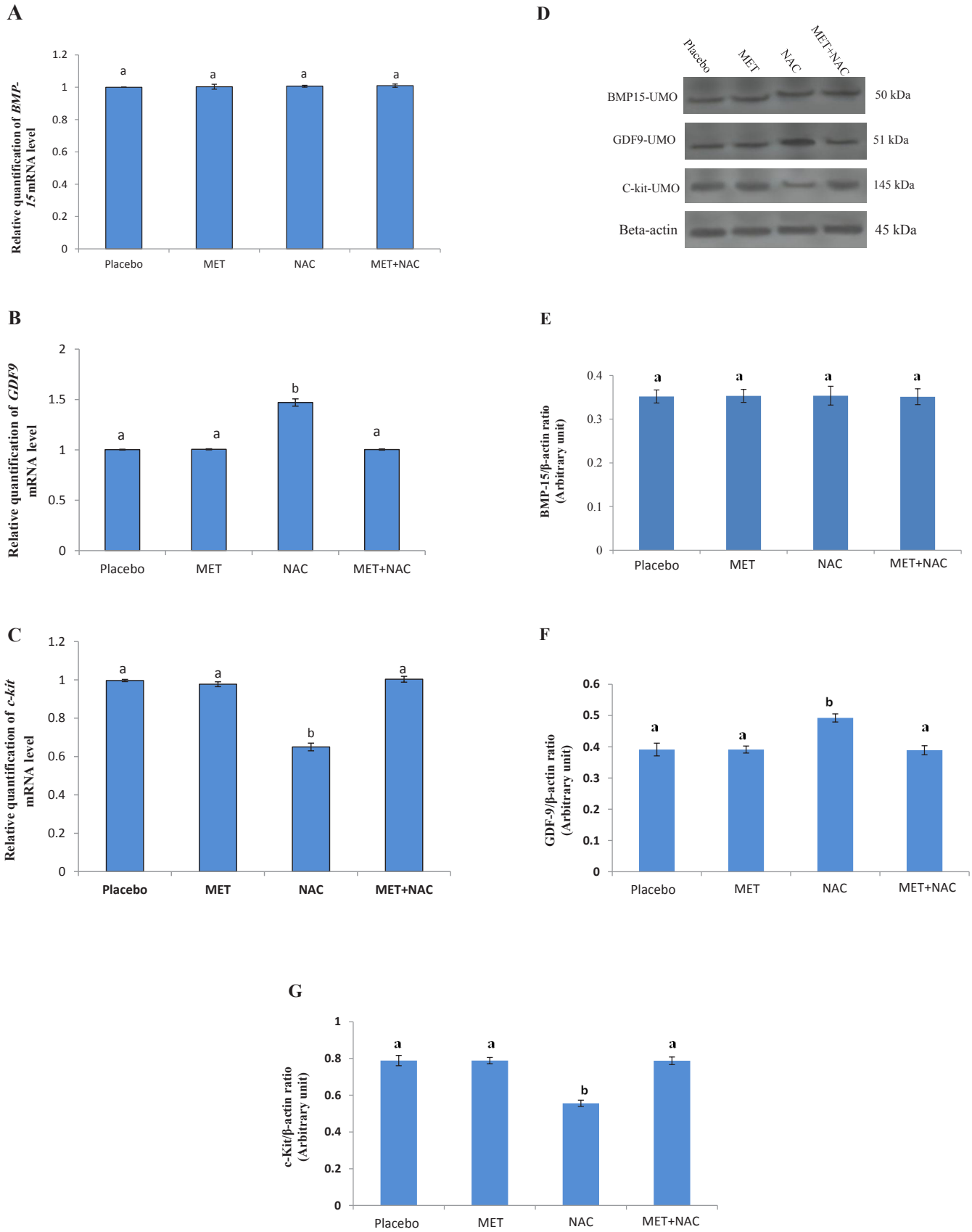

Fig.5: Effects of NAC and MET on *BMP-15*, *GDF-9* and *c-kit* mRNA and protein expression in unfertilized mature oocytes (UMO, MII oocytes) of PCOS patients. Results of reverse transcriptase real-time polymerase chain reaction (PCR) for mRNAs of **A.** *BMP-15*, **B.** *GDF-9*, **C.** *c-kit* in MII oocytes, **D.** Immunoblots of BMP-15, GDF-9 and c-kit from oocyte cell lysates. Densities of **E.** BMP-15, **F.** GDF-9, and **G.** c-kit protein bands in the experimental groups are shown. Means without a common letter are significantly different (P<0.05).
NAC; N-acetylcysteine, MET; Metformin, and PCOS; Polycystic ovarian syndrome.

Discussion

A typical characteristic of PCOS patient commonly observed during induction stimulation for ART cycles is increased number of low quality oocytes which is mainly related to state of endocrine disorder in these individuals (25). Considering the indispensable role of OSFs in oocyte development and maturation, many researches have shown impaired expression of OSFs particularly GDF-9, BMP-15 and c-kit, may account for low quality oocyte in PCOS undergoing ovarian stimulation (26). This may explain, at least a part of the folliculogenesis disorders found in these patients (27-29). Background literature in this filed is very discrepant. Some authors have reported reduce expression of GDF-9 with no significant alteration in the expression of BMP-15 (8), while others have shown no alteration in expression of these two factors both at RNA and protein level (9) in oocyte of PCOS individuals. The exact reason of such discrepancy is not well understood.

In continue to our previous study, we demonstrated that unlike MTE and NAC+MET groups, the administration of NAC compared to placebo group, improves the maturation and quality of oocytes and also embryo development in PCOS patients undergoing ICSI (18). Therefore, in this we aimed to evaluate whether NAC could alter BMP-15, GDF-9 and c-kit levels, as the main OSFs in the oocytes of PCOS patients in comparison to MET and MET+NAC.

Compelling evidence suggest that GDF-9 and BMP-15 members of the TGFβ superfamily are exclusively expressed in the oocyte and their expression increases as follicle development progresses (30). During postnatal ovarian development, c-kit mRNA and protein are localized in the oocytes (6), and in this regard Brankin et al. (31) has shown a relation between KL/c-kit interaction with antrum formation, steroidogenesis and oocyte quality. Furthermore, genetic and descriptive studies have implicated the involvement of c-kit receptor and its ligand, KL, in oocyte growth (32).

Low GDF-9 levels is associated with abnormally increased KL level in PCOS, which could lead to abnormal ovarian features such as enlarged oocytes and increased follicle numbers (7, 32). In PCOS patients, the GDF-9 mRNA level within the oocytes is lower than in oocyte derived from normal individuals (8), and it is believed that there is a negative association between GDF-9 expression and KL/c-kit expression. Tuck (7) believes that excess androgens may act to further reduce the inhibitory effect of GDF-9, thus resulting in an abnormal increase in the KL/c-kit protein level in PCOS individual. Considering the inverse relationship between c-kit and GDF-9 in PCOS (7, 8, 32), therefore, improving the expression of GDF-9 is expected to cause a reduction in c-kit levels.

Our results displayed a significant increase in the expression of GDF-9 in the unfertilized mature oocytes of PCOS patients after administration of NAC compared to MET, indicating that NAC, as an anti-oxidant/anti-apoptotic agent, could enhance the expression of GDF-9 through inhibiting the activity of NF-kB and AP-1 transcription factors, therefore affecting the activity of MAP-kinase signaling and related genes expression (33), which may be able to alleviate PCOS follicular disorders and prevent follicular developmental detention and atresia.

Our study, in agreement with aforementioned studies, also showed a significant decrease in the expression of c-kit in the oocytes of PCOS patients and also the soluble c-kit protein in the FF following administration of NAC compared to control. In addition, evidence has indicated the relationship between KL/c-kit system with MAPK pathway and/or PI3K/Akt pathway, which are both necessary for follicle development (34). It is likely that NAC decreases the expression of c-kit through interference in MAPK pathway, all of this could be the underlying reason in the role of NAC in preventing follicular developmental detention and atresia and alleviation of follicular disorders in PCOS patients.

Although the FF content may be an invaluable hallmark for PCOS diagnosis, but the NAC ability to modulate these intra-ovarian factors of the oocyte may have interesting pharmacological perspectives for clinical management of PCOS patients. According to literature (7, 8, 32), there is an inverse relationship between c-kit and GDF-9. Therefore, improves expression of GDF-9 by NAC treatment, is expected to follow by a reduction in c-Kit and indeed MET appears to mask this effect of NAC, how this masking effect is performed, remains to be elucidated.

In this regard it has been shown that with increased follicular size and E2 production, the amount of soluble c-kit protein in human FF also increase (35), which is consistent with the correlation observed in this study between soluble c-kit with the FF volume, E2, and androstenedione concentrations.

It has been demonstrated that the excess secretion of anti-mullerian hormone (AMH) in the FF of PCOS patients may directly inhibit the production of OSFs such as GDF-9 and BMP-15, which can explain the low levels of OSFs in PCOS oocytes (36). Our findings showed a reduction in the AMH level in the FF of NAC treated group (18). Although this reduction was not statistically significant but may be considered as an underlying reason for the increased levels of GDF-9 in the NAC treated patients. Moreover, in agreement with other findings (8, 9), our study revealed no significant difference in the level of BMP-15 mRNA among the studies groups.

Conclusion

Considering the fact that NAC improves oocyte maturation and embryo quality, and decreases the rate of immature oocytes in women with PCOS while being a safe and well-tolerated agent, we suggest the administration of NAC as an alternative to other insulin-sensitizing agents like MET. Therefore, the present study argues that NAC possibly improves the oocyte quality of PCOS patients compared to MET through modulating the c-kit and GDF-9 expression, indicating that NAC supplement may be a therapeutic alternative to the insulin-sensitizing agents in PCOS management.

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Author's Contributions

E.C., M.S.M., S.M.A.S.; Participated in study design, data collection and evaluation, drafting and statistical analysis. M.H.N.E., E.C.; Performed follicle collection and prepared oocytes for ICSI pertaining to this component of the study. E.C., M.S.M., M.H.N.E.; Contributed extensively in interpretation of the data and the conclusion. B.A., E.C.; Conducted molecular experiments and RT-qPCR analysis. All authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript and approved the final draft.

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Original Article

The Effects of Dienogest on Macrophage and Natural Killer Cells in Adenomyosis: A Randomized Controlled Study

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Abstract

Background: Progestin has been used for symptomatic treatment of adenomyosis, although its effect on the immune system has not been studied. The aim of this study was to investigate the changes of macrophage and natural killer (NK) cell infiltration in tissues obtained from women with adenomyosis who did or did not receive oral progestin dienogest.

Materials and Methods: In this randomized controlled clinical trial study, 24 patients with adenomyosis who required hysterectomy were enrolled. Twelve patients received dienogest 28-35 days before surgery, and the other 12 patients were not treated with any hormones. The endometrial and myometrial tissue samples were immediately collected after hysterectomy, and immunohistochemistry for a macrophage marker (CD68) and a NK cells marker (CD57) was performed.

Results: The number of CD57 cells was significantly increased in endometrial glands of the treated group compared to the untreated group (P=0.005) but not in stroma in the endometrium of the treated patients (P=0.416). The difference in the number of CD68 cells was not statistically significant between treated and untreated groups in the endometrial glands (P=0.055) or stromal tissues (P=0.506).

Conclusion: Administration of oral progestin dienogest to patients with adenomyosis increased the number of uterine infiltrating NK cells in glandular structure of eutopic endometrium. The differential effects of progestin on NK cells depended on the site of immune cell infiltration. The effects of oral progestin on uterine NK cells in adenomyosis have the potentials to be beneficial to pregnancies occurring following discontinuation of treatment in terms of embryo implantation and fetal protection (Registration number: TCTR20150921001).

Keywords: Adenomyosis, Dienogest, Macrophages, NK Cells, Progestins

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Introduction

Adenomyosis is a common gynecologic disease in premenopausal and late reproductive age women. Frequent symptoms include progressive dysmenorrhea, chronic pelvic pain, dyspareunia, abnormally heavy menstrual bleeding, and interval menstrual bleeding. The severity and frequency of the symptoms correlate with the extent and depth of the ectopic endometrium in the myometrium, which follows the endometriosis pathology and progression of the disease (1). Generally, diffuse globular enlargement is the common sign of adenomyosis and the size of uterus is typically not larger than 12 weeks gravid size. This enlargement of the uterus is due to invasion of the myometrium by endometrial glands and stroma (2). A definitive diagnosis of adenomyosis can only be made at histopathology following hysterectomy.

Currently, the etiology and pathology of adenomyosis have yet to be elucidated, however, many theories have been proposed; for example, the invagination of basalis endometrium into the myometrium and the subsequent inflammation. Adenomyosis uteri repetitively exhibit exaggerated and non-synchronized uterine contractions, which induce a micro-fracture at the endometrial-myometrial junction zone (EMJZ). This micro-trauma at EMJZ causes the displacement of endometrium into the surrounding myometrium, where the myometrial cells proliferate and undergo metaplasia, resulting in the thickening of EMJZ (3, 4). The aberrant immunologic activity in adenomyosis is seen by immunohistochemistry with an increase in the number of infiltrating

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macrophages. These could activate T and B cells to produce antibodies and cytokines that may destroy the EMJZ (5). Macrophages transform from the circulatory monocyte cells and serve many functions including phagocytosis, antibody-dependent cell-mediated cytotoxicity, and presenting antigens to lymphocytes. Natural killer (NK) cells, on the other hand, are cytotoxic lymphocytes that play a role in innate immune response. They can remove infected cells without recognition, and thus the killing process is an immediate response to virus-infected cells and tumor formation (6, 7).

Abnormal immune responses appear to play a role in adenomyosis. Expression of human leukocyte antigen (HLA) class II is recognized as the first step in the activation of macrophages and/or T lymphocytes by presenting foreign antigens to these cells. HLA class II expression is shown to be increased in the glandular cells of eutopic and ectopic endometrium of adenomyosis (6, 7). At this point, the definitive treatment for adenomyosis is hysterectomy (5), but it is not the treatment of choice for patients who wish to remain fertile. Oral contraceptive pills, high doses of progestin, levonorgestrel-releasing intrauterine device (LNG-IUD), gonadotropin releasing hormone agonist (GnRH-a), and danazol are hormonal treatment options for both adenomyosis and endometriosis (1). The use of pre-operative GnRH agonists in patients with myomas, endometriosis and adenomyosis was tested by Khan et al. (8). They found a decrease in MCP-1 level, as well as a reduction in the number of macrophages in endometrial and myometrial layers of patients with adenomyosis compared to an untreated group. Progestin is considered a good choice for long-term treatment of adenomyosis because it causes minimal side effects. The administration of a progestin, depending on the dose, may not cause a hypoestrogenic state.

Four generations of synthetic progestogens are now available to clinicians. A new progestin called dienogest (DNG) (17a-cyanomethyl-17b-hydroxyestra-4, 9-dien-3-one) has specific anti-androgenic effects (9). It has good bioavailability and strong progestational effect due to its high selectivity to the progesterone receptor. It has been reported as one of the progestogens employing the strongest action on the endometrium (10). Its half-life is 9-10 hours, and it is approximately 90% secreted by the kidneys. The therapeutic dose of DNG for treatment of endometriosis is 2 mg daily providing a blood level of 10-7 mol/L. Although the blood level of dienogest after oral administration is very low, the symptoms of endometriosis can be cured (9). Dienogest has been shown to slightly increase the number and activity of NK cells in peritoneal fluid and spleen of rats, and significantly decrease IL-1β production by peritoneal macrophages (11). The objective of our study was to investigate the effect of DNG on immune cells focusing on macrophages and NK cells in women with adenomyosis.

Materials and Methods

A randomized control trial study was conducted from 1 February 2015 to 31 May 2016 at the Department of Obstetrics and Gynecology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. The study was approved by the Ethical clearance Committee on human rights related to researches involving human subjects (protocol #045801). The inclusion criteria were pre-menopausal women aged 20-50 years, suffering from dysmenorrhea with or without hypermenorrhea, undergoing either abdominal hysterectomy or laparoscopic hysterectomy for adenomyosis, and diagnosed adenomyosis by ultrasonography. The exclusion criteria were patients who had used i. Pill or oral exogenous hormones for 3 months or ii. GnRH agonist or depot medroxyprogesterone acetate injection for 6 months before surgery. Twenty-four eligible patients were recruited into the study. The ultrasonographic diagnostic criteria for adenomyosis included a globular shape with diffuse uterine enlargement, myometrial anterior-posterior asymmetry, poorly described areas in the myometrium, a heterogeneous myometrial echotexture, sub-endometrial echogenic linear striations with or without small myometrial cysts, or hemorrhagic foci within the heterotopic endometrial layer (12). The sample size was calculated according to measurement outcomes and the mean number of NK cells and macrophages from Khan's study with α of 0.05, β of 0.80 and suspecting 10% loss. The desired sample size was 12 for each arm. Therefore, a total of 24 subjects were needed for this present study.

The subjects were divided into two groups via block randomization with a block size of 4. A group of 12 patients received 2 mg/day dienogest for 28-35 days before surgery, and the other 12 underwent operation without prescribing the drug (control group). The phase of the menstrual cycle was determined by the subjects' last menstrual period and confirmed by endometrial histology. Patients with liver disease, kidney disease, autoimmune disease, coagulopathy, endometriotic cysts, or under hormonal therapy were excluded. Exclusion of patients with pelvic endometriosis was not possible; therefore, it was the potential limitation for the present study.

Tissue collection and preparation

The myometrial tissue had gross features of adenomyosis, that is, hypertrophic swirls of smooth muscle separating duller prominent trabeculated patterns and gray foci of endometrium. The tissue was excised in 1cmx1cmx1cm dimentions immediately after hysterectomy. The endometrial tissue sample was also collected in block pattern size 1 cm×1 cm×1 cm from the myometrial layer. All collected biopsy specimens were fixed in formalin and prepared for sectioning as paraffin-embedded tissue blocks. 3-µm thick sections of the samples were then prepared for subsequent histopathological and immunohistochemical studies.

Immunohistochemistry

Immunohistochemical analysis was performed with anti-CD68 antibody for macrophages and anti-CD57 and anti-CD56 for NK cells. Both primary rabbit monoclonal antibodies clone PGM-1 and clone 123C3.D5 against CD68 and CD57, respectively, were from DAKO (Glostrup, Denmark), and the primary mouse monoclonal antibody clone NK-1 against CD56 was from Thermo Fisher Sci-

entific (Waltham, USA). Primary antibodies CD68, CD57, and CD56 were used at a dilutions of 1:100, 1:150, and 1:2400, respectively. The 3-μm thick paraffin-embedded tissue sections were deparaffinized in xylene and then rehydrated. Slides were incubated for 60 minutes at 60°C and treated with Bond Dewax Solution (Leica Biosystems, Bannockburn, IL). Epitope retrieval was performed by incubating the slides in Bond Epitope Retrieval Solution for 20 minutes at 100°C. Immunohistochemical analysis was performed using the Bond Polymer Refine Detection kit (Leica Biosystems, Bannockburn, IL), a 3-step indirect immunoperoxidase technique. Briefly, primary antibody was applied for 45 minutes at room temperature. Peroxide block (3% hydrogen peroxide) was then applied for 5 minutes and rinsed with Bond Wash Solution. Post Primary Polymer was applied for 9 minutes. Polymer Poly-HRP IgG was applied for 7 minutes and rinsed with Bond Wash Solution and deionized water, then diaminobenzidine chromogen was applied for 4 minutes. Slides were counterstained with hematoxylin for 5 minutes. Appendix tissue was used as positive control. The negative control showed an absence of specific staining (12).

The number of CD68 and CD57 brown spots were counted in 20 different fields (200×200 microns) for each person (magnification: ×200) under light microscopy. The number of CD68 and CD57 positive cells were calculated and expressed as the mean positive cells per mm^2. The results in each biopsy specimen were recounted and confirmed by a second observer who did not know the patient's history. Double-labeling immunohistochemical method for CD57 and CD56 was also performed. The primary antibody anti-CD57 was the first antibody identified by diaminobenzidine chromogen, while the primary antibody anti-CD56 was the second antibody identified by mixed red refine chromogen. The procedure was similar to the described protocol above including deparaffinization, epitope retrieval and a 3-step indirect immunoperoxidase technique. However, before counterstaining each slide with hematoxylin, the second antibody (anti-CD56 primary antibody) was applied for 40 minutes at room temperature. Post Primary Polymer AP was applied for 20 minutes. Polymer Poly-AP IgG was applied for 20 minutes and rinsed with Bond Wash Solution and deionized water before the Mixed Red Refine chromogen was applied for 10 minutes. Slides were then counterstained with hematoxylin for 5 minutes.

Statistical analysis

Each parameter is presented as either the mean ± SD or medians (25, 75%) depending on the distribution of data. The clinical characteristics were compared by chi-square test or student's t test for differences between two groups. The differences in numbers of macrophages and NK cells between the two groups were analyzed by the non-parametric Mann-Whitney U-test; a value of $P<0.05$ was considered statistically significant. The data were analyzed by IBM SPSS Statistics for Windows, version 19.0 (Armonk, NY: IBM Corp).

Results

Twenty-four elibible patients were enrolled and randomly divided into treated and untreated groups. There were no dropouts after treatment of dienogest for 28-35 days, therefore, complete data from 24 participants were available for analysis (Fig.1). The demographic characteristics of the participants; for example, age, BMI, indication for and type of surgery were not significantly different (Table 1). The number of participants with proliferative and secretory menstrual phases in the DNG and untreated groups were not statistically different.

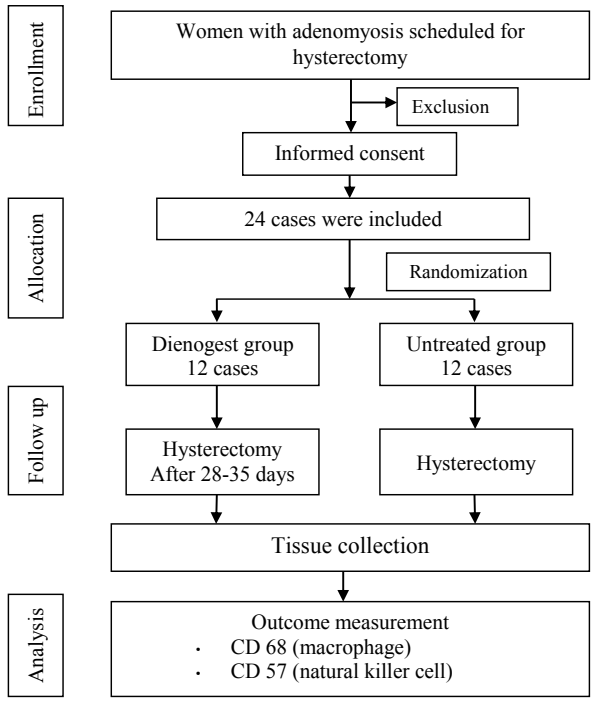

Fig.1: Study design for the effects of dienogest on macrophages and natural killer cells in adenomyosis clinical trials.

Table 1: Demographic data of women with adenomyosis

Characteristic	Dienogest n=12 mean ± SD	Untreated n=12 mean ± SD	P value
Age (Y)	43.8 ± 5.16	45.4 ± 4.48	0.458
BMI (kg/m^2)	25.9 ± 4.78	24.6 ± 2.99	0.878
Phase	n (%)	n (%)	
Proliferative phase	5 (41.66%)	6 (50%)	1.000
Secretory phase	7 (58.33%)	6 (50%)	1.000
Indication for surgery			
Dysmenorrhea	7 (58.33%)	8 (66.66%)	1.000
AUB	5 (41.66%)	4 (33.33%)	1.000
NSAIDs usage	11 (91.66%)	10 (83.33%)	1.000
Type of operation			
Laparotomy	11 (91.7%)	12 (100%)	1.000
Laparoscopy	1 (8.3%)	0	1.000

BMI; Body mass index, AUB; Abnormal uterine bleeding, and NSAIDs; Nonsteroidal anti-inflammatory drug.

Effect of dienogest on endometrial gland and stroma of endometrium

NK cell infiltration, as shown by CD57-positive brown

spots (Fig.2), was significantly increased in glands in treated versus untreated groups [P=0.005, median (range 25-75%), 4.37 (0.31-14.68) vs. 0 (0, 0)] (Fig.2D), but not in stroma of the endometrium of the treated group [P=0.416, median (range 25-75%), 21.87 (10-68.81), 10 (8.75-15.93)] (Fig.2F, Table 2). NK cells were confirmed by double immunostaining for CD57 and CD56 (Fig.2G, H). Macrophage infiltration, as demonstrated by CD68-positive brown spots (Fig.3), was also increased, but was not statistically significant in treated versus untreated groups [P=0.055, median (range 25-75%), 4.37 (0-9.06), 0 (0-1.56)] (Fig.3D, Table 2).

Table 2: Macrophage and natural killer (NK) cell infiltration in endometrial glands and stroma in endometrial tissue in adenomyosis patients

	Glands			Stroma		
	Untreated n=12	**Dienogest n=12**	**P value**	**Untreated n=12**	**Dienogest n=12**	**P value**
Macrophage (cells/mm^2)	0 (0-1.56)	4.37 (0-9.06)	0.055	44.37 (22.18-50.93)	64.37 (20.62-92.5)	0.506
NK cells (cells/mm^2)	0 (0)	4.37 (0.31-14.68)	0.005	10 (8.75-15.93)	21.87 (10-68.81)	0.416

Data as presented as [median (range 25-75%)].

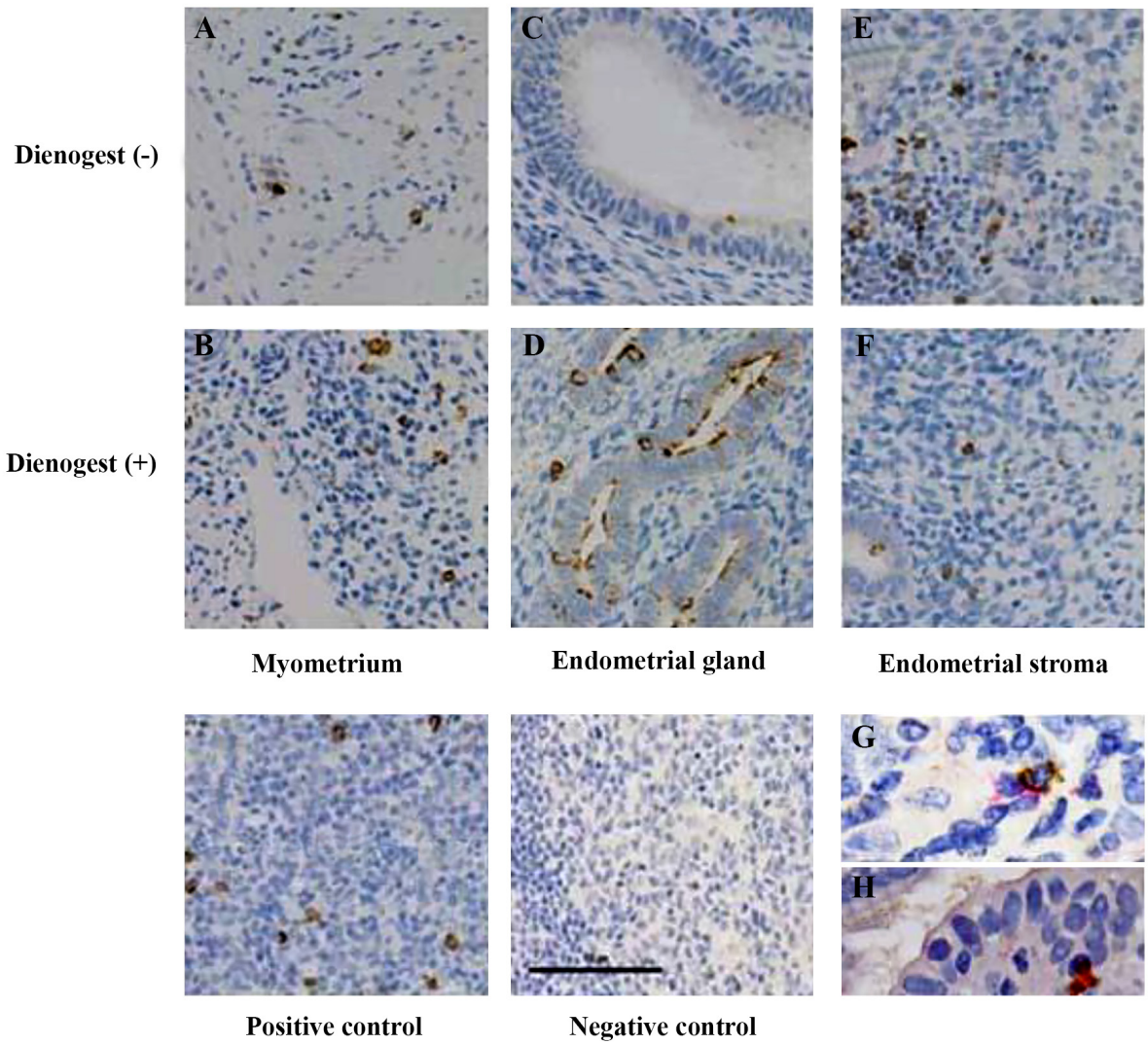

Fig.2: Immunohistochemistry staining for CD57 in eutopic and ectopic endometrium. The accumulation of CD57 is indicated by a dark brown coloration in the nucleus; all nuclei were counter-stained with hematoxylin. **A.** Ectopic endometrium in myometrium of adenomyosis women in untreated group, **B.** Ectopic endometrium in myometrium of adenomyosis women in dienogest-treated group, **C.** Endometrial gland in endometrium of untreated group, **D.** Endometrial gland in endometrium of dienogest-treated group, **E.** Endometrial stroma in endometrium of untreated group, **F.** Endometrial stroma in endometrium of dienogest-treated group, **G.** Double staining for CD57 and CD56 in endometrium, and **H.** Double staining for CD57 and CD56 in myometrium.

Effect of dienogest on the myometrium surrounding the ectopic endometrium

Infiltration of macrophages and NK cells in the myometrium of women with adenomyosis was also investigated. No differences were seen between DNG-treated and untreated groups (Figs.2, 3, Table 3).

The decrease of endometrial thickness after dienogest treatment

The uterine endometrial thickness was more significantly reduced after DNG treatment for 30 ± 2.76 days (mean ± SD) when compared to the untreated group [median (25-75%), 37.5 (33.37-60.12), 94.5 (67.75-143.25), respectively] (P=0.021).

Table 3: Macrophage and natural killer (NK) cells infiltration in ectopic endometrium in myometrial tissue in adenomyosis patients

	Untreated n=12	Dienogest n=12	P value
Macrophages (cells/mm^2)	8.75 (4.06-17.18)	5 (3.75-13.43)	0.663
NK cells (cells/mm^2)	5.62 (4.68-19.06)	11.25 (9.68-16.87)	0.354

Data as presented as [median (range 25-75%)].

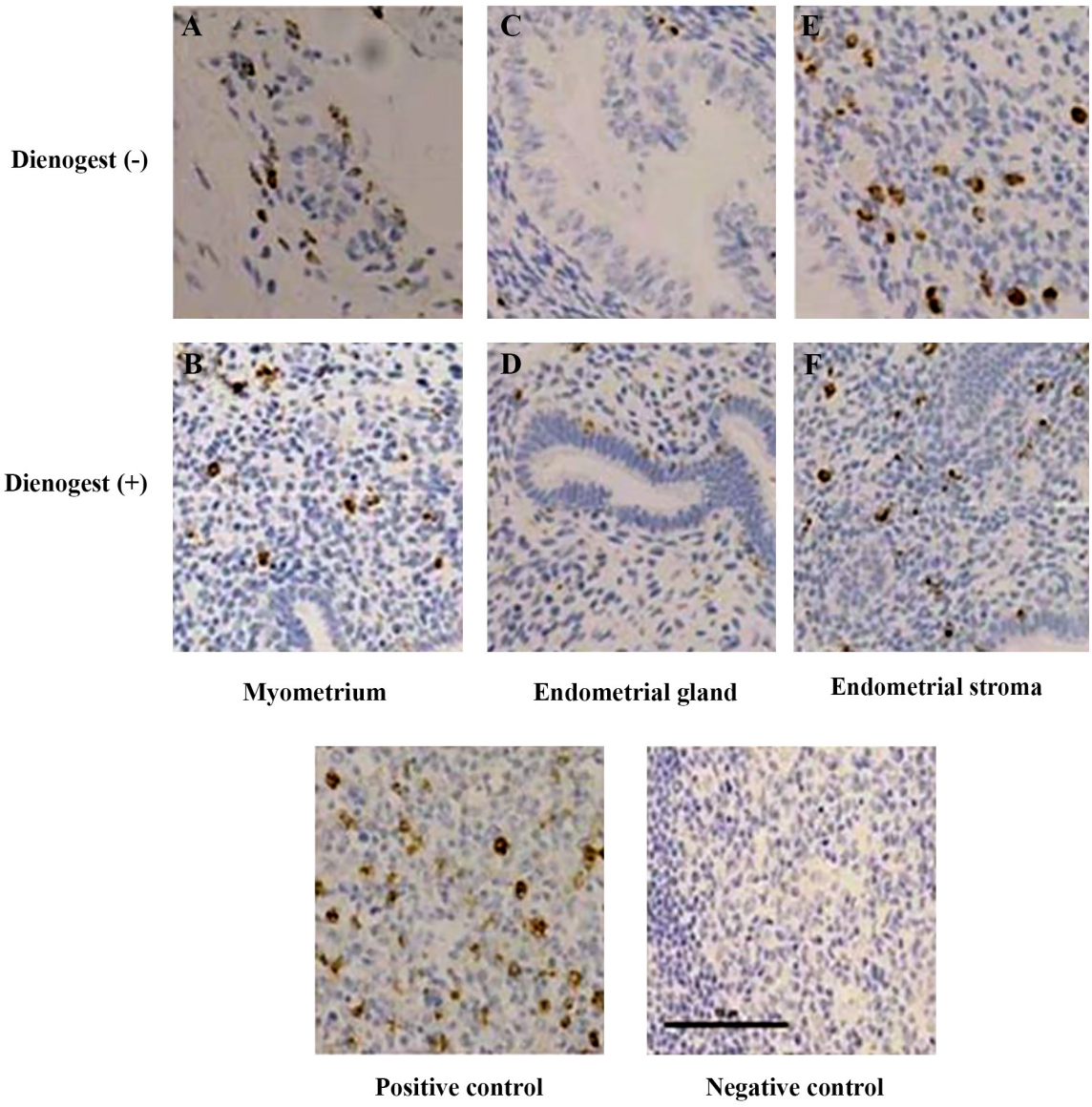

Fig.3: Immunohistochemistry staining for CD68 in eutopic and ectopic endometrium. The accumulation of CD68 is indicated by a dark brown coloration in the nucleus and all nuclei were counter-stained with hematoxylin. **A.** Ectopic endometrium in myometrium of adenomyosis women in untreated group, **B.** Ectopic endometrium in myometrium of adenomyosis women in dienogest-treated group, **C.** Endometrial gland in endometrium of untreated group, **D.** Endometrial gland in endometrium of dienogest-treated group, **E.** Endometrial stroma in endometrium of untreated group, and **F.** Endometrial stroma in endometrium of in denogest-treated group.

Discussion

This randomized controlled trial compared the numbers of infiltrating macrophages and NK cells in the eutopic and ectopic endometrium in women with adenomyosis after either one month of treatment with DNG or no treatment. The results showed that oral DNG increased the number of NK cell infiltration in glandular structure of eutopic endometrium but not in ectopic endometrium. However, no differences were observed in the number of macrophages and NK cells in stromal tissue of eutopic endometrium or ectopic endometrial tissue of the two study groups. This suggests that progestin has a direct effect on endometrium in addition to its systemic effect, thus may improve local immunity in the endometrium.

The histopathological characterization of adenomyosis is similar to endometriosis, except for the site of endometriotic lesions. Immune dysfunction and inflammation play significant roles in both endometriosis and adenomyosis. Various immune alterations occur in endometriosis that are also present in adenomyosis. We noted an increase in the number and activity of macrophages secreting proinflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) in women with endometriosis. It has been shown that patients with adenomyosis have both HLA-DR and HLA-G expression in eutopic and ectopic endometrium (7, 14). The expression of HLA-G corresponding to major histocompatibility complex (MHC) class I increases the survival of endometrial cells from the host's immunosurveillance (13). Ota and Igarashi (7) and Ota et al. (15) reported an increased number of macrophages in ectopic and eutopic endometrium in women with adenomyosis. Recently, Zhihong et al. (16) did a prospective case-control study by collecting the endometrial tissue from patients with adenomyosis during the implantation window after ovarian stimulation, and then performed immunohistochemistry and real time-polymerase chain reaction (RT-PCR). The results showed an increase in cytokine IL-6, monocyte chemoattractant protein-1 (MCP-1) and the number of macrophages. MCP-1, which is produced by uterine NK cells is a cytokine that induces the migration of immune cells to target tissue.

NK cells play a role in destroying viruses and tumor cells but not normal host cells. The killing activity depends on the activity of killer cell inhibitory receptors (KIRs) and MHC molecules (17). The reduction of NK cell activity in peripheral blood and peritoneal fluid has been shown in endometriosis (18, 19). The NK cell cytotoxicity may also be reduced in eutopic endometrium in adenomyosis, because the expression of CD94a (surface marker for KIR) is increased in the endometrium versus the myometrium in women with adenomyosis (17). There are a few classical NK cells CD56+CD16+ in both eutopic and ectopic endometrium in adenomyosis (20). Here, we used CD57-a terminally sulfated carbohydrate epitope (glucuronic acid 3-sulfate)-to identify NK cells. This marker identifies the final stage of NK cell maturation. Therefore, these matured CD57+ NK cells may have strong cytotoxic activity, but weak proliferative property (21). Additionally, we verified NK cells via dual immunohistochemistry staining for CD56 and CD57.

Several previous studies have demonstrated progesterone and progestin-modulated immune responses (22-26). The cyclical change of progestin in endometriums of nonpregnant women suggested hormone regulation, specifically progesterone (27). Our results showed that DNG increased the number of mature NK cells at the glandular epithelium of eutopic endometrium, which is comparable to Klinger's findings. Oral combined contraceptive pills containing DNG have been demonstrated to increase the number of lymphocytes, monocytes and granulocytes in patients treated for one cycle (22). A similar result was seen in an animal study, where administration of DNG in rats with endometrial tissue auto-transplantation slightly increased the NK cell activity and number in peritoneal fluid cells and spleen cells (11).

Progestin treatment including megestrol acetate for endometrial hyperplasia and endometrial cancer-induced immune suppression can increase the number and activity of NK cells and other immune cells (24). NK cells are the important immune cell type in the gestational decidua. The regulation of uterine NK cells by progesterone and progestin are well-known during pregnancy (27, 28). However, the role of uterine NK cells remains unclear. Many are present during implantation, and they may be involved in the implantation mechanism (23). The potential role of uterine NK cells is that it supports the preparation of uterus for embryo implantation by producing many cytokines. Moreover, Le Bouteiller and Piccinni (27) found that uterine NK cells in early decidua could kill target cells, for example, infected maternal decidual cells, supporting the local immune responses to uterine infection (23, 27, 29). Therefore, DNG administration in patients with adenomyosis may improve the implantation process and protect the fetus from infection to pregnancies that occur after discontinuation of treatment.

Different types of progestin may affect immune responses differently (23, 30). However, there is no direct comparative study on the effect of different progestins on NK cells. This would be an interesting topic for further studies. Progesterone recruites uterine NK cells by increasing the chemokine C-X-C motif ligand (CXCL)10 and CXCL11, which has been demonstrated in an in vitro study using an endometrial organ culture system (31). In addition, hormone replacement therapy has been shown to change NK cell activity (32). In this study, DNG enhanced the NK cell number only at the glandular structure of the endometrium. There was no increase in NK cell numbers in stromal tissue of eutopic endometrium or in the ectopic endometrium. In the study conducted by Mehasseb et al. (33), they examined the expression pattern of progesterone receptor (PR)-A and PR-B and foci lesion of adenomyosis by immunohistochemistry in the endometrium of control and adenomyosis subjects. The expression of both PRs was lower in the stroma, and the

inner and outer myometrium in the adenomyotic samples compared to glands. Therefore, the differential effect of DNG on various tissues may have been mediated by differential expression of PRs on adenomyotic tissue and eutopic endometrium.

DNG did not affect macrophage infiltration on eutopic and ectopic endometrium or on the myometrium from patients with adenomyosis. The effect of DNG on macrophage infiltration was different from that of GnRHa shown by Khan et al. (8). GnRHa decreased the infiltration of CD68-expressing cells in the endometrium of women with endometriosis and adenomyosis. It is possible that the local actions of DNG and GnRHa on endometriosis or adenomyosis lesions were different. The highlight of our study is that, patients were randomly assigned to two study groups by block randomization with a block size of 4. There was an equal number of participants in each study group and the cohorts had homogeneous features. This maintained the balance of the study groups and reduced selection bias. In this study we had aimed to investigate the effect of progestin on mature NK cells by immunohistochemistry, which indirectly reflect NK cell cytotoxicity. However, further studies on functional NK cells and other characteristics of NK cells, i.e. proliferation or apoptosis, are still needed.

Conclusion

Progestin DNG administration causes an increase in uterine mature NK cells in glandular structure of eutopic endometriumn in patients with adenomyosis. The immunomodulating effect of progestin on adnenomyosis may be beneficial for implantation and fetal protection to pregnancies occurring after treatment. The enhancing effect of progestin on NK cells is differentially expressed depending on the site of immune infiltration.

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Author's Contributions

S.P.; Conducted the study including patient recruitment and data collection, and drafted the manuscript. Y.T., S.L.; Participated in acquisition of data and helped to draft the manuscript. N.R., M.S.; Gave technical support and conceptual advice. W.W., K.D.; Performed the experiments. A.S.; Conceived, designed the study, analyzed data and revised the manuscript. All authors read and approved the final manuscript.

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Original Article

Accuracy of Two-Dimensional Transvaginal Sonography and Office Hysteroscopy for Detection of Uterine Abnormalities in Patients with Repeated Implantation Failures or Recurrent Pregnancy Loss

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Abstract

Background: We sought to compare diagnostic values of two-dimensional transvaginal sonography (2D TVS) and office hysteroscopy (OH) for evaluation of endometrial pathologies in cases with repeated implantation failure (RIF) or recurrent pregnancy loss (RPL).

Materials and Methods: This prospective study was performed at Royan Institute from December 2013 to January 2015. TVS was performed before hysteroscopy as part of the routine diagnostic work-up in 789 patients with RIF or RPL. Uterine biopsy was performed in cases with abnormal diagnosis in TVS and/or hysteroscopy. We compared the diagnostic accuracy values of TVS in detection of uterine abnormalities with OH by receiver operating characteristic (ROC) curve analysis.

Results: TVS examination detected 545 (69%) normal cases and 244 (31%) pathologic cases, which included 84 (10.6%) endometrial polyps, 15 (1.6%) uterine fibroids, 10 (1.3%) Asherman's syndrome, 9 (1.1%) endometrial hypertrophy, and 126 (15.9%) septate and arcuate uterus. TVS and OH concurred in 163 pathologic cases, although TVS did not detect some pathology cases (n=120). OH had 94% sensitivity, 95% specificity, 62% positive predictive value (PPV), and 99% negative predictive value (NPV) for detection of endometrial polyps. In the diagnosis of myoma, sensitivity, specificity, PPV, and NPV were 100%. TVS had a sensitivity of 50% and specificity of 98% for the diagnosis of myoma. For polyps, TVS had a sensitivity of 54% and specificity of 80%. Area under the ROC curve (AUROC) was 70.69% for the accuracy of TVS compared to OH.

Conclusion: TVS had high specificity and low sensitivity for detection of uterine pathologies in patients with RIF or RPL compared with OH. OH should be considered as a workup method prior to treatment in patients with normal TVS findings.

Keywords: Diagnosis, Hysteroscopy, Ultrasonography, Uterine

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Introduction

Intrauterine pathologies are present in 25-50% of infertile patients (1). Structural abnormalities of the uterine endometrial cavity affect reproduction outcomes because they interfere with implantation or cause spontaneous abortions (2). Therefore, accurate diagnosis of any endometrial pathology in the patient is an important step prior to beginning the assisted reproductive technology (ART) cycles (2, 3). During the last decades, hysterosalpingography (HSG), hysteroscopy, sonohysterography, and transvaginal sonography (TVS) have been developed to evaluate the uterine cavity; each has their own advantages and disadvantages (4-7). TVS is universally considered the initial, non-invasive procedure for assessment of intrauterine pathologies (8). Hysteroscopy allows for direct and three-dimensional (3D) visualization, and sampling of the uterine cavity. Although considered the gold standard (9), it is not as affordable and comfortable as TVS, which has relatively lower patient discomfort (4). Since the introduction of the hysteroscopic technique, the procedure has undergone considerable mod-

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ifications, leading to an increase in patient compliance and tolerance. Fiberoptics, smaller caliber of the endoscopes, use of simpler distention media, and availability of safer local infiltrative anesthetics have all contributed to the increased use of this technique to evaluate the uterine cavity in the office setting (10, 11). Diagnostic or office hysteroscopy (OH), though increasingly used for uterine cavity evaluation, is still underutilized (11). Some studies have evaluated the diagnostic values of two-dimensional (2D) and/or 3D TVS compared with hysteroscopy (1, 4-8, 12). El-Mazny et al. (1) compared TVS and OH for evaluation of intrauterine pathologies in patients scheduled for ART. They have concluded that the TVS was specific (100%), but not sensitive (41.7%) compared to OH. However, the value of OH as a routine evaluation in the management of infertile women is a debatable topic. The goal of this prospective study was to evaluate the diagnostic validity of 2D TVS and outpatient OH in the detection of uterine cavity pathologies in recurrent implantation failure (RIF) or recurrent abortion cases.

Materials and Methods

We conducted this prospective cohort study at Royan Institute between December 2013 and January 2015. The Institution Review Board and the Ethics Committee of Royan Institute approved this study. All patients signed a consent form to give permission to use their treatment outcomes confidentially without mentioning the name. All TVS evaluations were performed free for participants. We included all patients with primary and secondary infertility, 20 to 40 years of age, who were diagnosed with failed *in vitro* fertilization (IVF), intrauterine insemination (IUI), or recurrent pregnancy loss (RPL). Exclusion criteria were as follows: history of previous surgery and pathology in the uterus, or patients with heterogenic or echogenic endometrium attributed to bleeding.

RIF was defined as the lack of clinical pregnancy after transfer of at least four good-quality embryos in a minimum of three fresh or frozen cycles in women under the age of 40 years (13). RPL referred to two or more failed clinical pregnancies as recorded by ultrasonography or histopathologic evaluations in infertile women. During the study period, we initially evaluated all eligible patients by TVS, then OH in the same month. Uterine biopsy was performed in cases with abnormal diagnosis according to TVS and/or hysteroscopy. For example, in complex situations like submucous myoma, endometrial polyps and extensive Asherman's syndrome we scheduled several hysteroscopic procedures and performed therapeutic interventions after obtaining informed consent from the patients.

Uterine assessment

Patients underwent TVS in the follicular phase of the menstrual cycle (days 5-13) when the menstrual bleeding stopped and before the diagnostic hysteroscopy evaluation. TVS and OH were carried out in the same cycle. All sonographic evaluations were performed by an expert radiologist (F.A.) using an Aloka α-10-color doppler with a transvaginal 6 MHz probe. Uterine cavity abnormalities that included polyp lesions, uterine myoma, septate and arcuate uterus, adhesion, and endometrial hypertrophy were evaluated. We have defined a polyp as a round or oval echogenic lesion with intact endometrial-myometrial junction located in the endometrial cavity. Submucosal fibroma is a benign lesion that originates from the smooth muscle layer and the accompanying connective tissue of the uterus. It is observed in sonography as a mixed or hypoechoic mass lesion that originates from the myometrium and interrupts the endometrial layer. Septum is a form of congenital malformation that divides the uterine cavity by a longitudinal short or long wall whereas the outside of the uterus has a normal shape. Abnormal adhesion is detected as an irregular endometrial line in ultrasound and observed as a fibrous band which separates the endometrial cavity. Endometrial hypertrophy is detected as thickening of the endometrium on sonography which represents excessive proliferation of the endometrium cells (14).

An expert gynecologist performed the OH the next day by using a rigid hysteroscope (Oblique Telescope 30°, diameter: 2 mm, length: 26 cm, KARL STORZ GmbH & Co., Germany) assembled in a 4.2 mm diameter diagnostic sheath with an atraumatic tip. A high-intensity cold light source and fiberoptic cable were used to clarify the uterine cavity. Normal saline (0.9%) was applied as the distention medium, with pressure maintained between 100-120 mm Hg using a pressure adjustable cuff system to achieve the lowest adequate pressure to distend the uterine cavity. This practically painless procedure does not require the use of analgesics or sedatives.

The patient was placed in the dorsolithotomy position and a pelvic examination was performed to detect the size of the uterus and its direction. No speculum or tenaculum were needed as the vaginoscopic ''no touch'' technique was applied. Once this was accomplished, the hysteroscope with its light source was placed at the level of the ectocervix, and guided into the endocervical canal. At the entrance of uterine cavity, a systematic observation was performed that included the uterine cornua, tubal ostia, uterine fundus, lateral, anterior, and posterior uterine walls. The uterine cavity and endocervical canal were re-evaluated during withdrawal of the instrument.

A video system was used for patient observation and to document the procedure for future reference. The patients were under observation for a minimum of 30 minutes to assess for possible side effects and complications. The gynecologist who performed the diagnostic hysteroscopy was unaware of the TVS results in order to minimize performance bias. OH were recorded on a special data form that included the following items: i. Appearance and figure of the endocervical canal (endocervicitis-determined by congestion and hypertrophy of the mucosal lining; mucous polyp-associated with contact bleeding and excessive discharge), ii. Endometrial appearance (endometritis-congestion, hyperemia, hemorrhages, and adhesions; hyperplastic endometrium-thickened and easily indented by pressure, with or without multiple polyps), iii. Figure

of the uterine cavity, and iv. Existence and situation of structural lesions (myomas, polyps, adhesions, and congenital anomalies).

Statistical analysis

All data were recorded in the Statistical Package for the Social Sciences (SPSS) version 20 (SPSS Inc., Chicago, IL, USA) and analyzed using appropriate accuracy indices. Diagnostic accuracy was assessed by sensitivity, specificity, positive and negative predictive values (PPV, NPV) calculated using 2×2 tables for each method. The agreement between the two methods for the detection of uterine lesions was calculated by the Kappa coefficient. Histopathologic results were the gold standard. Cases with abnormal OH had only a uterine pathologic assessment. Because the results of the sonography test affect whether the gold standard (pathology) procedure is used to verify the test result, verification bias exists when sonography results are compare with pathology findings. We have used previous methods (15-17) to adjust the verification bias. The McNemar test was used to compare marginal homogeneity for results of 2D TVS and OH. Diagnostic accuracy value for results of the 2D TVS compared OH findings was calculated through the receiver operating characteristic (ROC) curve.

Results

All planned procedures were completed successfully. There were no complications recorded during or after the procedures. During the study period, we evaluated 789 patients with both 2D TVS and OH. In the TVS examination, 545 (69%) cases were normal and 244 (31%) had pathologic findings that included 84 (10.6%) polyp lesions, 15 (1.6%) uterine fibroids, 10 (1.3%) Asherman's syndrome, 9 (1.1%) endometrial hypertrophy, and 126 (15.9%) septate and arcuate uterus. TVS and OH results were in agreement in 163 pathologic cases. However TVS could not detect some pathology (n=120). The McNemar test showed that the diagnostic values for detection of Asherman's syndrome, endometrial hypertrophy, arcuate and septate uterus significantly differed between the two methods (Table 1).

Area under ROC curve (AUROC) was almost acceptable 70.69% for the accuracy of 2D TVS compared with OH (Fig.1).

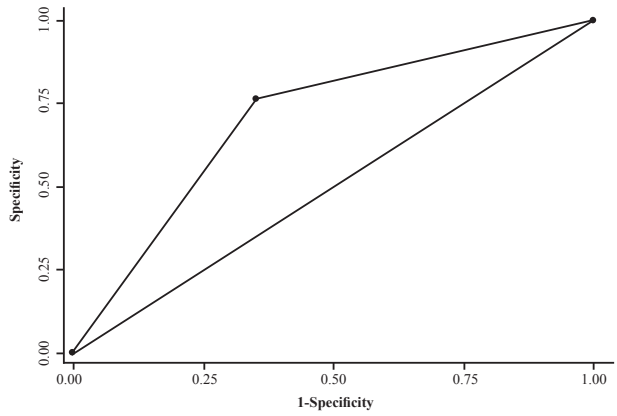

Fig.1: Area under ROC curve (AUC=0.70) for accuracy of two dimensional trans-vaginal sonography in compare to office hysteroscopy.

Polyp lesions

There was a significant agreement between TVS and OH in the diagnosis of uterine polyps. The Kappa coefficient of 0.5 indicated an intermediate match. The results showed that TVS had 54% sensitivity and 80% specificity to detect polyps, with a reported PPV of 19% and NPV of 95% (Table 1).

Uterine fibroids

The results demonstrated a sensitivity of 50% and specificity of 98% for TVS to diagnosis myoma, with a PPV of 20% and NPV of 99%. There was a significant agreement between TVS and OH in the diagnosis of uterine fibroids. The Kappa coefficient was 0.3 and this agreement level was weak.

Table 1: Total finding by TVS and OH in 789 patients

	2D TVS n (%)	OH n (%)	SN (%)	SP (%)	PPV (%)	NPV (%)	Kappa (%)	P value[a]
Normal finding	545 (69)	506 (64.1)						
Polyps	84 (10.6)	97 (12.3)	54	80	19	95	50	0.18
Submucosal myoma	15 (1.6)	15 (1.9)	50	98	20	99	30	1.00
Asherman's syndrome	10 (1.3)	23 (2.9)	30	97.4	13	96.2	16	0.02
Endometrial hyperplasia	9 (1.1)	19 (2.4)	66.7	98.2	31.6	99.6	42	0.02
Arcuate uterus	87 (11)	65 (8.2)	29.9	94.4	40	91.6	27	0.03
Septate uterus	39 (4.9)	64 (8.1)	61.5	94.7	37	97.9	45	0.01
Total pathologic findings	244 (31)	283 (35.9)						

2D TVS; Two-dimensional transvaginal sonography, OH; Office hysteroscopy, SN; Sensitivity, SP; Specificity, PPV; Positive predictive value, NPV; Negative predictive value, and [a]; P value was computed using the McNemar test statistic.

Endometrial hypertrophy

TVS and OH provided consistent results for the diagnosis of endometrial hypertrophy. The Kappa coefficient was 0.42 and this agreement level was intermediate. The results revealed that a sensitivity of 66.7% and specificity of 98.2% for TVS to diagnose endometrial hypertrophy. TVS had a PPV of 31.6% and NPV of 99.6%.

Asherman's syndrome

There was a significant agreement between TVS and OH in the diagnosis of Asherman's syndrome. The Kappa coefficient was 0.16; however, this matching level was weak. The analysis showed a sensitivity of 30% and specificity of 97.4% for TVS to diagnose Asherman's syndrome. TVS had a calculated PPV of 13% and NPV of 96.2%.

Septate and arcuate uterus

There was significant agreement between TVS and OH in the diagnosis of septate uterus. The consistent level was intermediate with an obtained Kappa coefficient of 0.45. The results demonstrated a sensitivity of 61.5% and specificity of 94.7% for TVS to diagnose septate uterus. TVS had a PPV of 37.5% and NPV of 97.9%. The agreement between TVS and OH to diagnose arcuate uterus was significant. The consistent level was weak with a Kappa coefficient of 0.27. We obtained a sensitivity of 29.9% and specificity of 94.4% for TVS to diagnose arcuate uterus. The PPV and NPV were calculated as 40 and 91.6%.

Discussion

Different types of uterine lesions (polys, fibroma, congenital anomalies and acquired disease) can play an important role in female reproductive failures. Various methods are used to diagnose these uterine pathologies. Hysteroscopy is an endoscopic evaluation of the uterine cavity with video recording capabilities, which enables a second opinion (1). This test can be performed in a clinic office without the need for anesthesia (18). Direct visual imaging of the uterine cavity by this method allows for the diagnosis of cancer, as well as polyps and submucosal myomas (19). Diagnostic hysteroscopy is considered a gold standard method for evaluation of the uterine cavity, with the capability for uterine pathology treatment particularly for women with RIF and RPL, as well as other infertile women (13, 20, 21). TVS is a noninvasive technique to evaluate the uterine cavity (14). The present study aims to evaluate the diagnostic value of TVS performed preceding to routine hysteroscopy to determine if TVS can alleviate the number of diagnostic hysteroscopies commonly performed in women with normal uterine cavities.

Our results demonstrated the following: sensitivity (54%), specificity (80%), PPV (19%), and NPV (95%) of TVS for the diagnosis of endometrial polyps. These results for detection of myoma were: sensitivity (50%), specificity (98%), PPV (20%), and NPV (99%). Our results agreed with a number of studies (1, 4, 12, 22). Babacan et al. (4) reported that TVS has a sensitivity of 54% and specificity of 84% for detecting endometrial polyps. Cepni et al. (12) found that TVS had a sensitivity of 58% and specificity of 94% for detection of intra-cavity fibromas. These researchers determined that TVS had a sensitivity of 72% and specificity of 50% for diagnosis of endometrial polyps. Bonnamy et al. (22) reported that TVS had a sensitivity of 57% and specificity of 69% for detection of intrauterine masses. Wanderley et al. (23), observed that TVS has a diagnostic accuracy of 65.9% for polyps, 78.1% for myoma, and 63.2% for endometrial hyperplasia. However, other studies reported different results (5, 6, 24).

Niknejadi et al. (14) reported that TVS had sensitivity of 89.2% and specificity of 99.6% for endometrial fibroids. Soares et al. (24) and Loverro et al. (6) demonstrated that TVS had a sensitivity of 75-85% and specificity of 90-100% for the diagnosis of endometrial polyps. Balić and Balić (25), in a retrospective study, reported that TVS and hysteroscopy had identical sensitivity (100%) for diagnosis of endometrial polyps, whereas hysteroscopy had higher specificity (92.3%) than TVS (56.4%). The authors concluded that the agreement between hysteroscopy and histology was good, whereas there was moderate agreement between TVS and histology. On other hand, Krampl et al. (26) stated that TVS had a sensitivity of 23% and specificity of 93% for the diagnosis of intracavitary lesions in patients with abnormal uterine bleeding. The TVS sensitivity in their study was less than other studies. Fedele et al. (27) found that TVS had a misdiagnosis rate of 4.2% and was less effective than hysteroscopy for detection of polyps; however, they reported a sensitivity of 91% and specificity of 100% in TVS for detection of uterine adhesions. They concluded that TVS was a noninvasive, relatively inexpensive, possibly effective method to screen for uterine adhesions in high risk population.

In the present study, we demonstrated that OH had a sensitivity of 94% and specificity of 95% for detection of endometrial polyps. Both values were 100% for diagnosis of myoma. Grimbizis et al. (8) showed that OH had a sensitivity of 97% and specificity of 91% for diagnosis of endometrial polyps, as well as a sensitivity of 100% and specificity of 98% for detection of myoma. Niknejadi et al. (9) reported excellent specificity (91.2%), good sensitivity (88.2%), an 81.4% PPV, and a 94.6% NPV for TVS in detection of uterine polyps.

Our finding presented that 2D TVS has a low sensitivity and high specificity for diagnosis of septate and arcuate uterus. Recently, some studies evaluated the accuracy of 3D TVS for diagnosis of uterine anomalies (28-34). Szkodziak et al. (34) concluded that HSG was not an optimal procedure to diagnose uterine anomalies, whereas 3D TV USG could precisely determine uterus anomalies and might be considered as alternative to MRI. Ludwin et al. (33) reported that 3D-SIS was the same as hysteroscopy performed in conjunction with laparoscopy (HL) with the highest accuracy and also there was no significant differ-

ence in diagnostic value between 3D TVS with 2D SIS and 3D SIS or between expert 2D TVS and 3D TVS with 2D SIS. Despite the high diagnostic value of these ultrasound devices, is endoscopy necessary for differential diagnosis of common uterine abnormalities? The Thessaloniki ESHRE/ESGE consensus have recommended 3D-TVS for the diagnosis of female genital anomalies in high-risk, symptomatic women and in any asymptomatic patient suspected of having an anomaly discovered during a routine workup. This consensus suggested that the different diagnostic tools should be applied in an accurate manner and performed by experts to prevent mis-, over- and underdiagnoses. The role of a combined ultrasound evaluation and OH should be prospectively examined in future researches (35).

In the present study, we did not obtain endometrial biopsies for histologic evaluation in patients who had normal hysteroscopy and 2D TVS. However, we used statistical methods to adjust for verification bias to determine a diagnostic value of 2D TVS for endometrial abnormalities.

Conclusion

The present study noted that both methods have demonstrated high specificity; however, in our experience, OH was significantly more sensitive than 2D TVS for detection of uterine pathologies in patients with RIF and recurrent abortion. It seemed that the OH should be considered as workup method prior to the treatment cycle even in women with normal HSG and/or TVS.

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Author's Contributions

M.Sh.; Conceived the study. M.Sh., F.A.; Are responsible for the design of the research and performed the Transvaginal Sonography and Office Hysteroscopy. M.O.; Collected the study participations. A.A., M.Ch; Analyzed the data and interpreted the results of the experiments and prepared the figures and drafted the manuscript. A.A., M.Sh., F.A.; Edited and revised the manuscript. All authors read and approved the final manuscript.

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Original Article

Quality of Life and Its Influencing Factors of Couples Referred to An Infertility Center in Shiraz, Iran

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Abstract

Background: Infertility adversely affects quality of life (QoL). The present study aims to evaluate QoL and its associated factors among infertile couples.

Materials and Methods: In this cross-sectional study, the Fertility QoL (FertiQoL) instrument was used to measure QoL among 501 volunteer couples who attended the Infertility Clinic at the Mother and Child Hospital, Shiraz, Iran. We used an additional questionnaire to assess participants' demographic and clinical characteristics. The relationship between the scores of QoL to the sociodemographic and treatment data was analysed.

Results: The subjects with lower income levels had lower relational, mind/body, emotional, and total core scores. Female participants without academic education had lower scores in the emotional subscale, while the male participants showed lower scores in emotional, mind/body, relational, social, and total QoL domains. Subjects who had undergone any type of treatment, including pharmacological treatment, intrauterine insemination (IUI), intra-cytoplasmic sperm injection (ICSI), and *in vitro* fertilization (IVF) showed significantly lower scores in the environmental domain. Participants with lower infertility duration obtained significantly greater QoL scores. Finally, tolerability, emotional, and environmental domains were significantly more desirable when the infertility problem was related to a male factor.

Conclusion: Infertile couples with shorter duration of infertility and male etiology have higher QoL. Lower academic education, lower income levels, or prior unsuccessful treatments are associated with lower QoL.

Keywords: Education, Income, Infertility, Quality of Life, Treatment

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Introduction

Child bearing, one of the naturally desired human goals, is mandatory for survival of the human species. An estimated 72.4 million couples worldwide experience primary or secondary infertility (1). The reported infertility rate for different countries ranges from 5-30% (2). A meta-analysis has calculated an average infertility rate in Iran of 10.9% of the population (3). Infertility induces numerous psychological, economic, ethical, and cultural consequences that result in diminished self-confidence and quality of life (QoL) (4). Infertility and its related diagnostic or therapeutic modalities that include pharmacological treatment (oral pareneral administration), intrauterine insemination (IUI), *in vitro* fertilization (IVF), and intra-cytoplasmic sperm injection (ICSI) induce a heavy burden on affected couples. According to the World Health Organization (WHO) guidelines, ''QoL is an individuals' perception of their position in life in the context of culture and value systems in which they live'' (5). A large number of studies have investigated QoL in infertile couples with non-specific questionnaires, such as World Health Organization Quality of Life form (WHOQOL-BREF) and Health Survey Short Form (SF-36) (6, 7).

A few studies used the Fertility QoL (FertiQol) questionnaire as a fertility-specific QoL assessment of infertile individuals. The FertiQol questionnaire has been shown to be a valid, reliable measure for the impact of infertility on QoL (8-10). According to a substantial body of literature, infertility negatively affects QoL and appears to lead to mental problems, such as anxiety, depression, frustration, isolation, disturbed identity, and lack of attraction (11-13). Infertility is a critical issue for extended Iranian families (14). A study in Tehran has investigated QOL of infertile couples and reported significantly higher

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QoL in infertile men compared to infertile women (15). Until now, few studies have investigated factors that impact QoL in infertile couples. Of particular importance is how the factors that predict QoL vary in different infertile populations. Recognition of these associated factors is essential for program planning to increase the QoL of the infertile population (16, 17). The present study aims to assess the QoL of infertile couples with particular emphasis on the influences of related factors on their QoL.

Materials and Methods

This cross-sectional study selected subjects by simple random sampling from infertile couples who attended the Infertility Clinic of the Mother and Child Hospital, Shiraz, Iran from February 2014 to March 2015. Couples who did not achieve pregnancy after at least one year of timed unprotected sexual intercourse were invited to voluntarily participate in this study. Overall, 501 eligible couples agreed to participate. All procedures were performed in accordance with the ethical standards of the Institutional and/or National Research Committee and with the 1964 Declaration of Helsinki, its later amendments or comparable ethical standards.

The Ethics Committee of Shiraz University of Medical Sciences approved this study (code: 92-01-50-7132). At first, participants received a complete explanation about the nature of the study and each participant signed a written informed consent. All participants were requested to complete a data gathering form that contained structured questions about demographic information, socioeconomic status, and fertility characteristics. Interviews were also performed by the researchers and trained assistants for further explanations if needed and to assist the illiterate participants for their verbal consent and appropriate completion of the questionnaire in the Infertility Clinic. We administered the international FertiQol questionnaire to assess participants' QoL (9). The questionnaires gathered information about age, educational and economic status, type of medications, causes of infertility, and duration of infertility. All parameters were considered to be the independent variable, whereas QoL was the dependent variable of the study. In this study, we used the Persian version of the FertiQoL instrument. The Persian version was previously proven to be a valid, reliable tool to evaluate QoL of infertile couples (10).

The FertiQoL questionnaire consists of core and treatment sections: 24 specific questions that cover mind/body, relational, social, and emotional domains in the core section and 10 questions on environment and tolerability domains in the treatment section. We divided the participants into three socioeconomic groups: low income (couples that made less than one million tomans per month); middle income (1-3 million tomans per month); and high income (more than 3 million tomans per month). Scores of the six subscales of FertiQoL instrument could range from 0 to 100, where higher scores indicated a better QoL.

Statistical analysis

The scores of QoL were calculated by the Researchers Excel scoring FertiQol online system. Statistical analyses were performed using SPSS 18.0 statistical software (SPSS, Inc., Chicago, IL, USA). Continuous variables were compared using the independent sample t test, Mann-Whitney U test, and one-way ANOVA. Additionally, categorical variables were compared using Pearson's chi-square test. The relationship between the scores of every scale and subscale of QoL with the sociodemographic and treatment data was analyzed. $P<0.05$ were considered significant.

Results

Out of the 501 eligible infertile couples, 499 couples properly completed the questionnaires. The mean age of the male participants was 32.30 ± 5.65 years and for female participants, it was 31.66 ± 6.13 years. The self-reported monthly incomes of 389 (78.1%) couples were equal or less than one million tomans, 87 (17.5%) couples earned 1-3 million tomans, and 11 (2.2%) couples earned more than 3 million tomans per month. In this survey, 11 cases did not report their monthly incomes. The participants' educational and fertility characteristics are listed in Table 1.

Table 1: Educational and fertility characteristics of the participants

Variable	n (%)
Cause of infertility	
Male	147 (29.5)
Female	132 (26.5)
Both	45 (9.0)
Unexplained	130 (26.1)
Duration of infertility	
<5 Y	302 (60.6)
5-10 Y	101 (20.3)
>10 Y	63 (12.7)
Duration of tducation (male)	
<9 Y	89 (18.5)
9-11 Y	221 (46.0)
>11 Y	170 (35.4)
Duration of education (female)	
<9 Y	67 (13.9)
9-11 Y	217 (45.2)
>11 Y	196 (40.8)

The study showed that participants with a shorter duration of infertility obtained better scores in the total treatment domain ($P=0.04$). Individuals with lower monthly income levels (below 1 million tomans) had lower scores in the total FertiQol, mind/body, and emotional subscales. The same subjects also obtained lower scores in the total treatment and tolerability domains; however the differences were not statistically significant ($P>0.05$). Education had a significant impact on the couples' QoL. Specifically, we noted significantly lower emotional subscale

scores among the women with lower academic degrees (Table 2). The men with lower academic degrees also obtained significantly lower scores in the total FertiQol, mind/body, relational, social, and emotional domains. Total FertiQol, total treatment, tolerability, environmental, and emotional subscales had better scores when the male factor was the cause of infertility compared to the conditions where females or both sexes were the causes for infertility.

Treatment of infertility, including pharmacological treatment (oral or parenteral medications), as well as IUI, IVF, and ICSI had lower scores for total treatment, tolerability, and environmental domains compared to the cases that did not use medications (P<0.05).

Discussion

In the present study, we have sought to evaluate the sociodemographic and clinical variables that influenced QoL in infertile couples. In the general Iranian population, a woman's social standing is strongly tied to maternal status or the possibility of pregnancy. Motherhood experience increases the total QoL score. FertiQoL is a reliable, sensitive instrument to evaluate QoL in infertile couples. In a recently published study, Maroufizadeh et al. (10) have evaluated the validity and reliability of the Persian version of the FertiQol instrument. They concluded that the Persian version performed similar to the original English version. They tested the factor structures of the FertiQol Instrument and suggested removal or modifications for Q15 and T2 to gain a higher loading on internal consistency of the relational and environmental factors.

The FertiQol instrument covers QoL with respect to treatment quality and tolerability (9). Despite the advantages over other techniques, a few studies have used the FertiQoL instrument to assess QoL in terms of infertility (6-8). In a review, Mousavi et al. (7) assessed different general and specific questionnaires used to evaluate QoL in infertile people. They found that researchers used 10 general and 2 specific questionnaires to assess QoL of infertile patients in the literature. They concluded that the two most frequently used general questionnaires were SF-36 and WHO-QOL. FertiQoL and the Fertility Problem Inventory, as specific tools, were seldom used.

Evaluation of QoL in both infertile partners was one of the strong points of this study. Our findings showed that total FertiQol, total treatment, tolerability, environmental, and emotional subscales had better scores when the male partners were the causes of infertility. Several investigators previously evaluated the role of male factor infertility on the QoL of couples (18, 19) and found less negative effects of infertility on the men's and couples lives as evidenced by

Table 2: The effects of demographic and other variables on quality of life (QoL) in infertile couples

Study variable		Emotional subscale (mean ± SD)	Relational subscale (mean ± SD)	Mind-body subscale (mean ± SD)	Social subscale (mean ± SD)	Total Core score (mean ± SD)	Treatment Environment subscale (mean ± SD)	Treatment Tolerability subscale (mean ± SD)	Total Treatment score (mean ± SD)	Total FertiQol score (mean ± SD)
Treatment	Pharmacological	51.9 ± 22.2	58.7 ± 17.2	58.1 ± 24.1	61.6 ± 18.2	60.1 ± 18.1	53.6 ± 13.7	55.5 ± 20.8	54.2 ± 14.7	58.4 ± 15.6
	Surgery	50.2 ± 24	57.8 ± 14.6	57.6 ± 23.1	59.9 ± 19.3	59.2 ± 18.3	53.3 ± 16.2	54.6 ± 20.8	53.8 ± 15.1	57.3 ± 15.8
	IUI	45.7 ± 21.7	55.3 ± 13.4	52.8 ± 21.8	59.8 ± 17.0	55.7 ± 15.8	53.7 ± 15.6	51.5 ± 22	53 ± 15.3	54.8 ± 13.8
	IVF, ICSI	49.2 ± 18.8	57.9 ± 14.8	57.2 ± 21.6	61.3 ± 19.3	59.1 ± 15.7	54.1 ± 17.8	51.5 ± 19.1	52.9 ± 15.6	57.3 ± 13.0
	No medication	53.1 ± 23.1	58.7 ± 15.2	61.8 ± 26.6	59.3 ± 21.4	61.3 ± 18.6	61.4 ± 14.5	62.1 ± 24.8	62 ± 15.2	61.1 ± 16.2
	P value	0.183	0.224	0.179	0.945	0.220	0.021	0.036	0.005	0.159
Monthly income	Below 1 million T*	49.3 ± 22.1	56.3 ± 15.1	56.5 ± 23.4	59.7 ± 18.8	57.9 ± 17.1	55.2 ± 16.3	55.8 ± 22	55.5 ± 15.7	57.1 ± 15.1
	Above 1 million T*	58.4 ± 22.0	63.8 ± 14.7	67.5 ± 21.8	67.7 ± 15.8	67.1 ± 15.8	57.8 ± 14.7	59 ± 22.3	58 ± 15.2	64.6 ± 14.0
	P value	0.001	0.001	0.001	0.000	0.00	0.287	0.001	0.101	0.001
Duration of infertility	<5 Y	50.6 ± 21.9	58.8 ± 15.2	58.9 ± 23.2	62 ± 17.8	60.2 ± 16.7	56.4 ± 15.1	57.6 ± 22.1	56.8 ± 14.8	59.1 ± 14.7
	5-10 Y	48.5 ± 22.4	54.7 ± 15.6	53.4 ± 22.8	59 ± 19.5	56.4 ± 18.0	54.9 ± 18.2	52.3 ± 21.2	54 ± 17.6	55.7 ± 15.6
	≥10 Y	52.3 ± 23.7	55.9 ± 14.8	60.1 ± 24.4	58.8 ± 21.1	59.2 ± 18.5	51.2 ± 16.2	52.8 ± 20.6	52 ± 14	57.1 ± 15.3
	P value	0.549	0.073	0.092	0.257	0.166	0.064	0.050	0.048	0.107
Duration of education	Men									
	<9 Y	45.5 ± 22.8	52.7 ± 14	55.2 ± 24	57.7 ± 19.3	55.2 ± 17.3	57.6 ± 14.7	56.1 ± 23.5	57.1 ± 16.5	55.4 ± 15.4
	9-11 Y	49.3 ± 21.5	56.9 ± 15.3	56.7 ± 23.7	60.2 ± 19.1	58.3 ± 17.1	55.2 ± 15.9	56.7 ± 22.3	56.1 ± 15.1	57.6 ± 15.1
	>11 Y	55.7 ± 22.6	61.2 ± 15.2	62.8 ± 22.2	64.2 ± 17.4	63.7 ± 16.7	55.5 ± 16.9	56.9 ± 21.2	55.8 ± 16	61.5 ± 14.5
	P value	0.001	0.000	0.014	0.015	0.00	0.471	0.907	0.848	0.003
	Women									
	<9 Y	46 ± 22.0	53.9 ± 13.3	55.5 ± 23.3	59.3 ± 19.2	56.1 ± 16.3	57.3 ± 13.9	62.4 ± 23.1	59.6 ± 15.4	56.8 ± 14.55
	9-11 Y	49.5 ± 21.6	56.1 ± 15.5	56.2 ± 24	59.3 ± 19.5	57.8 ± 17.5	56.6 ± 16.2	55.8 ± 22.5	56.4 ± 15.7	57.3 ± 15.2
	>11 Y	54.7 ± 22.8	61.2 ± 14.9	62.7 ± 22.3	64.5 ± 17.4	63.6 ± 16.7	54.3 ± 16	55.9 ± 20.8	54.8 ± 15.2	61.1 ± 14.8
	P value	0.012	0.00	0.008	0.010	0.00	0.237	0.072	0.093	0.015
Causes of infertility	Male factor	53.5 ± 21.2	58.4 ± 14.9	61.2 ± 22.7	61.6 ± 18.2	61.4 ± 16.3	59.1 ± 14.5	59.8 ± 21.2	59.3 ± 14.1	60.7 ± 14
	Female factor	47.7 ± 23.0	57.5 ± 15.9	55.4 ± 23.6	60.5 ± 19.6	57.7 ± 18.0	54.1 ± 16.3	51.8 ± 21.1	53.1 ± 15.4	56.4 ± 15.7
	Both	46 ± 19.9	56.3 ± 15.4	54.5 ± 21.5	58.6 ± 20.7	56.4 ± 16.6	52.9 ± 14	53.4 ± 21.9	53.2 ± 15.3	55.4 ± 14.6
	Unexplained	53.3 ± 22.7	58.7 ± 14.7	61.1 ± 24	63.5 ± 16.9	61.8 ± 17.2	54.2 ± 16.4	57.4 ± 22	55.4 ± 15.4	59.8 ± 15.3
	P value	0.034	0.793	0.067	0.351	0.085	0.016	0.010	0.005	0.036

IUI; Intrauterine insemination, IVF; *In vitro* fertilization, ICSI; Intra-cytoplasmic sperm injection, and *; Toman.

significantly higher QoL compared to those with female factor infertility (20-22). Huppelschoten et al. (23) concluded that infertile women had lower fertility-related levels of QoL and were at increased risk for developing emotional problems compared to their partners. However, Chachamovich et al. (24) examined infertile couples' QoL and found no differences between male and female partners. Rashidi et al. (15), in a cross-sectional study, assessed infertile couples' QoL using the SF-36 and concluded that the causes of infertility did not have significant effects on health-related QoL in infertile couples. These differences might be related to the use of a fertility-specific instrument (FertiQoL) in the study by Huppelschoten et al. (23) and the current study compared to the generic QoL assessment instrument by Chachamovich et al. (24) and Rashidi et al. (15). Therefore, further research should be performed to confirm the impact of male or female infertility on couples' QoL.

Karabulut et al. (25) reported that tertiary education was related to higher scores in the total, emotional, and environment domains of QoL. In other previous studies that used SF-36 for health-related QoL and WHOQOL-BREF for general QoL, the women with lower educational levels scored worse in the vitality, environment, and mental health domains (16). Similarly, our study showed significantly lower emotional scores in women with lower educational levels. The scores of the total FertiQol, mind/body, relational, social, and emotional domains were also significantly lower among the men with lower academic education. The current study results showed that couples with shorter duration of infertility had higher scores in the total treatment domain, which confirmed results from two previous studies (25, 26). However Chachamovich et al. (16) used WHOQOL-BREF and discovered no difference among the groups with different infertility durations. According to Rashidi et al. (15), SF-36 questionnaire results indicated that duration of infertility had no significant effects on health-related QoL in infertile couples. These differences might be due to different instruments used for the surveys or differences in the study groups' characteristics. The subject women in the study by Chachamovich et al. (16) had higher educational levels compared to the women that participated in the current study.

Researchers have noted an association between low adherence to fertility treatments and psychological imbalances (27, 28). Boivin et al. (29) discovered that women with a moderate number of treatments exhibited more stress compared to their counterparts that received no treatment or who underwent treatment for a significant amount of time. Our results showed lower QoL scores in the subjects who had undergone treatment compared to those who had not yet began treatment. Ragni et al. (26) noticed that IVF treatment was accompanied by lower scores in the mental health domain. In another study, the couples who had IVF treatments had greater emotional disturbances and anxiety compared to the control group (30). Similarly, Chachamovich et al. (16) revealed that awaiting IVF was associated with lower scores in the vitality and psychological domains. They observed that other treatments did not share a similar influence on the QoL domains. However, our study findings indicated that treatments such as IUI, IVF, and ICSI had comparable impacts on the QoL domains.

Our findings indicated that QoL scores were higher in infertile couples with shorter durations of infertility and male factor infertility. Lower education status and income levels, in addition to prior unsuccessful treatments were associated with lower QoL scores. We believe that comprehensive evaluation of influencing factors on the QoL of infertile couples using a fertility-specific questionnaire might help policy makers to detect and appropriately plan for infertile couples to receive the necessary suitable economical, psychosocial, and medical supports and to increase the accessibility of the treatment modalities. We propose complete insurance coverage of infertility treatments, at least for couples who do not have any offspring, in order to decrease the economic burden. An expert psychological support team should be readily available in infertility clinics for the affected couples to increase their living-skills and sense of satisfaction with life.

Conclusion

Infertile couples with shorter duration of infertility and male etiology have higher QoL. Lower academic education, lower income levels, or prior unsuccessful treatments are associated with lower QoL.

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Author's Contribution

B.N.J., M.M., S.F., T.P.; Contributed in the study design, data collection, data analysis and interpretation. A.S.; Contributed in the study design, data analysis and interpretation. All authors participated in the critical revision and approval of the final draft before submission also they all approved the final manuscript before publication.

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Original Article

The Effects of Exercise on Expression of CYP19 and StAR mRNA in Steroid-Induced Polycystic Ovaries of Female Rats

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Abstract

Background: Polycystic ovarian syndrome (PCOS) is the most frequent female endocrine disorder that affects 5-10% of women. PCOS is characterized by hyperandrogenism, oligo-/anovulation, and polycystic ovaries. The aim of the present research is to evaluate the expression of steroidogenic acute regulatory protein (StAR) and aromatase (CYP19) mRNA in the ovaries of an estradiol valerate (EV)-induced PCOS rat model, and the effect of treadmill and running wheel (voluntary) exercise on these parameters.

Materials and Methods: In this experimental study, we divided adult female Wistar rats that weighed approximately 220 ± 20 g initially into control (n=10) and PCOS (n=30). Subsequently, PCOS group were divided to PCOS, PCOS with treadmill exercise (P-ExT), and PCOS with running wheel exercise (P-ExR) groups (n=10 per group). The expressions of StAR and CYP19 mRNA in the ovaries were determined by quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). Data were analyzed by one-way ANOVA using SPSS software, version 16. The data were assessed at α=0.05.

Results: There was significantly lower mRNA expression of CYP19 in the EV-induced PCOS, running wheel and treadmill exercise rats compared to the control group (P<0.001). Treadmill exercise (P=0.972) and running wheel exercise (P=0.839) had no significant effects on CYP19 mRNA expression compared to the PCOS group. mRNA expression of StAR in the ovaries of the PCOS group indicated an increasing trend compared to the control group, however this was not statistically significant (P=0.810). We observed that 8 weeks of running wheel and treadmill exercises could not statistically decrease StAR mRNA expression compared to the PCOS group (P=0.632).

Conclusion: EV-induced PCOS in rats decreased CYP19 mRNA expression, but had no effect on StAR mRNA expression. We demonstrated that running wheel and moderate treadmill exercise could not modify CYP19 and StAR mRNA expressions.

Keywords: Cytochrome P450 Family 19, Valerate, Exercise, Polycystic Ovarian Syndrome, Steroidogenic Acute Regulatory Protein

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Introduction

Polycystic ovarian syndrome (PCOS) is a common endocrine disorder in premenopausal women characterized by ovulatory dysfunction, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovarian morphology (1, 2). The clinical manifestations of PCOS include menstrual abnormalities, hirsutism, acne, and anovulatory infertility (3, 4). Although PCOS is a prevalent disease among reproductive-aged women, the etiology of this disorder remains elusive. The PCOS ovaries are enlarged bilaterally. Follicular development arrests at the stage where the selection of the dominant follicle should normally happen. As a result, a large number of small antral follicles (4-7 mm diameter) gather in the ovaries of PCOS women; rarely, a dominant follicle presents for ovulation. The mechanism responsible for arrested follicular growth in PCOS is undiscovered (1, 5). Several causes have been attributed to this defect. The majority of evidence points to the high plasma free testosterone levels and low levels of estradiol in PCOS ovaries, which appears to result from dysregulation of steroidogenesis (6). Estradiol concentrations were low in small antral follicles and began to increase in some of the 7 mm follicles of control women (7). A healthy follicle 8 mm in diameter efficiently converts androstenedione to estradiol. Conversely, atretic and/or cystic follicles have a high androstenedione to estradiol ratio. Aromatization of androgen to estradiol in dominant follicles is conducted in the granulosa cells (GC) by aromatase enzyme (8, 9).

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Aromatase (CYP19) is a key steroidogenic enzyme that separately catalyzes the conversion of testosterone and androstenedione to estradiol and estrone. Aromatase is encoded by the CYP19 gene located on the long arm of chromosome 15 at position 15q21.1. Presumably, the increase in P450arom expression and estradiol production occurs in follicles that would have become large dominant follicles. Some investigations indicate that the gene which codes for CYP19 can be included as a major determinant of risk for PCOS (10). It has been assumed that GCs obtained from medium-sized follicles of women with PCOS generate very little estrogen. Therefore, estradiol production is low in PCOS follicles because of the lack of mature follicle development that produces a large amount of estradiol to increase P450arom mRNA expression (11, 12).

Hyperandrogenism is the central feature of PCOS. A chronic excessive androgen of ovarian and/or adrenal origin can result from a widespread biochemical feature such as hyperinsulinemia and hyperandrogenism in PCOS women. Intra-ovarian hyperandrogenism may be causatively linked with anovulation in PCOS. Some or all patients with PCOS may have an intrinsic, and possibly primary, abnormality of androgen biosynthesis (3, 4). One feasible cause for such an abnormality is the production of the steroidogenic acute regulatory protein (StAR). StAR mRNA has first been detected in mouse embryos and in the adrenal cortex, ovaries, testis, and kidneys (13, 14). This gene encodes StAR that plays a pivotal role by raising the conversion of cholesterol into pregnenolone. This initiates the process of steroidogenesis by facilitating the delivery of cholesterol from the outer mitochondrial membranes to the inner mitochondrial membranes of the cell. StAR has a crucial role in the regulation of steroidogenesis (15).

Physiological changes in the synthesis of steroid hormones are closely linked with alterations in StAR expression. It has been demonstrated in the ovary that StAR expression highly correlates with steroidogenic activity, because the increased production or concentration of StAR may result in abnormal steroidogenesis found in PCOS (16). Pharmacological intervention and lifestyle modification are considered treatments for PCOS and normalize hyperandrogenism and anovulation. Lifestyle intervention in PCOS includes dietary intervention and physical activity (17, 18). The most preferred, effective treatment of PCOS is physical activity. It is a key component of any lifestyle modification. Moderate regular exercise undoubtedly affects fertility and assisted reproductive technology (ART) outcomes (19, 20). Physical activity and exercise lead to fluctuation in hormone levels. Numerous evidence shows that exercise can modify body weight, improved ovulatory function, circulating androgen levels, and insulin sensitivity in women with PCOS (21). There are no studies, to our knowledge, that have assessed the effects of 8 weeks of moderate intensity (28 m/minute, 0% grade) treadmill exercise and running wheel exercise (voluntary) on StAR and CYP19 mRNA expressions in rats with PCOS. In order to test this hypothesis, we examined the ovaries from rats with PCOS to evaluate the effects of exercise on weight changes and expression of relative concentrations of StAR and CYP19 mRNA in the estradiol valerate (EV)-induced PCOS rat model.

Materials and Methods

Animals and protocols

In this experimental study used 40 female Wistar rats (220-240 g) acquired from Shahid Beheshti University (Iran). The rats were kept at the animal house under standard laboratory conditions (12 hour light/12 hour dark cycles and controlled temperature of 21-22ºC and 55-65% relative humidity with free access to food pellets and tap water. In order to conduct a comparative evaluation, we divided the rats into two group of 10 animals per group: control group which not receive any injection (n=10) or other manipulations and an experimental PCOS group (n=30) that received one injection of 4 mg/kg body weight EV dissolved in 0.2 ml olive oil. We recorded the weight and fertility for all of the rats. At 60 days past the EV injection, rats in PCOS group were randomly divided into three groups of 10 animals per group: PCOS (n=10), PCOS plus exercise on a treadmill (P-ExT, n=10) and PCOS plus exercise on a running wheel (P-ExR, n=10). The treatment lasted for 8 weeks. Rats in the control and PCOS groups did not participate in any exercise program. At 8 weeks after the intervention, we measured their estrous cycles daily for 21 consecutive days. The Ethics Committee at the Neuroscience Research Center of Shahid Beheshti University of Medical Sciences (Tehran, Iran) approved the study procedures.

Physical exercise

Chronic voluntary exercise (running wheel)

The P-ExR rats were placed in the wheel running device for 4 hours/day with free access to water and no food. This exercise was voluntary in nature compared to the forced treadmill exercise. Running wheel distance was documented daily and we measured their body weights two times per week. This group included 10 rats that ran a total distance of 1200 m within a period of 8 weeks.

Physical exercise on a treadmill

The P-ExT rats were placed on the motorized treadmill for 5 days to walk for 10 minutes at 5 m/minute for training purposes. The treadmill training was performed between 8:00 and 12:00 daily. We did not change the grid during exercise. The training group was given exercise training for five days/week for 8 weeks. Each session began with 12 m/minute for 5 minutes to prepare the rats for the main training session. In the first week, the rats were

trained on the treadmill at 10 m/minute, with a running time of 10 minutes/day. In the second week, we increased the speed to 15 m/minutes for 30 minutes/day. In the third week, the speed was increased to 20 m/minutes for 45 minutes/day. In the fourth week, the speed was increased at 28 m/minutes and increased the duration to 60 minutes/day (Table 1). For the last 4 weeks, we kept the speed and duration constant. This condition corresponded to a moderate intensity of approximately 65-70% maximal oxygen consumption (22).

Table 1: The treadmill exercise program

Week	Duration (minutes)	Speed (m/minute)
1	10	10
2	30	15
3	45	20
4	60	28
5	60	28
6	60	28
7	60	28
8	60	28

Quantitative real-time reverse transcriptase polymerase chain reaction

After anesthesia, we immediately removed the rats' ovaries and froze them in liquid nitrogen. We used 5 ovaries from each group for quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR). The tissue samples were stored at -80°C until RNA extraction.

RNA extraction

Total RNA was extracted using pureZol RNA isolation reagent according to the manufacturer's instructions (Bio-rad, USA). The quantity and purity of RNA were measured by a nanodrop spectrophotometer (NanoDrop-1000, Thermo Scientific, USA) with an OD260 to calculate the concentration and 260/280 to assess sample purity.

cDNA synthesis

We determined that 5 µg of RNA was the final concentration needed to synthesis cDNA. If the concentration of RNA was 2000 ng in 1 µl, we used 2.5 µl RNA for the 5 µg RNA concentration. After DNase treatment, 5 µg of total RNA was reversed to cDNA by RevertAid reverse transcriptase (M-MuLV RT, 1 µL), random hexamer primers (1 µl), dNTPs (2 µL), and RiboLock RNase-inhibitor (0.25 µL) for 10 minutes at 25°C, followed by 60 minutes at 42°C in a final volume of 20 µL. The reaction was terminated by heating at 70°C for 5 minutes.

Real-time polymerase chain reaction

We designed the real-time PCR primers according to the primer-BLAST tool (Table 2). The primers were synthesized by Bioneer Company (Republic of Korea). Real-time PCR was performed in duplicate for each sample on a Corbett Rotor-Gene 6000 (Adelaide, Australia). In each reaction, 1 µL of cDNA, 0.4 µL of forward and reverse primers, and 7.5 µL of SYBR Green PCR Master Mix 2X (Ampliqon, Denmark) were added with RNase-free water to make a final volume of 20 µL. All primers were used at an optimized concentration of 50 µM. Real-time PCR was performed in three steps: i. Initial denaturation (10 minutes at 95°C); ii. A three-step quantification and amplification program (15 seconds at 95°C followed by 20 seconds at 56°C and 40 seconds at 72°C) for 40 cycles; and iii. Melting curve (5 minutes at 72°C). Reactions with no template were included as negative controls, which showed no evidence of product amplification or primer dimers. The specificity of the real-time PCR reactions was verified by the generation of a melting curve analysis. The target genes were normalized with the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The relative mRNA level of each target gene was calculated by the $2^{-\Delta\Delta CT}$ method, where Δ_{CT} (cycle threshold)=CT of target gene-CT of housekeeping and ΔΔCT= ΔCT of the target gene in the FH group-ΔCT of the target gene in the control group.

Statistical analysis

Data were expressed as mean ± SD. All the data were analyzed using one-way ANOVA followed by post hoc Tukey's test using the SPSS software (version 16). In all cases, $P<0.05$ was considered significant.

Table 2: Primers used for quantitative real-time polymerase chain reaction

Primer name	Primer sequence (5′→3′)	Product length (bp)	Gene bankaccession no.
CYP19	F: CGTCATGTTGCTTCTCATCG R: TACCGCAGGCTCTCGTTAAT	100	NM_017085.2
StAR	F: GCCTGAGCAAAGCGGTGTC R: CTGGCGAACTCTATCTGGGTCTGT	100	NM_031558.3
GAPDH	F: TGCCGCCTGGAGAAACCTGC R: TGAGAGCAATGCCAGCCCCA	172	NM_017008.4

Results

Effect of exercise on body weight

The body weight of rats from the PCOS group increased significantly compared to the control and exercise groups. At the end of the experiment, the body weights of the exercise groups were meaningfully less compared to the PCOS group (P<0.001) (Table 3). There was a substantial effect by the EV injection on the weights of the ovaries, while there was no effect on exercise. The ovaries of both the PCOS and the PCOS exercise groups weighed less than the ovaries of the control group.

Table 3: Effects of the treadmill and running wheel exercises on body and ovarian weights

Variable	Control	PCOS	P-ExT	P-ExR
Body weight (g)	239.8 ± 2.48	255 ± 2.99	210.3 ± 1.87[a]	213.7 ± 1.71[a]
Ovary weight (mg)	84.5 ± 1.33	40.3 ± 1.54[b]	42.8 ± 0.9[b]	40.0 ± 1.18[b]

Significant differences are indicated by letters. Data are mean ± SEM, P<0.05, n=10 in each group. [a]; Compared to PCOS group, [b]; Compared to control group, PCOS; Polycystic ovarian syndrome, P-ExT; PCOS rats that exercised on a treadmill, and P-ExR; PCOS rats that exercised on a running wheel.

Steroidogenic acute regulatory protein (StAR) and CYP19 mRNA expressions

Ovarian expression of aromatase (CYP19)

There was significantly lower mRNA expression of *CYP19* in the EV-induced PCOS, P-ExR, and P-ExT rats compared to the control group (P<0.001). There was no significant effect of treadmill exercise (P=0.972) or running wheel exercise (P=0.839) on CYP19 mRNA expression compared to the PCOS group (Fig.1).

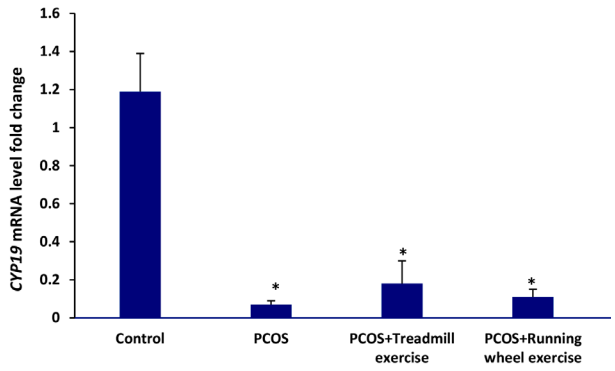

Fig.1: Effect of exercise (running wheel and treadmill) on the expression of ovarian aromatase (*CYP19*) mRNA in the polycystic ovarian syndrome (PCOS) group in estradiol valerate (EV)-induced PCOS rats. Values are mean ± SD. *; P<0.05 vs. control (Tukey post-hoc).

Ovarian expression of steroidogenic acute regulatory protein (StAR)

The PCOS group had a higher level of *StAR* mRNA expression compared to the control group although this finding was not statistically significant (P=0.810). The 8 weeks of running wheel and treadmill exercises could not statistically decrease StAR mRNA expression compared to the PCOS group (P=0.632) (Fig.2).

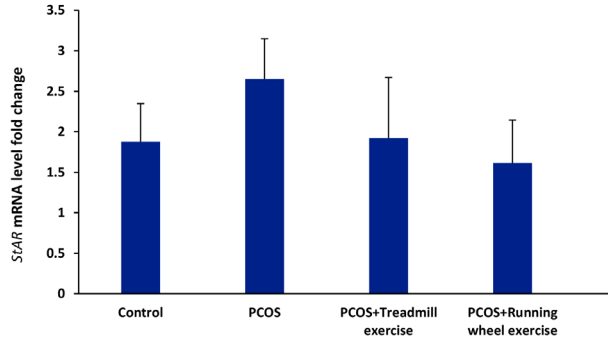

Fig.2: Effect of exercise (running wheel and treadmill) on the expression of ovarian steroidogenic acute regulatory protein (*StAR*) mRNA in the polycystic ovarian syndrome (PCOS) group in estradiol valerate (EV)-induced PCOS rats. Values are mean ± SD.

Discussion

PCOS is a term for unexplained hyperandrogenism associated with variable degrees of cutaneous and anovulatory symptoms, and obesity (23, 24). In PCOS, follicular development arrests at the stage when the GCs of the growing follicles would normally begin to express the aromatase enzyme and secrete estradiol. However, several abnormalities of PCOS include a significant increase in androgens and luteinizing hormone (LH), and reduced follicle-stimulating hormone (FSH) (25). In PCOS, hypersecretion of LH leads to excessive androgen levels. On the other hand, FSH is vital for maturing follicles through FSH receptors (FSHRs) in GC of antral follicles which contributes to aromatase transcription (26).

In PCOS, the expression of FSHR in GC appears to be up-regulated (27), which is believed to be responsible for the observed hyper-responsiveness of PCOS GC to FSH both *in vitro* and *in vivo* (28, 29). Although FSH hypersensitivity hastens estrogen production, PCOS women are unable to sustain their estrogen levels. One of the reasons for insufficient production of estradiol in PCOS follicles is the inadequate amount of aromatase to stimulate bioactivity and increase aromatase mRNA expression (28). Takayama et al. (30) reported that immunohistochemical studies of polycystic ovaries did not show any aromatase activity in antral follicles of various sizes. Erickso et al. (12) demonstrated that GC obtained from medium-sized follicles of women with PCOS had little aromatase activity. Similarly, Jakimiuk et al. (11) showed that when compared to the control follicles, all PCOS follicles contained low levels of P450arom mRNA estradiol and lower aromatase stimulating bioactivity.

Yang et al. (31) reported that freshly isolated GC from luteinized follicles in PCOS had significantly reduced aromatase mRNA and protein. The results of this study have supported that testosterone is a key factor responsible for down-regulation of aromatase in PCOS. At the average level in small follicles in PCOS patients, testosterone down-regulated both mRNA and protein levels of aromatase in cultured non-PCOS luteinized GC. Consistent with previous studies, this research demonstrated that decreased levels of P450arom mRNA in PCOS rats com-

pared to the control group. The 8 weeks of running wheel and treadmill exercises could not increase CYP19 mRNA expression. When LH affects its receptor on the theca cell, StAR initiates the process of steroidogenesis by transporting cholesterol from the outer to the inner mitochondrial membrane (32, 33).

StAR has a crucial role in binding to cholesterol. It is the first and rate limiting step of steroidogenesis, hence, it has been considered a major candidate for high levels of steroid hormones in PCOS patients (14, 34). Jakubowski (35) reported that StAR was one of the candidate genes involved in PCOS. Kahsar-Miller et al. (15) revealed that alteration in the StAR gene could cause PCOS as a reason in the earliest steps of androgen biosynthesis. They observed that the relative concentration and distribution of StAR in the PCOS ovary did not differ significantly from the normal ovary. However, they reported that the StAR protein was located primarily in the thecal and GC of follicular cysts. The StAR protein content in the entire ovary as determined by Western blot analysis tended to be greater in patients with PCOS than controls.

Wickenheisser et al. (36) demonstrated that StAR promoter activity was similar in PCOS and normal control cells. There were similar levels of StAR mRNA in both PCOS and normal theca cells. Nelson et al. (37) did not observe elevated StAR mRNA expression in PCOS compared to normal cells. Urbanek et al. (38) reported no association between PCOS or hyperandrogenemia and the StAR locus. In accordance with the previous studies, mRNA expression of StAR in the ovary of the PCOS group was not very different, though partially elevated, compared to the control group. However, the treadmill and running wheel exercises failed to significantly affect StAR mRNA expression.

Intervention studies to see if exercise can modify StAR and CYP19 mRNA expression in women with PCOS are lacking both in human and animal, as the study in animal models. There is no study about the effect of exercise on StAR and CYP19 mRNA expression in PCOS. Knowler et al. (39) have recently demonstrated that a 24-week training period that included 30 minutes bicycling three times a week in PCOS women resulted in a significant decrease in fasting insulin and insulin resistance. Vigorito et al. (40) reported that bicycling for 40 minutes 3 times a week for 3 months in obese patients with PCOS led to an improvement in insulin sensitivity. Qiu et al. (41) demonstrated that 2 weeks of swimming in the testosterone propionate-induced PCOS rat improved insulin sensitivity, decreased serum androgen levels, and recovered normal ovarian morphology. Homa et al. (42) reported that voluntary exercise improved estrous cycle in prenatally-androgenized female mice.

Conclusion

The current study findings have indicated that EV-induced PCOS in rats is linked to decreased CYP19 mRNA expression with no effect on StAR mRNA expression. We have determined that voluntary exercise (running wheel exercise) and moderate treadmill exercise could not alter CYP19 mRNA and StAR mRNA expressions. However, we only measured two genes; hence, future studies could survey other genes considered vital in the steroidogenesis pathway in PCOS. It seemed that exercise might affect PCOS via another pathway. This investigation has provided a new way to investigate the effect of exercise on expression of critical genes in PCOS.

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Author's Contributions

F.A.; Experimental design, data collection and evaluation. H.Kh.; Providing materials for experiment and contributed extensively in interpretation of the data and the conclusion. M.H.; Data analysis and approving the final version of this paper for submission. M.H.; Data analysis. A.A.; Scientific consulor.

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Original Article

Congenital Malformations in Singleton Infants Conceived by Assisted Reproductive Technologies and Singleton Infants by Natural Conception in Tehran, Iran

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Abstract

Background: Multiple pregnancies occur more frequently in assisted reproductive technology (ART) compared to normal conception (NC). It is known that the risk of congenital malformations in a multiple pregnancy are higher than single pregnancy. The aim of this study is to compare congenital malformations in singleton infants conceived by ART to singleton infants conceived naturally.

Materials and Methods: In this historical cohort study, we performed a historical cohort study of major congenital malformations (MCM) in 820 singleton births from January 2012 to December 2014. The data for this analysis were derived from Tehran's ART linked data file. The risk of congenital malformations was compared in 164 ART infants and 656 NC infants. We performed multiple logistic regression analyses for the independent association of ART on each outcome.

Results: We found 40 infants with MCM 29 (4.4%) NC infants and 14 (8.3%) ART infants. In comparison with NC infants, ART infants had a significant 2-fold increased risk of MCM (P=0.046). After adjusting individually for maternal age, infant gender, prior stillbirth, mother's history of spontaneous abortion, and type of delivery, we did not find any difference in risk. In this study the majority (95.1%) of all infants were normal but 4.9% of infants had at least one MCM. We found a difference in risk of MCMs between *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). We excluded the possible role of genotype and other unknown factors in causing more malformations in ART infants.

Conclusion: This study reported a higher risk of MCMs in ART singleton infants than in NC singleton infants. Congenital heart disease, developmental dysplasia of the hip (DDH), and urogenital malformations were the most reported major malformations in singleton ART infants according to organ and system classification.

Keywords: Assisted Reproductive Technology, Congenital Malformations, Embryo Transfer, *In Vitro* Fertilization, Sperm Injections, Embryo Transfer

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Introduction

A highly contested subject exists for assisted reproductive technologies (ART) and congenital malformations in infants (1). The higher risk of congenital malformations in ART infants in comparison with infants from normal conception (NC) is one of the greatest concerns for these children (2). The authors of the present paper have previously assessed 400 ART infants for the incidence of major congenital malformations (MCM) without a control group. We determined a 7% frequency of disorders, which was 2-3% higher than infants in the public population. There was no significant difference between two groups of intracytoplasmic sperm injection (ICSI) and *in vitro* fertilization (IVF) in that study (P=0.08) (3). In our previous study, 309 (43%) infants were from multiple pregnancies. There

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was a greater risk of congenital malformations in the multiple pregnancies compared to the single pregnancies (4). The incidence of congenital malformations in ART infants compared with NC infants in a number of studies showed a rate of congenital malformations in ART infants 2 times higher than the control group (5, 6). There was no difference observed between ICSI and IVF in the incidence of congenital malformations (7-9).

Others reported no difference in a comparison between ART infants and NC infants (1, 5, 10-12). Unlike the above studies, some studies were solely carried out to determine the differences in the incidence of congenital malformations in single ART infants compared to single NC infants. This group of studies reported a greater incidence of congenital malformations in ART infants compared to NC infants (5, 13-15). The incidence of congenital malformations was equal between ICSI and IVF groups in some studies (3-5, 7-9, 11, 13). Although, in one paper, there were more congenital malformations in the ICSI group (1.58%) compared to the IVF group (1.11%, P=0.052) (12). The contradictory results mentioned in the above articles have led us to study the presence of MCM in single ART infants and compare the results with single NC infants. In addition, we compared the incidence of MCM between ICSI and IVF infants.

Materials and Methods

This was a historical cohort study of MCM in 820 births from January 2012 to December 2014. We compared the incidence of MCMs among 168 ART infants (exposed group) to 652 NC infants (non-exposed group). We assessed approximately 4 NC infants for each ART infant. This retrospective record linkage cohort study used the following data set. The ART database (exposed group) was obtained from the Child Health and Development Research Center (CHDRC) which is a subset of the Iranian Academic Center for Education, Culture, and Research (ACECR). All mothers underwent treatment at Royan Institute for Reproductive Biomedicine (RI-RB). The exposed and unexposed infant data were gathered from CHDRC. We have defined MCM according to the International Classification of Disease-11 (ICD-11).

In Tehran, the CHDRC is the center which issues health certificates for children from birth until 16 years of age. Hence, numerous infants from various districts of Tehran voluntary are referred to this center in order to obtain full visiting rights and are followed for several years. We obtained demographic information and the results from two visits that included infant's sex, mother's age, reproductive technology, mothers' history of stillbirth and abortion, type of delivery, and complete medical records. The inclusion criteria consisted of infants followed by CHDRC after two examinations at the center (during the first 6 months of age and between 6 and 18 months of age); no major genetic disease in the infant's family history; no exposure to X-ray radiation; no abdominal trauma during pregnancy; resident of Tehran; first born; singleton child; no drug or medicine usage by the mother during pregnancy; and no parental family relationships.

Statistical analysis

We used descriptive statistics to determine the prevalence of MCMs in both the singleton ART and NC groups. Multiple logistic regression analyses (backward model) with SPSS-21 software were used to estimate the odds ratio (OR) with 95% confidence interval (CI) to establish a relationship between ART for each outcome. Independent variables consisted of type of delivery, infant's sex, mother's age, reproductive technology, prior stillbirth, and history of spontaneous abortion. We entered mothers' age, mother's history of stillbirth and abortion, and type of delivery to the model to determine if these were confounding factors. For all of the mentioned outcomes, we performed stratified analyses to control for the bias of confounding effects. In the present study, all MCM both groups were assessed according to ICD-11 criteria (i.e., infants that needed surgery up to the age of 1 year and developed a defect in organ function). The Research Ethics Committee of ACECR and Royan Institute Institutional Review Board approved this study.

Results

Of 820 singleton infants identified, we selected 168 ART infants (exposed group) and 652 NC infants (control, non-exposed group) from the CHDR Center during 2012 to 2014. The prevalence rate of MCM in singleton ART and singleton NC groups, a comparison of MCM between the exposed and unexposed singleton infants, and a separate comparison of MCM for IVF and ICSI singleton infants (Table 1). This table shows the variables as maternal age and infant's sex in ART infants compared with NC infants. There were no statistically significant differences in the rate of malformations for age groups and infant's sex. In the two groups, NC mothers had an average age of 28 years (28.6 ± 4.4); while for ART mothers it was 31.2 years (31.2 ± 4.8). There were 51% boys and 49% girls in both groups. According to ICD-11, hypospadias, inguinal hernia, severe PDA+VSD, stenosis of the lacrimal duct until age one year, urethral reflux more than grade 3, hydronephrosis, undescended testis (until one year of age), severe amblyopia, club foot, developmental dysplasia of the hip (DDH) that required surgery, and rickets resistant to conventional therapy were considered major malformations. MCM analysed according to specific risk factors in both singleton ART and singleton NC infants (Table 1), in addition to MCM for IVF and ICSI.

MCM analysed according to specific risk factors in both singleton ART and singleton NC infants (Table

2). We found a nonsignificant, increased risk of MCM (P=0.052, 95% CI: 1.01-3.78). When we entered confounding factors-history of abortion during pregnancy, prior stillbirths, and delivery methods in the both univariate and multivariate models, the effects on significance and risk of MCM was P=0.047 with a 95% CI of 1.01-3.78. We sorted the prevalence of MCM in both groups of infants (Table 3).

Table 1: The effects of demographic and other variables on quality of life (QoL) in infertile couples

Variable	NC	ART			Total
		ART	ICSI	IVF	
	n (%)	n (%)	n (%)	n (%)	n (%)
All infants	652 (80)	168 (20)	134 (15.9)	34 (4.1)	820 (100)
Maternal age (Y)					
<35	531 (81.4)	124 (73.8)	100 (74.6)	24 (70.6)	655 (79.4)
>35	121 (18.6)	44 (26.2)	34 (25.4)	10 (29.4)	165 (20.6)
Delivery					
Normal	110 (16.8)	5 (3)	3 (2.2)	2 (5.9)	115 (14)
Cesarean	542 (83.2)	163 (97)	131 (97.8)	32 (94.1)	705 (86)
Sex					
Boy	337 (51.7)	81 (48.2)	68 (50.7)	13 (38.3)	418 (51)
Girl	315 (48.3)	87 (51.8)	66 (49.3)	21 (61.7)	402 (49)
History of abortion					
No	542 (83.1)	23 (13.7)	12 (9)	11 (32.4)	565 (69)
≥1	110 (16.9)	145 (86.3)	122 (91)	23 (67.6)	255 (31)
History of stillbirth					
No	644 (98.8)	164 (97.6)	132 (98.5)	32 (94.1)	808 (98.5)
≥1	8 (1.2)	4 (2.4)	2 (1.5)	2 (5.9)	12 (1.5)
Major congenital malformations (MCM)					
No	623 (95.6)	154 (91.6)	124 (92.5)	30 (88)	777 (94.8)
Yes	29 (4.4)	14 (8.4)	10 (7.5)	4 (12)	43 (5.2)

ICSI; Intracytoplasmic sperm injection, ART: Assisted reproducive technologies, and IVF: *In vitro* fertilization.

Table 2: Rate of major congenital malformations (MCM) compared in singleton assisted reproductive technology (ART) and singleton normal conception (NC) infants

Variable	MCM		OR (95% CI) (Crude)	P value (Crude)	OR (95% CI) (Adjusted)*	P value (Adjusted)
	No	Yes				
Reproductive technology				0.059		0.047
Normal	623 (95.6%)	29 (4.4%)	Reference		Reference	
ART	154 (91.7%)	14 (8.3%)	1.89 (0.98-3.66)		1.89 (1.01-3.66)	
Sex				0.87		0.79
Boy	397 (95%)	21 (5%)	Reference		Reference	
Girl	380 (94.5%)	22 (5.5%)	1.09 (0.59-2.02)		1.08 (0.58-2.01)	
Maternal age (Years)				0.56		0.73
<35	622 (95 %)	33 (5%)	Reference		Reference	
≥35	155 (93.9%)	10 (6.1%)	1.22 (0.59-2.52)		1.14 (0.54-2.37)	
History of stillbirth				0.48		0.74
No	766 (94.8%)	42 (5.2%)	Reference		Reference	
≥1	11 (91.7%)	1 (8.3%)	1.96 (0.21-13.15)		1.41 (0.17-11.38)	
History of abortion				0.129		0.65
No	540 (95.6%)	25 (4.4%)	Reference		Reference	
≥1	237 (92.9%)	18 (7.1%)	1.64 (0.88-3.06)		1.21 (0.53-2.76)	
Delivery				0.25		0.34
Normal	107 (97.3%)	3 (2.7%)	Reference		Reference	
Cesarean	670 (94.4%)	40 (5.6%)	2.13 (0.65-7.01)		1.80 (0.53-6.04)	

***; Adjusted for all variables in Table 1. OR; Odds ratio and CI; Confidence interval.**

Table 3: Prevalence of major congenital malformations (MCM) in singleton assisted reproductive technology (ART) and singleton normal conception (NC) infants

Reproductive technologies	NC	ART
Disease	**n (%)**	**n (%)**
Congenital heart disease (PDA+VSD)	-	3 (1.9)
Developmental dysplasia of the hip (DDH)	-	2 (1.2)
Urethral stenosis	5 (0.8)	2 (1.2)
Hypospadias	6 (0.9)	1 (0.6)
Undescended testis	6 (0.9)	2 (1.2)
Lacrimal duct stenosis	6 (0.9)	1 (0.6)
Fusion labia	2 (0.3)	-
Craniosynostosis	-	2 (1.2)
Hydronephrosis+urethral reflux	-	1 (0.6)
Cleft lip and palate	-	1 (0.6)
Hermaphroditism	2 (0.3)	-
Down syndrome	1 (0.1)	-
Club foot	1 (0.1)	-
Total malformations	29 (4.4)	15 (1.82)
Total infants	652 (79.5)	168 (20.5)

Discussion

Approximately 20% of pregnant women, were 35 years or older, which was relatively similar in both the ART and NC groups. However, between the two IVF and ICSI groups, there were more mothers aged 35 years or older in the IVF group. In terms of method of delivery, 86% had a cesarean section, which was elevated in the control group. In the present study, there were 14 out of 168 (8.3%) ART infants and 29 out of 652 (4.4%) NC infants with MCM. This implied that the number of congenital disorders in singleton ART infants was two times that of singleton NC infants, however this finding was not statistically significant in univariate analysis (OR=1.95, 95% CI: 1.01-3.78, P=0.052). Analysis of the confounding factors-history of abortion, prior stillbirths, and delivery methods according to univariate and multivariate models showed a significance level of P=0.047 and 95% CI: 1.01-3.78 for the risk of MCM. The above findings supported the results of four review studies and meta-analysis until 2013 with regards to single ART infants. These four papers reported higher numbers of major congenital disorders in single ART infants compared with single NC infants (4, 8, 9, 15).

Studies reported a two-fold greater risk of emergence of congenital disorders in single ART infants compared with single NC infants (5, 6, 14, 15). The above findings indicated that the possibility of major congenital disorders in single ART infants was higher than single NC infants. In contrast, Moses et al. (1), Yan et al. (12), and Bassiouny et al. (10) did not report any significant difference between control and single ART groups. In the present study, 8.9% of all single ART infants had one major congenital disorder. The ratio of ICSI to IVF was twice (8.2 vs. 32.6%) which indicated a significant difference (P=0.047). The majority of studies in this field did not report any significant differences between single ICSI and single IVF in terms of congenital disorders (4, 8, 9). In a study by Yan et al. (12) there were 1.58% congenital disorders in the ICSI group compared to 1.11% in the IVF group, which showed a significant difference (P=0.052, OR=1.42, 95% CI: 0.99-2.03) (12). The history of abortion in both groups of ART mothers (89.6%) was much higher than NC mothers (17.1%). There was no significant difference between the two ART groups in history of abortion (P=0.89). After adjustments for maternal age (ART mothers become pregnant approximately 5 years later than NC mothers) and infant's sex, stillbirth, abortion, and type of delivery, we found no difference in risk (OR=1.95, P=0.047, 95% CI: 1.01-3.78).

In the current study, the most common disorders in single ART infants were congenital cardiac diseases (1.9%), genitourinary system disorders (3.5%), DDH (1.2%), lacrimal duct obstruction (0.6%), craniosynostosis (1.2%), and lip and palate cleft (0.6%). The most prevalent disorders were related to the cardiovascular and urogenital systems. In NC infants, the most common disorders were related to the urogenital system and lacrimal duct obstruction. In our previous study with ART infants, we observed a higher frequency with congenital cardiac diseases, urogenital system disorders, and musculoskeletal disorders (3).

The prevalence of systems and organs involved in congenital disorders varied amongst different studies. Perhaps the reasons for these differences were due to careful and continuous examinations, differences in the numbers of cases, and use of different equipment for examinations and diagnoses (i.e., kidney or brain sonography). Yan et al. (12) reported that the cardiovascular system (0.29%), central nervous system (0.2%), and limb disorders (0.13%) were the most common involved sites, whereas Hansen et al. (5) stated that musculoskeletal disorders (3.8%), urogenital disorders (2.7%), and cardiovascular disorders (1.3%) were the most frequently observed. Midrio et al. (2) reported a high prevalence of anorectal disorders (OR=13.3, 95% CI: 4-39.6) in ART infants.

Conclusion

Some studies reported a slightly increased risk of MCM in ART infants. We have observed a higher risk of MCM in ART singleton infants compared to NC. In singleton ART infants, congenital heart disease, DDH, and urogenital malformations were the most commonly observed MCMs. More cardiovascular and endocrine malformations were observed in singleton ART infants compared to singleton NC infants. Therefore, we recommend that ART infants should undergo more precise examinations with regards to the above body systems.

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Author's Contributions

M.F.; Designed and performed experiments, contributed substantially to the conception and design of the study, drafted or provided critical revision of the article, co-wrote the paper, the acquisition and analysis of the data, interpretation, provide final approval of the version to publish. R.M.K., S.A.Sh.F.; Designed and performed experiments, contributed in the research, drafted or provided critical revision of the article and co-wrote the paper, provide final approval of the version to publish. P.B.; Drafted or provided critical revision of the article, interpretation and co-wrote the paper, provide final approval of the version to publish. M.A., A.V.T.D.; Designed and performed experiments, contributed in the research, agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work, co-wrote the paper, provide final approval of the version to publish.

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Original Article

Comparison of *In Vitro* Fertilization/Intracytoplasmic Sperm Injection Cycle Outcome in Patients with and without Polycystic Ovary Syndrome: A Modified Poisson Regression Model

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Abstract

Background: Polycystic ovary syndrome (PCOS) is a frequent condition in reproductive age women with a prevalence rate of 5-10%. This study intends to determine the relationship between PCOS and the outcome of assisted reproductive treatment (ART) in Tehran, Iran.

Materials and Methods: In this historical cohort study, we included 996 infertile women who referred to Royan Institute (Tehran, Iran) between January 2012 and December 2013. PCOS, as the main variable, and other potential confounder variables were gathered. Modified Poisson Regression was used for data analysis. Stata software, version 13 was used for all statistical analyses.

Results: Unadjusted analysis showed a significantly lower risk for failure in PCOS cases compared to cases without PCOS [risk ratio (RR): 0.79, 95% confidence intervals (CI): 0.66-0.95, P=0.014]. After adjusting for the confounder variables, there was no difference between risk of non-pregnancy in women with and without PCOS (RR: 0.87, 95% CI: 0.72-1.05, P=0.15). Significant predictors of the ART outcome included the treatment protocol type, numbers of embryos transferred (grades A and AB), numbers of injected ampules, and age.

Conclusion: The results obtained from this model showed no difference between patients with and without PCOS according to the risk for non-pregnancy. Therefore, other factors might affect conception in PCOS patients.

Keywords: Intracytoplasmic Sperm Injection, *In Vitro* Fertilization, Polycystic Ovary Syndrome, Pregnancy

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Introduction

The International Committee for Monitoring Assisted Reproductive Technology and the World Health Organization (WHO) define infertility as the failure to achieve a clinical pregnancy for 12 months or more of regular unprotected intercourse (1-3). Infertility is recognized as one of the main public health concerns by WHO (4, 5). There are 72.4 million (low estimate: 40.2 million, high estimate: 120.6 million) currently infertile women aged 20-44 years, from which 10.98 million reside in more developed countries and 61.4 million reside in less developed countries (5). The National Infertility Study (2004-2005) has reported that Iran, as a less developed country, has a prevalence rate of current primary infertility of 3.4% and lifetime primary infertility of 24.9% (6).

The prevalence of infertility is increasing worldwide; hence, assisted reproductive technology (ART) and its success rate are critical (7). Infertility is a multi-factorial disorder with different etiologies. One cause is polycystic ovary syndrome (PCOS) (8). PCOS is one of the most frequent hormonal disorders among reproductive age women with a prevalence rate of 5-10%. PCOS consists of reproductive, metabolic, and cardiovascular components (9). The prevalence rate of PCOS in Iran has been determined as 7.1% by the National Institute of Health (NIH) definition, 11.7% by the Androgen Excess Society criteria (AES), and 14.6% according to the Rotterdam consensus (Rott) (10). PCOS is one of the prevalent causes of infertility due to anovulation (11). Approximately 90 to 95% of women who refer to infertility clinics with anovulation have PCOS (12-14). Because the etiology of PCOS is not defined, its therapy remains mostly symptomatic and empirical (15). An increased risk gestational diabetes and other pregnancy complications has been shown in women with PCOS (16). Previous studies reported no difference

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between women with and without PCOS in clinical pregnancy rate (PR) (17-19). This study aimed to determine the association between PCOS and *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) cycle outcome in infertile women who referred to Royan Institute, Tehran, Iran between January, 2012-December, 2013.

Materials and Methods

This historical cohort study consisted of infertile women who referred to Royan Institute for Reproductive Biomedicine, Stem Cell Biology and Technology Center, Tehran, Iran between January 2012 and December 2013. Patients were recruited according to their medical records in the institute's files. The study population consisted of subjects who presented with primary or secondary infertility that visited the clinics more than once and received IVF/ICSI treatment regardless of the cause of infertility.

We defined the relationship between PCOS status and clinical pregnancy based on clinical criteria. This cohort study was divided into 2 groups: women with and without PCOS. The outcome variable was clinical pregnancy, which was determined as the presence of a fetal heartbeat by ultrasound performed at 6 to 8 weeks after the last menstrual period. Diagnosis of PCOS was established based on the presence of two of the following Rott criteria: oligomenorrhea (defined by more than six cycles with a length of more than 35 days) and/or anovulation; clinical (presence of hirsutism with a score of 8 or more, persistence of acne during the third decade of life or later, or the presence of androgenic alopecia); biochemical signs of hyperandrogenism; and polycystic ovaries (PCO) visualized on ultrasound with the presence of 12 or more follicles that measured 2-9 mm in diameter in each ovary and/or ovarian volume more than 10 cm^3.

The potential confounding factors that we considered in the analysis were: treatment modality (IVF/ICSI); unusual vaginal discharge (diagnosed based on clinical symptoms and sexual history); hyperandrogenism; ART protocol (long, antagonist, short, other); history of ART cycle; age; body mass index (BMI); number of grade A embryos transferred; number of grade AB embryos transferred based on an evaluation of the number of blastomeres, blastomere symmetry, and the number of anucleated fragments; the number of injected ampules; human chorionic gonadotropin (hCG) administration day; and number of oocytes injected. These variables were recorded in the patients' medical files.

Statistical analysis

The study was designed to have 80% power to detect a 15% difference in clinical pregnancy, with two-sided alpha levels of 0.05. According to sample size calculation for independent proportions there should be a minimum of 150 participants in each group. Descriptive data was expressed as percentages, mean, and SD. First, we examined the individual association of the PCOS status and pregnancy failure by log binomial regression model; next, any potential confounder predictors were entered into the model. Convergence problems may arise with binomial regression models, therefore we modeled the outcome by Poisson Regression Model. On the other hand, use of Poisson regression tends to provide conservative results. Finally, we have used a modified Poisson regression model to estimate the risk ratios (RRs) and 95% confidence intervals (CIs) after adjustment for potential confounders. Royston fractional polynomials (20, 21) were used for all continuous measures in the model in order to examine the proper scale of variables. Stata software (version 13, Stata Corp., College Station, TX, USA) was used for all statistical analyses.

The Board of Research Ethics at Royan Institute approved this study (code: EC/89/1046). All subjects provided informed consent to participate and they received assurance that the results would be published statistically without any personal data.

Results

Out of 996 infertile women who referred to Royan Institute as a referral center for ART in Iran, 13 (1.31%) were lost to follow up and 983 cases were successfully followed. The cases appropriately followed had a PR of 35.20%. Patients underwent ultrasound imaging (sonography) at the start of the treatment process, which was administered by a radiologist. The results showed that 115 (11.72%) had PCOS. The remaining 866 (88.28%) cases did not have PCOS. The mean (SD) age of clinically pregnant women was 34.27 (4.92) years. The mean age for the non-pregnant group was 36.15 (5.43) years. The difference in the mean age was statistically significant (P=0.001). There was no meaningful difference (P=0.90) in BMI of clinically pregnant (25.54 kg/m^2, SD: 3.92) compared to non-pregnant women (25.57 kg/m^2, SD: 3.85 (Table 1).

There was no significant association between infectious discharge, stimulator drug type, hyperandrogenism, history of ART cycle, BMI, hCG administration day, the injected oocyte number, and ART cycle outcome. The results showed a negative association between age and the injected ampule number, and risk of non-pregnancy. Cases that underwent the long protocol had a better outcome. The numbers of grade A and AB embryos transferred, after adjustment for potential confounders, showed a negative association with ART cycle outcome. Fractional polynomial regression was checked for age, BMI, ampule number, hCG day, and injected oocyte number. The results indicated that the linear form was the best scale for these continuous variables. Crude analysis revealed that the risk of non-pregnancy in women who underwent ART in patients with PCOS was 0.53%; for those without PCOS, it was 0.66% (RR: 0.79, 95% CI: 0.66-0.95, P=0.014). Hence, there was a significantly lower risk of non-pregnancy in PCOS cases compared to cases without PCOS. After adjustment for confounder variables (Table 2), we observed no difference between the risk of non-pregnancy in women with and without PCOS (RR: 0.87, 95% CI: 0.72-1.05, P=0.15).

Table 1: A comparison of independent variables based on outcome status

Variable	Clinical pregnancy	Non-pregnancy	P value
PCOS¶			
No	291 (33.60)	575 (66.40)	0.006
Yes	54 (46.96)	61 (53.04)	
Stimulate medicine ¶			
HMG	29 (25.66)	84 (74.34)	0.001
Pure FSH	34 (37.78)	56 (62.22)	
Gonal F	114 (48.31)	122 (51.69)	
HMG+Gonal F	121 (28.34)	306 (71.66)	
Others	47 (41.23)	67 (58.77)	
Infectious discharge ¶			
Yes	59 (32.78)	121 (67.22)	0.462
No	286 (35.66)	516 (64.34)	
ART protocol ¶			
Long	290 (37.37)	486 (62.63)	0.001
Antagonist	35 (28.46)	88 (71.54)	
Short	4 (10.81)	33 (89.19)	
Other	5 (18.52)	22 (81.48)	
Hyperandrogenism ¶			
Hirsutism	38 (43.18)	50 (56.82)	0.102
None	307 (34.34)	587 (65.66)	
History of ART cycle¶			
Yes	210 (33.76)	412 (66.24)	0.217
No	136 (37.67)	225 (62.33)	
Age (Y)*	34.27 (4.92)	36.15 (5.43)	0.001
BMI*	25.54 (3.92)	25.57 (3.85)	0.908
Embryo transferred grade A*	0.26 (0.68)	0.13 (0.46)	0.001
Embryo transferred grade AB*	0.97 (1.07)	0.63 (0.92)	0.001
Ampule number*	26.23 (10.01)	30.82 (14.18)	0.001
hCG administration day*	12.38 (2.64)	12.35 (2.49)	0.878
Injected oocyte number*	8.12 (3.90)	7.03 (4.24)	0.001

¶; Number (%), *; Mean (SD), PCOS; Polycystic ovary syndrome, ART; Assisted reproductive treatment, BMI; Body mass index, hCG; Human chorionic gonadotropin, HMG; Human menopausal gonadotropin, and FSH; Follicle-stimulating hormone.

Table 2: Crude and adjusted RR for factors associated with clinical pregnancy (dependent variable) which remained in the final modified Poisson regression model

Variable	Crude estimate			Adjusted estimate*		
	RR	95% CI	P value	RR	95% CI	P value
PCOS	0.79	0.66-0.95	0.014	0.87	0.72- 1.05	0.15
Age (Y)	1.02	1.01-1.03	0.001	1.01	1.01-1.02	0.001
Embryo transferred grade A	0.84	0.75-0.94	0.004	0.80	0.71-0.90	0.001
Embryo transferred grade AB	0.87	0.83-0.92	0.001	0.86	0.82-0.91	0.001
Infectious discharges	0.95	0.85-1.07	0.45	0.91	0.81-1.01	0.10
ART protocol	1.12	1.07-1.18	0.001	1.06	1.01-1.11	0.02
Stimulator drug type	0.99	0.95-1.03	0.82	0.97	0.93-1.01	0.16
Ampule number	1.008	1.006-1.011	0.001	1.004	1.001-1.007	0.008

***; Adjusted for the other variables in the Table, PCOS; Polycystic ovary syndrome, ART; Assisted reproductive treatment, RR; Risk ratio, and CI; Confidence interval.**

Discussion

The present study measured pregnancy outcome in PCOS and non-PCOS infertile patients who underwent IVF/ICSI. A number of studies investigated the pregnancy outcome in PCOS women who underwent infertility treatments. Most studies were performed in Western countries. Our study differed from other reports in several key ways. First, we have performed this study in one of the countries in Asia, located in the Middle East. Asian women are slightly distinct from Western women in terms of PCOS, obesity, and their complications (22). In the current study, the clinical pregnancy was considered the ART outcome in a comparison between PCOS and non-PCOS infertile women treated by IVF/ICSI. A large number of studies attempted to determine other indicators (implantation and live birth rates) in addition to the clinical PR in infertile women with and without PCOS (23). The effectiveness of a novel modified ART protocol compared with a conventional ART protocol, ultra-long agonist (ULA) protocol versus long agonist (LA) protocol, and gonadotropin-releasing hormone (GnRH) antagonist versus GnRH agonist long protocols on PCOs females who underwent ART has been evaluated in a few studies (17, 24, 25).

Retrospective and prospective researches have reported adverse pregnancy outcomes that include miscarriage rate, multiple PR, abortion rate (26), prevalence of preterm delivery, and pregnancy induced hypertension in these women (22). However, the distinction or novelty of the current study was the use of modified Poisson regression when the outcome of interest (clinical pregnancy) was dichotomous. In an assessment of risk of pregnancy failure, interpretation of the odds ratio, obtained from logistic regression, as a RR leads to its potential exaggeration. Numerous reports have proposed that the RR is preferred over the odds ratio for most prospective investigations. It was demonstrated that application of logistic regression to prospective studies is uncritical and naive conversion of an adjusted odds ratio to a RR has compounded the difficulties, such as invalid confidence limits and inconsistent estimates for the RR, which was not reduced with increasing sample size (15, 27).

Our findings showed no significant difference in PR in patients with and without PCOS who underwent IVF/ICSI. We obtained this result after adjustments for confounder variables of age, type of ART protocol, and quality of the transferred embryo. A study on 189 infertile patients with PCOS, 129 PCOS, and 142 without PCOS or PCOS (control) who underwent IVF-ET indicated no significant differences in the clinical PR between the PCOS group (202 cycles, 51.0%), PCO group (134 cycles, 53.0%), and control group (150 cycles, 46.0%) (18). Another study analyzed clinical and biological features of PCOS in patients enrolled in ICSI cycles compared to normo-ovulatory women. The clinical PR per transfer (31.5 vs. 22.2%, not significant) did not differ statistically in the two groups (19).

Findings obtained from a systematic review and meta-analysis of 7 published studies (755 patients) reported no significant difference in PR between the GnRH antagonist group and the GnRH agonist group (17). However, another study (24) evaluated the effectiveness of ULA and LA. The results indicated that the ULA protocol yielded significant higher clinic PR (70.2%) compared to the LA protocol (50.8%) in women with elevated BMI and PCOS who underwent IVF/ICSI. In the current study, the final modified Poisson regression model (adjusted model) demonstrated a significant difference in pregnancy outcome in patients who underwent IVF/ICSI according to the type of ART protocol used. We were unable to follow up pregnancy complications, live births, and infants as other outcomes of ART. A long-term follow up of newborns of infertile patients who underwent IVF/ICSI, particularly PCOS women, should be performed in the future.

Conclusion

This paper proposed the use of modified Poisson regression to estimate the risk of pregnancy failure. The results obtained from this model showed no difference between patients with and without PCOS according to the risk of non-pregnancy.

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Author's Contributions

A.A.H., R.O.S.; Contributed to conception and design. A.A.H., R.O.S., M.A.M., M.S., A.E., A.G.; Contributed to all experimental work, data and statistical analysis, and interpretation of data. R.O.S.; Were responsible for overall supervision. A.A.H., M.S., S.V., A.E., A.G.; Drafted the manuscript, which was revised by R.O.S. and M.A.M. All authors read and approved the final manuscript.

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Original Article

Estimation of Day-Specific Probabilities of Conception during Natural Cycle in Women from Babylon

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Abstract

Background: Identifying predictors of the probabilities of conception related to the timing and frequency of intercourse in the menstrual cycle is essential for couples attempting pregnancy, users of natural family planning methods, and clinicians diagnosing for possible causes of infertility. The aim of this study is to estimate the days in which the likelihood of conception happened by using first trimester ultrasound fetal biometry in natural cycles and spontaneous pregnancy, and to explore some factors that may affect them.

Materials and Methods: This study is retrospective cohort study, with random sampling. It involved 60 pregnant ladies at first trimester; the date of conception was estimated using: i. Crown-rump length biometry (routine ultrasound examinations were performed at a median of 70 days following Last menstrual period or equivalently 10 weeks), ii. Date of last menstrual cycle. Only women with previous infertility and now conceiving naturally with a certain date of Last menstrual period were selected.

Results: The distribution of conception showed a sharp rise from day 8 onwards, reaching its maximum at day 13 and decreasing to zero by day 30 of Last menstrual period. The older and obese women had conceive earlier than younger women but there was insignificants difference between the two groups (P>0.05). According to the type of infertility, the women with secondary infertility had conceived earlier than those with primary infertility. There was a significant difference between the two groups (P<0.05).

Conclusion: Day specific of conception may be affected by factors such as age, BMI, and type of infertility. This may be confirmed by larger sample size in metacentric study.

Keywords: Last Menstrual Cycle, Crown-Rump Length, Conception, Ultrasound

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Introduction

Identifying predictors of the probabilities of conception in relation to the timing and frequency of intercourse in the menstrual cycle is essential for couples attempting pregnancy, users of natural family planning methods, clinicians diagnosing for possible causes of infertility, and reproductive epidemiologists (1, 2).

Wide variation in the timing of ovulation has been found in prospective studies related to hormonal and physiological changes such as basal body temperature or ultrasound (3). In pregnant women, the date of conception may be predicted from early fetal growth using sonographic biometry. This method has been proved to be more reliable than last menstrual period for dating the onset of pregnancy (4) and now is considered as a method of choice for dating conception in routine practice (5). However precise knowledge of the timing of conception, however, has important clinical implications: in this context, as for counseling regarding fertility, hormonal ovulation monitoring methods have been made commercially available to help optimizing the chances of conception (6). Timing of ovulation is also important for the follow-up of pregnancies regarding growth monitoring, screening for birth defects and management of delivering (7). Such study has not been conducted in our society. So this study aims to estimate the days in which the likelihood of conception happened by using first trimester ultrasound fetal biometry in natural cycles and spontaneous pregnancy, and to explore some factors that may affect them.

Materials and Methods

This study is a retrospective cohort study, with random sampling. The study conducted through the period from January 2014-August 2015, involving 60 selected pregnant ladies at first trimester at attending privet ante natal care clinic. Among all patients attended to privet ante natal care clinic in Babylon Province (3000 patients), 312 patients were infertile. Two hindered fifty one of those infertile women fit our inclusion criteria, and only 60 women were randomly selected by excel program. These ladies have already infertility problem

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and now conceived naturally without any ovulation induction or any hormonal treatment for the past 6 months. All pregnant women have been asked about medical and surgical histories, Physical and ultrasound examination were done in this regard. All the ultrasound examinations performed by single investigator (H.A) using ultrasound machine (Medison, Korea). Date of conception was estimated using crown-rump length biometry [the embryo is measured along its longest axis to obtain the crown-rump length (CRL) measurement] (8). Routine ultrasound examinations were performed at a median of 70 days following last menstrual peroid (LMP) (or equivalently 10 weeks).

Inclusion criteria are as the fillowing: i. Female with history of infertility who have conceived spontaneously without ovulation induction medication, ii. Only women with a certain date of LMP, iii. Singleton pregnancy, and iv. No associated diseases like diabetes mellitus or hypertension. Exclusion criteria are as the fillowing: i. Multiple pregnancy, ii. Diabetes mellitus, protein urea, oligohydrominous, iii. Leaking liquor and vaginal bleeding, iv. Congenital abnormality, v. Uncertain date of LMP, and vi. History of amenorrhea, breast feeding or contraceptive usage or any other hormonal treatment.

The pregnant ladies are subgroup according to the age (more or less than 35 years), body mass index [BMI, normal weight (<24.4 kg/m^2)], overweight (25-29.9 kg/m^2 or obese >30 kg/m^2) (9) and according to the type of infertility (primary or secondary infertility). The predictive model used to predict date of conception depend on fetal age in days and CRL in mm, expected using the following equation (10).

Fetal age (days)=21.564+2.224 ´ CRL-0.342 ´ ln mm, (CRL) is the measurement of the length of human embryo the top of the head (crown) to the bottom of the buttocks (rump). It is typically determined from ultrasound and can be used to estimate gestational age.

Statistical analysis of data was done by using SPSS version 17. We used independent t test to estimate differences between two groups in continuous variable. Also we used one-way ANOVA to evaluate differences of means among multiple groups. Linear regression analysis used to determine the odds ratio as a measure of the association between factors that affect on date of conception. Results are reported as (mean ± SD) unless indicated. P<0.05, was considered statistically significant (11). All participant signed an informed written consent of their wish of participation, the results of our work. Medical approval by the scientific committee of College of Medicine/University of Babylon.

Results

Most patients aged less than 35 years (78.3%) and about half of them were in normal weight (53.3%) (Table 1).

Table 1: General characteristics of the studied women

Demographic characteristics	n (%)	Total
Type of infertility		60 (100%)
Primary	32 (53.3%)	
Secondary	28 (46.7%)	
Age (Y) 27.25 ± 6.87		60 (100%)
< 35	47 (78.3 %)	
≥35	13 (21. 7%)	
Body mass index (BMI, Kg/m^2) 25.36 ± 4.54		60 (100%)
Normal	32 (53.3%)	
Overweight	18 (30.0%)	
Obese	10 (16.7%)	

The lowest gestational age (GA) values were those estimated by the US method, and the difference with the LMP was significant (P<0.001). The mean ± SD date of conception from LMP was (14.08 ± 5.92, Table 2).

Table 2: Gestational age at by the last menstrual period (LMP) and ultrasound (U/S), and date of conception from LMP

Date in days	Mean ± SD	Lower	Upper	P value
LMP	72.07 ± 9.70	60	105	<0.001**
U/S	58.13 ± 9.29	47	78	
Date of conception from LMP	14.08 ± 5.92	8	42	

This distribution showing a sharp rise from 8 days onwards, reaching its maximum of 18% at 13 days and decreasing to zero by 30 days following LMP (Fig.1).

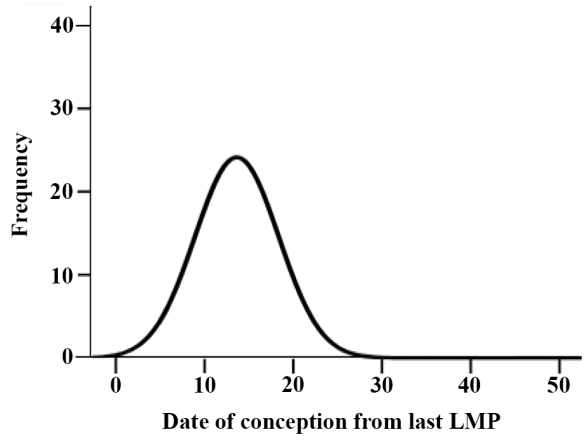

Fig.1: Day-specific frequency of conception in studied women.
LMP; Last menstrual period.

According to the age, the older women had conceived earlier than younger women but there was insignificant difference between two groups (P>0.05). Women aged >35 year displayed more variation in timing of onset of pregnancy, with an increased likelihood of pregnancies occurring earlier in the cycle (Table 3). Obese women displayed more variation in timing of onset of pregnancy, with an increased likelihood of pregnancies occurring earlier in the cycle (Table 4). According to the type of infertility, the women with secondary infertility had conceived earlier than women with primary infertility. There was a significant difference

between two groups (P<0.05).

Table 3: Gestational age at by the last menstrual period (LMP) and ultrasound (U/S), and date of conception

Age groups	LMP (mean ± SD)	CRL (mean ± SD)	Date of conception from last LMP (mean ± SD)
<35 Y	73.24 ± 10.25	58.97 ± 9.93	14.50 ± 6.39
>35 Y	67.58 ± 5.45	54.99 ± 5.58	12.50 ± 3.39
P value	0.072	0.189	0.303

CRL; Crown-rump length.

Table 4: Gestational age at by the last menstrual period (LMP) and ultrasound (U/S), and date of conception

BMI groups	LMP (mean ± SD)	CRL (mean ± SD)	Date of conception from last LMP (mean ± SD)
Normal	73.35 ± 10.00	58.29 ± 9.26	15.47 ± 7.76
Overweight	71.22 ± 9.88	58.28 ± 10.39	12.84 ± 1.95
Obese	69.33 ± 8.45	57.28 ± 7.91	11.93 ± 1.68
P value	0.505	0.958	0.165

BMI; Body mass index and CRL; Crown-rump length.

Within the studied group, (46.7%) had secondary infertility. Compared with women with primary infertility, women with secondary infertility displayed more variation in timing of onset of pregnancy, with an increased likelihood of pregnancies occurring earlier in the cycle (Table 5). By linear regression analysis, type of infertility was the most important factor that effects on date of conception, primary infertility associated with delayed date of conception while increase age and BMI associated with prompt date of conception (Table 6).

Table 5: Gestational age at by the LMP and ultrasound, and date of conception according types of infertility

Types of infertility	LMP (mean ± SD)	CRL (mean ± SD)	Date of conception from last LMP
Primary	73.73 ± 11.52	58.42 ± 10.59	15.70 ± 7.61
Secondary	70.29 ± 7.04	57.84 ± 7.89	12.40 ± 2.63
P value	0.179	0.816	0.034*

LMP; Last menstrual period and CRL; Crown-rump length.

Table 6: Linear regression analysis for factors that affect on date of conception

Parameter	B	SE	T	Sig.	95% CI (for B)	
					Lower	Upper
Age (Y)	-0.053	0.153	1.308	0.197	-0.108	0.508
Wt (Kg)	0.03	0.966	0.31	0.975	-1.914	1.974
Ht (m)	0.32	0.870	0.378	0.707	-1.421	2.079
BMI (Kg/m^2)	-0.37	2.448	-0.150	0.881	-5.292	4.556
Follicle (cm)	0.113	0.402	0.291	0.772	-0.693	0.927
Primary/ secondary infertility	1.37	2.046	2.142	0.037	0.266	3.497

Wt; Weight, Ht; Height, BMI; Body mass index, and CI; Confidence interval.

Discussion

Many research groups had focused to detect the time of ovulation in fertile cycle. Although ovulation generally occurs at around 14 days following the first day of last menses, a wide variation in the timing of ovulation has been found in prospective studies. In pregnant women, the date of conception may be estimated from early fetal growth using sonographic biometry (12). This method has been proved more reliable than last menstrual period for dating the onset of pregnancies (4) and most national guidelines now consider early biometry as the method of choice for dating conception in routine practice (5, 6). Day-specific probabilities of conception are defined as the probability that conception occurs on a given day of the cycle (13), provided that the cycle is fertile. The distribution of date of conception in our study showing a sharp rise from 8 days onwards, reaching its maximum of 18% at 13 days and decreasing to zero by 30 days following LMP which was somehow like the results obtained by Wilcox et al. (14) who found that the maximum probability of conception was reached by day 12.

Women aged >35 displayed more variation in timing of onset of pregnancy, with an increased likelihood of pregnancies occurring earlier in the cycle. These are the same results obtained by Liu et al. (15), Who found that increase maternal age>35 are associated with a shortening of cycle length, and also earlier ovulation.

This study concluded that obese women displayed more variation in timing of onset of pregnancy, with an increased likelihood of pregnancies occurring earlier in the cycle. Minge et al. (16) found that oocytes from the obese mice displayed slower embryo development maintained through the blastocyst stage.

This study demonstrated that women with secondary infertility displayed more variation in timing of onset of pregnancy, with an increased likelihood of pregnancies occurring earlier in the cycle. This result may related to time of oocyte maturation and implantation. Factor that affect oocyte maturation may be related to time of LH surge; the signaling mechanism from the surrounding cumulus cells; and intrinsic oocyte factors. The possibility of an intrinsic oocyte factors remains the most appropriate probability cause for option as the cause of oocyte maturation continuity or arrest (17, 18).

Conclusion

Day specific of conception may be affected by factors such as age, BMI, and type of infertility. This should be approved by larger in size multicentric study. This study should broaden the perspective of future epidemiologic research in infertility and pregnancy monitoring because of the wider access to retrospective data and the potential bias in prospective studies of ovulation monitoring.

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Author's Contributions

H.A.t.; Participated in study design, data collection and evaluation, drafting. B.J.E.; Contributed extensively in interpretation of the data and the conclusion. Both authors performed editing and approving the final version of this paper for submission, also participated in the finalization of the manuscript and approved the final draft.

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Case Report

Pregnancy in Non-Communicating Rudimentary Horn of A Unicornuate Uterus

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Abstract

Diagnosis and management of pre-rupture stage of the pregnant horn are difficult and usually missed on a routine ultrasound scan. Also most cases are detected after rupture of pregnant horn. We presented a 28-year-oldG2 L1 woman with diagnosis of rudimentary horn pregnancy (RHP) at 14 weeks of gestation. We diagnosed her with a normal intrauterine pregnancy, whereas a pregnancy in a right-sided non-communicating rudimentary horn with massive hemoperitoneum was later discovered on laparotomy. RHP has a high risk of death for mother, so there must be a strong clinical suspicion for the diagnosis of RHP. Although there is a major advancement in field of diagnostic ultrasound and other imaging modalities, prenatal diagnosis has remained elusive and a laparotomy surgery is considered as a definitive diagnosis.

Keywords: Pregnancy, Rudimentary, Uterus

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Introduction

Rudimentary horn pregnancy (RHP) as a rare incidence, has been estimated at 1:76,000-1:160,000 pregnancies (1). It has also been reported that 75-83% of cases are the pregnancy in non-communicating rudimentary horn that is caused by transmigration of peritoneal sperm or fertilized ovum (2). Gynecological and obstetrical complications of pregnancy in unicornuate uterus with a rudimentary horn are as following: i. Spontaneous abortion, ii. Preterm labor, iii. Infertility, iv. Endometriosis, v. Hematometra, vi. Intrauterine growth restriction (IUGR), vii. Intra peritoneal bleeding, and viii.. Uterine rupture. Kidney abnormalities have also been reported in 31% of cases, while the patients were diagnosed after reaching their stable condition (3). Rupture of RHP is considered as a life threatening condition for mothers. We used timely laparotomy, excision of the horn and blood transfusion to save a 28-year-old G2 L1 woman who was initially diagnosed with a normal intrauterine pregnancy, but a 14-week pregnancy in a right-sided non-communicating rudimentary horn with massive hemoperitoneum was later discovered on laparotomy.

Case report

A 28-year-old G2L1 woman who was 14 weeks pregnant was admitted at Amiralmomenin Hospital, Zabol, Iran, in November 2015, with generalized abdominal pain, nausea and vomiting. The patient who had a previous cesarean section received early prenatal care two years ago and an ultrasound exam at 14 weeks of gestation (a day before she was admitted at the hospital). The patient suffering from hypovolemic shock, was extremely pale, and had a weak pulse of 120-130 beats per minute and a blood pressure of 80/60 mm Hg. Physical exam revealed impaired consciousness and agitation, generalized abdominal tenderness with sharp right lower quadrant (RLQ) pain, no vaginal bleeding, and a closed cervix. A portable ultrasound detected more than 2 liters of free fluid in the abdomen and pelvis that confirmed the presence of unicorn ate uterus and a 14-week pregnancy in right-sided rudimentary horn. After fluid resuscitation, the patient was transferred to the operating room for an emergency laparotomy. Her blood pressure was 90/60 mm Hg at the time of laparotomy.

During laparotomy, we founded that right-sided non-communicating rudimentary horn was already ruptured and the fetus with amniotic sac extruded into the peritoneal cavity with presence of about a 3-liter hemoperitoneum (Figs.1-3). The rudimentary horn was then excised (Fig.4) and the abdomen closed following hemostasis. Furthermore, patient received 3 unites of pack cell and recovered well after surgery. She was discharged with satisfactory condition on fifth post-operative day after the kidney anomalies ruled out. This study was approved by the Ethics Committee of Iran University of Medical Sciences, Tehran, Iran. Written informed consent was obtained from case.

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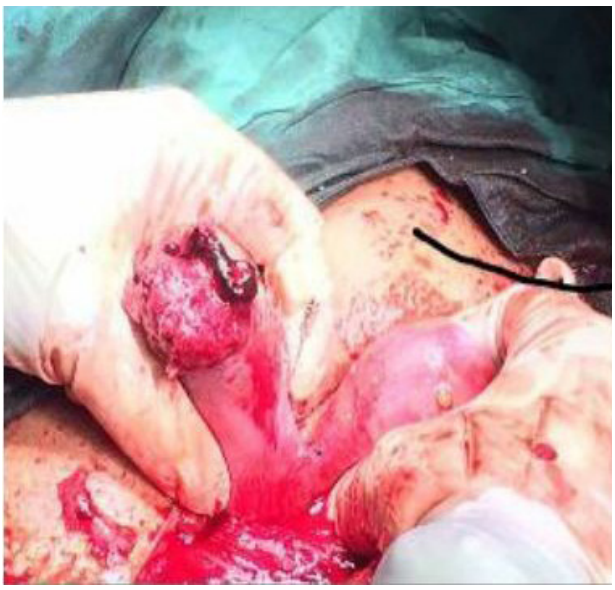

Fig.1: Rudimentary horn pregnancy still attached to the main horn.

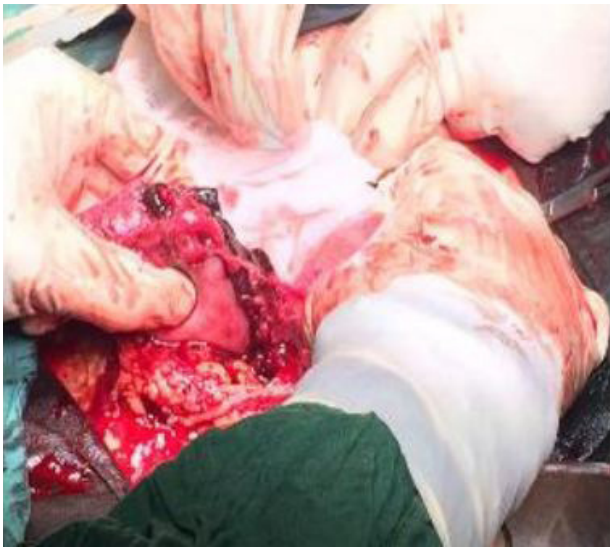

Fig.2: A 4-cm ruptured rudimentary horn with placenta partially protruding from it.

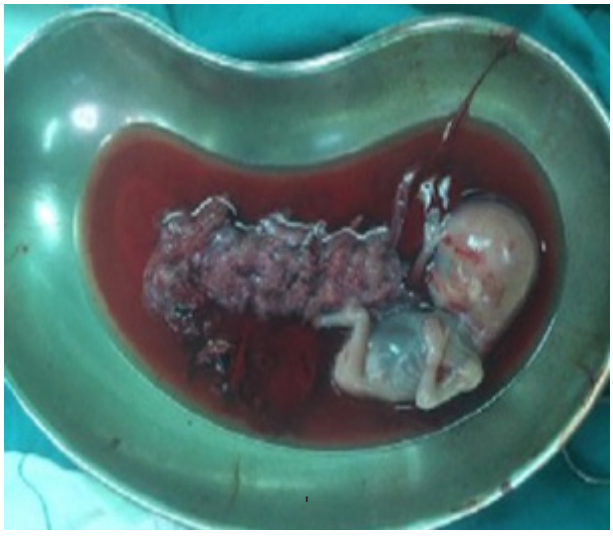

Fig.3: Fetus with placenta.

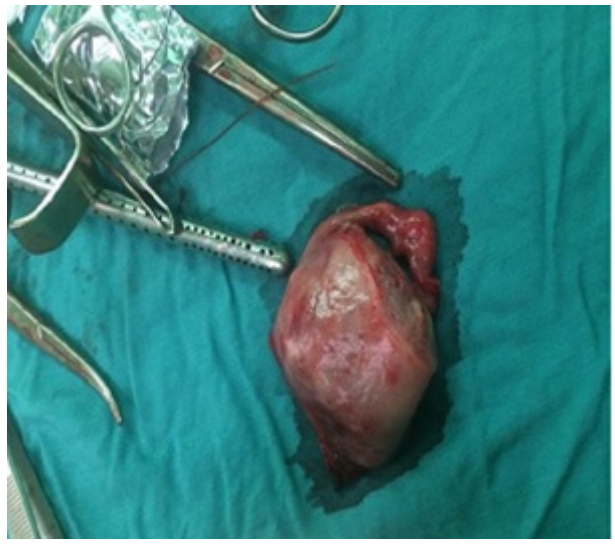

Fig.4: Excised rudimentary horn.

Discussion

Pregnancy in a non-communicating rudimentary horn is the results of developmental defect of one Müllerian duct or incomplete connection with Müllerian ducts on the opposite site that has been estimated at 1:76,000-1:160,000 pregnancies (1-4). The first case of uterine rupture following RHP was reported by Kanagal and Hanumanalu (5). It has been reported that the timing of a ruptured rudimentary horn that is mainly associated with horn musculature and its ability to hypertrophy is estimated between 5 and 35 weeks. The early diagnosis of RHP is likely to prevent maternal morbidity and mortality. The best management strategies for early diagnosis of RHP are as: i. Ultrasound, ii. Hysterosalpingography, hysteroscopy, laparoscopy, as well as iii. Magnetic resonance imaging (MRI) (5). It is noted that the sensitivity of ultrasound is 26%, although its sensitivity decreases when the maternal age increases (6).

However, RHP is likely to be missed by the most experienced radiologist. The most common ultrasound reports that leads to misdiagnose RHP are as follows: i. Tubal pregnancy, ii. Cornal pregnancy, iii. Intrauterine pregnancy, and iv. Abdominal pregnancy (7). It is difficult to confirm a rudimentary horn with thin myometrium diagnosis because of obscuring adjacent anatomical structures. The following diagnostic criteria for RHP were indicated by Tsafrir et al. (8) using ultrasonography: i. Pseudo pattern of asymmetrical bicornuate uterus, ii. Non-continuity between tissue surrounding the gestational sac and the uterine cervical canal, and iii. The presence of myometrial tissue surrounding the gestational sac. Furthermore, a hyper vascularization pattern like placenta accrete is considered as an indication for the diagnosis of RHP that is detected by both Color Doppler ultrasound and Doppler ultrasound. Samuels and Awonuga (9) have reported a uterine rupture after labor induction with misoprostol.

The application of different methods of labor induction for termination of RHP was unsuccessful and led to

a uterine rupture. The surgical approach is considered as the first management strategy. There are several reports of early diagnosis and laparoscopic excision of rudimentary horn (10-12). Edelman has reported a successful strategy treatment of RHP including the use of methotrexate (MTX) and laparoscopic excision of rudimentary horn in the first weeks of pregnancy (13). Emergency surgery after diagnosis even in cases of un-ruptured rudimentary horn has been recommended (3). Also, prophylactic removal of the rudimentary horn has been suggested (14). There is a report of RHP reaching a full-term delivery that led to live birth using cesarean section (15). The reproductive outcome of a unicornuate uterus is discussed in some articles. However, afew of them have discussed the reproductive outcome after resection of rudimentary horn. Those patients who have ever undergone resection of a rudimentary horn should be considered as a high-risk group in the fallowing pregnancy (16, 17). This case report had no ethical consideration for patient.

Conclusion

RHP has high risk of death for mother, so there must be a strong clinical suspicion for the diagnosis of RHP. Although there is a major advancement in field of diagnostic ultrasound and other imaging modalities, prenatal diagnosis has remained elusive and a laparotomy surgery is considered as a definitive diagnosis. Early diagnosis, timely resuscitation, laparotomy, and blood transfusion are the necessary management steps to save a patient.

Acknowledgements

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Author's Contributions

M.K., A.M.K.; Contributed to conception and design. K.S.; Were responsible for overall supervision. F.F.; Drafted the manuscript, which was revised by M.K. All authors read and approved the final manuscript.

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Case Report

The Many Guises of Endometriosis: Giant Abdominal Wall Endometriosis Masquerading as An Incisional Hernia

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Abstract

Endometriosis is defined by the presence of ectopic endometrial tissue outside the uterine cavity. Although it is a leading cause of chronic pelvic pain and infertility, its clinical presentation can vary, resulting in diagnostic and therapeutic challenges. Extrapelvic endometriosis is particularly difficult to diagnose owing to its ability to mimic other conditions. Endometrial tissue in a surgical scar is uncommon and often misdiagnosed as a granuloma, abscess, or malignancy. Cyclical hemorrhagic ascites due to peritoneal endometriosis is exceptionally rare. We report the case of a pre-menopausal, nulliparous 44-year-old woman who presented with ascites and a large abdominal mass that arose from the site of a lower midline laparotomy scar. Five years previously, she had undergone open myomectomy for uterine fibroids. Soon after her initial operation she developed abdominal ascites, which necessitated percutaneous drainage on multiple occasions. We performed a laparotomy with excision of the abdominal wall mass through an inverted T incision. The extra-abdominal mass consisted of mixed cystic and solid components, and weighed 1.52 kg. It communicated with the abdominopelvic cavity through a 2 cm defect in the linea alba. The abdomen contained a large amount of odourless, brown fluid which drained into the mass. There was a large capsule that covered the small and large bowel, liver, gallbladder, and stomach. Final histology reported a 28×19×5 cm mass of endometrial tissue with no evidence of malignant transformation. The patient recovered well post-operatively and has remained asymptomatic. Our case illustrates that, despite being a common disease, endometriosis can masquerade as several other conditions and be missed or diagnosed late. Delay in diagnosis will not only prolong symptoms but can also compromise reproductive lifespan. It is therefore paramount that endometriosis is to be considered early in the management of premenopausal women who present with an irregular pelvic mass or hemorrhagic ascites.

Keywords: Ascites, Endometriosis, Infertility, Laparotomy

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Introduction

Endometriosis is a histological diagnosis defined by the presence of endometrial glandular and stromal tissue outside the uterine cavity. It affects around 10% of women of reproductive age and is frequently implicated in infertility (1). It is rarely found before menarche and tends to regress after menopause. Although endometriosis is considered to be a benign condition, a well-established association exists between longstanding lesions and the development of clear cell and endometrioid carcinomas (2). The human endometrium is hormone-dependent and undergoes cyclical hyperplasia, secretion, and shedding. The ectopic endometrial foci in endometriosis respond to cyclical hormonal changes in the same way as the intrauterine endometrium, which results in focal bleeding, inflammation, and fibrosis. This manifests in symptoms which vary in frequency and intensity, including dysmenorrhea, menorrhagia, dyspareunia, and pelvic pain.

The etiology and pathogenesis of endometriosis are not fully understood. Several proposed theories include retrograde menstruation first described by Sampson (3), coelomic metaplasia, and mestastatic spread. More recent research has proposed that altered immunity, stem cells, and epigenetic changes are implicated in the disease process (4-7). The common sites for ectopic foci of endometrial tissue are the ovaries, fallopian tubes, vagina, cervix, rectovaginal septum, and the uterosacral ligaments (8). Extrapelvic implantation in a number of organs such as the gastrointestinal (GI) tract, lungs, pleura, kidneys, bladder, and brain has been reported (8, 9). The uterosacral ligaments and posterior cul-de-sac are the most common site of pelvic implantation while the GI tract is the most common extrapelvic site of endometriosis. Although endometriosis in a surgical scar is rare, there are several reports on endometriosis in Pfannenstiel incisions following cesarean sections (10-14). There is a paucity of literature that relates to abdominal wall endometriosis following other surgical procedures.

We report the case of a pre-menopausal, nulliparous 44-year-old woman who presented with ascites and a large abdominal mass that arose from the site of a lower midline laparotomy scar. After extensive investigation,

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the final histology confirmed endometriosis as the cause of her unusual clinical presentation.

Case report

A 44-year-old Nigerian female was admitted with a large, symptomatic abdominal mass. She was para 0, gravida 0 and in 2011 underwent open myomectomy through a lower midline abdominal incision for large, symptomatic fibroids. She also had a background of pulmonary tuberculosis (TB) which was treated in 1995, iron-deficiency anemia, and a benign goiter. We received the consent of all patients. Within the first few months following her open myomectomy she began to develop abdominal ascites, which was drained percutaneously on approximately three occasions in 2011-12. Owing to some personal circumstances, she did not attend her regular outpatient appointments and was eventually lost to follow-up. However, her abdomen continued to swell, with expansion of the skin and subcutaneous tissue that surrounded the scar.

She had no other relevant gynecological history and, aside from ferrous sulfate, she took no other regular medications. She was a non-smoker, denied alcohol use, and lived independently with her extended family. In 2016, five years following her original open myomectomy, she presented with a large abdominal mass that appeared to arise from the abdominal wall. This mass began to spontaneously discharge large volumes of brown fluid on the day of her admission. She was systemically well and gave no history of change in bowel habit or obstructive symptoms. She was not sexually active and had a normal menstrual history. She was not up-to-date with smear testing but denied intermenstrual bleeding. Her last menstrual period was one day previously.

On clinical examination she was thin, weighed 48 kg, with a body mass index (BMI) of 18. She was hemodynamically stable with a normal cardiorespiratory examination. She had a soft abdomen, with evidence of shifting dullness, and a large, irregular, firm mass that arose from the lower half of the abdomen overlain by her lower abdominal midline scar (Fig.1). It measured approximately 30×20×10 cm. The overlying skin was of variable thickness with several small puncti on its inferior aspect, one of which discharged brown, odorless fluid. The mass itself was non-tender. Digital rectal examination was unremarkable. Bowel sounds were normal and could not be detected on auscultation of the mass. The clinical impression was that this was a large incisional hernia with evidence of ascites. Laboratory blood results revealed a microcytic anemia (hemoglobin count 10.1 g/dL, mean cell volume 63.6 fL), normal white cell count, renal and liver functions, and a mildly elevated C-reactive protein of 33 mg/L. Abdominal and chest radiographs were unremarkable. A computed tomography (CT) scan of her abdomen and pelvis confirmed the presence of a large, extraperitoneal lobulated space-occupying lesion with a mixed cystic and solid appearance that extended at least 20 cm caudally (Fig.2). There was an 8×11×13 cm lesion in the uterus and a moderate amount of ascites, mainly in the lower abdomen and pelvis.

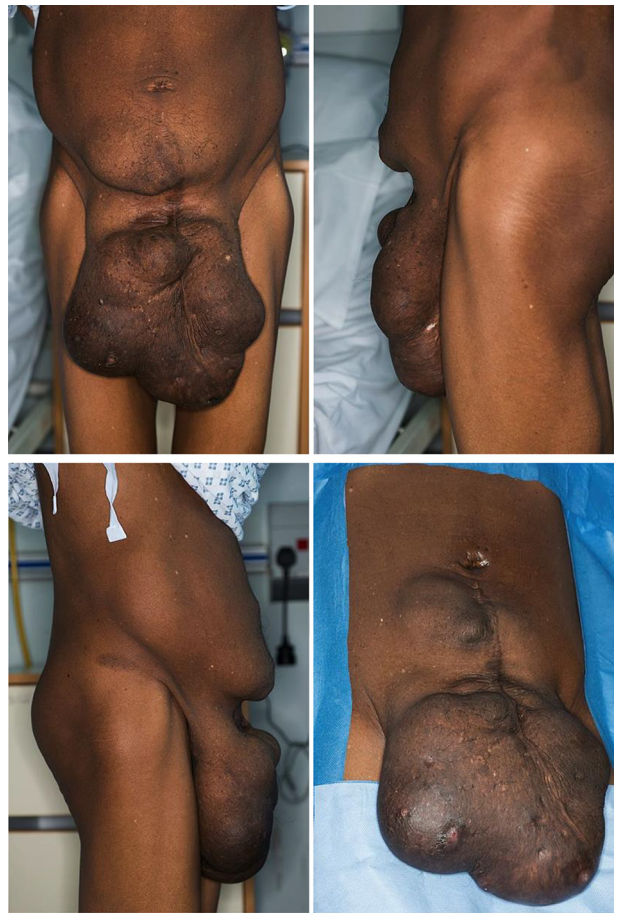

Fig.1: Pre-operative photographs of the multi-lobulated lesion with an overlying lower midline laparotomy scar from a previous open myomectomy. Multiple small puncti can be seen on the skin.

A follow-up pelvic ultrasound scan (USS) demonstrated a bulky uterus that measured 15×11.5×9.7 cm with gross pelvic ascites. The differential diagnosis was a possible uterine malignancy with peritoneal and abdominal wall carcinomatosis or disseminated TB, particularly in light of her previous pulmonary TB. Assessment of her tumor markers showed that both the CEA and CA19-9 were within normal range; CA 125 was elevated at 89.8 U/mL (0-35 U/mL). Her hepatitis (A, B, C, E), human immunodeficiency virus (HIV), autoimmune serology and sickle cell tests were all negative. She underwent ultrasound guided peritoneal biopsy for TB screening, the histology of which showed chronic inflammation with no evidence of granulomatous inflammation or malignancy. Additionally, her ascitic fluid was negative for *Mycobacterium tuberculosis*. Two weeks following her original admission she underwent a laparotomy with excision of the large abdominal wall mass. This was carried out through an inverted T incision that encircled the lower midline laparotomy incision and the mass, but preserved the umbilicus. The extra-abdominal mass was of mixed cystic and solid components, and weighed 1.52 kg. It communicated with the abdominopelvic cavity through a 2 cm defect

in the linea alba just below the umbilicus. The abdominopelvic cavity contained a large amount of odorless, brown fluid which flowed into the mass. There was an enlarged uterus bulky with fibroids. There were two large endometrioid cysts bilaterally in the region of the adnexae. The ovaries could not be clearly delineated and the fallopian tubes adhered to the pelvic walls. There was a large capsule that covered the small and large bowel, liver, gallbladder, and stomach. The mass was dissected free from the abdominal wall fascia and excised (Fig.3). The endometrioid cysts that contained altered blood were drained. The large abdominopelvic capsule was stripped off of its adherent viscera, but it was not safe to strip it off in its entirety.

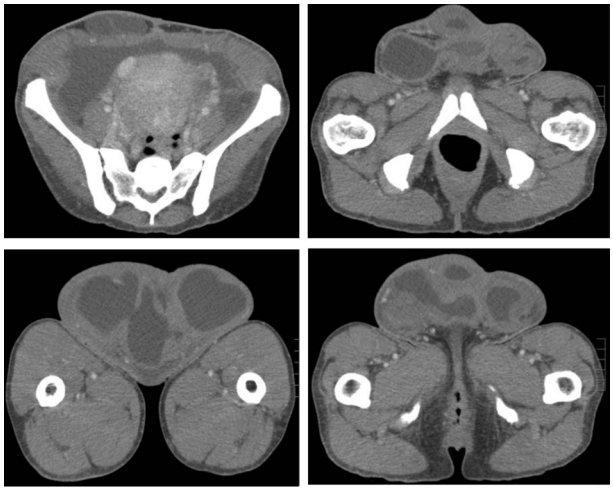

Fig.2: Axial sections from a computed tomography (CT) scan with intravenous contrast that demonstrated a large, extraperitoneal lobulated space-occupying lesion with mixed cystic and solid components.

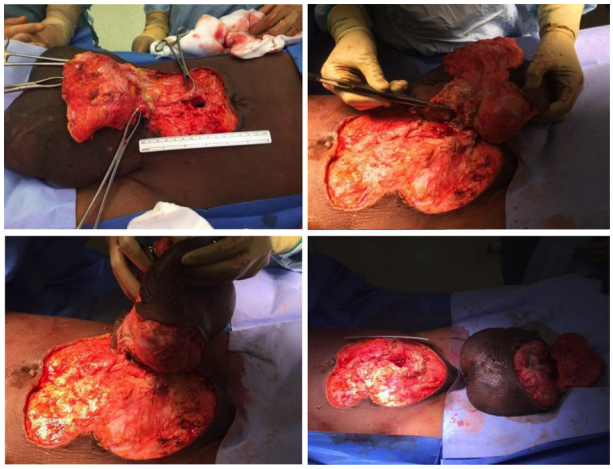

Fig.3: Findings at laparotomy; the extra-abdominal mass was of mixed cystic and solid components and communicated with the abdominopelvic cavity through a 2 cm defect in the linea alba just below the umbilicus. The specimen weighed 1.52 kg.

The fascial opening, which extended caudally by 5 cm, was closed with interrupted 1 Nylon sutures. The umbilicus was preserved. We left two large Robinson drains in the abdominopelvic cavity and two negative pressure (Redivac) drains remained in the subcutaneous space. The skin was closed with horizontal mattress sutures using 2-0 Vicryl Rapide. A negative pressure incision management system (PICO dressing) was applied to the wound. The final histology confirmed a 28×19×5 cm mass of endometrial tissue with no evidence of malignant transformation. The patient made a good post-operative recovery and was discharged one week following surgery with the abdominal drains and planned gynecological follow-up. She was assessed on a weekly basis in the General Surgery Outpatient Department in general surgery outpatients. All drains were removed 10 days following discharge (Fig.4). The patient provided written informed consent for publication of this case report and accompanying images. She remains well.

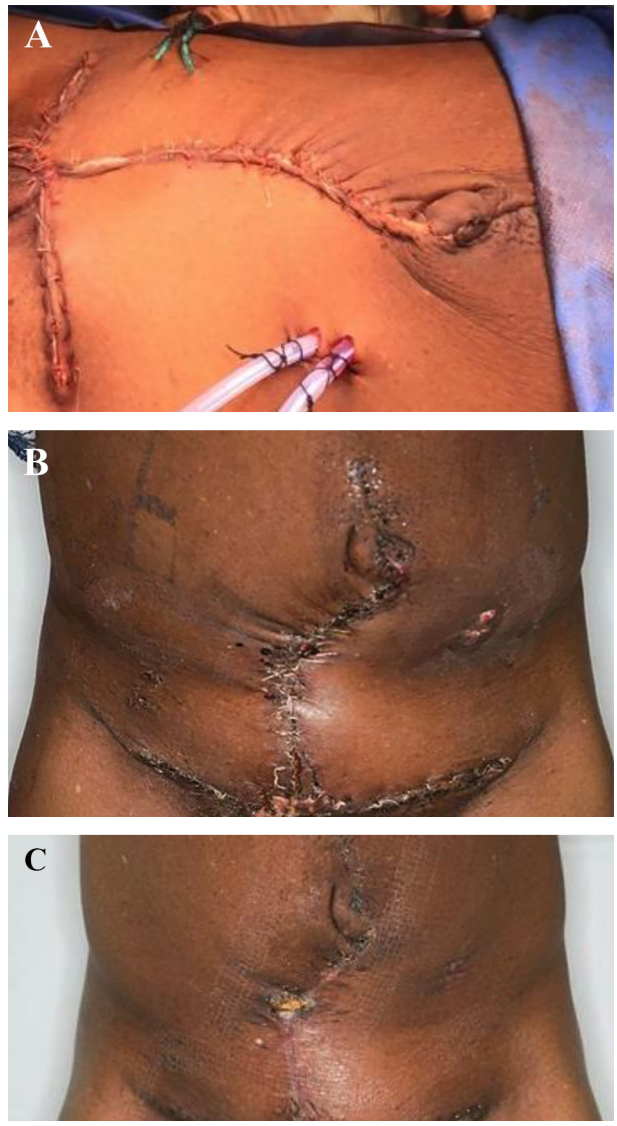

Fig.4: The skin was closed with horizontal mattress sutures using 2/0 Vicryl Rapide. **A.** The umbilicus was preserved. Two large Robinson drains were left in the abdominopelvic cavity, and two negative pressure (Redivac) drains were left in the subcutaneous space, **B.** Two weeks postoperative after all the drains had been removed, and **C.** Five weeks postoperative.

Discussion

Several theories have been proposed to explain the pathogenesis of ectopic endometrial tissue (15). Endo-

metriosis was first described by Rokitansky in 1860. Adenomyosis- the presence of endometrial tissue within the myometrium- was then described in more detail by the pathologist, Iwanoff, and the surgeon, Cullen. They believed that metaplasia of embryonic cells caused the ectopic endometrial tissue (16). The theory of coelomic metaplasia has remained as one of the leading models of endometriosis. It is based on the idea that peritoneal cells can differentiate into endometrial cells following chronic inflammatory stimulus (17).

Another leading theory is retrograde menstruation. In 1927, Sampson published his work on peritoneal and ovarian endometriomas. He proposed that reversed menstrual flow through the fallopian tubes led to implantation of endometrial cells into the peritoneal cavity, with subsequent endometriosis (3). This explanation has been debated, however, as it has not explained pre-pubertal endometriosis. The presence of ectopic endometrial tissue has even been identified in human female fetuses of different gestational ages (18). Sampson's theory does not explain the similar incidence of retrograde menstruation in women with and without endometriosis (19). The metastatic theory is often favored, although it has never been scientifically demonstrated. This theory suggests that endometrial tissue can be transported to adjacent locations via surgical intervention, hematogenous or lymphatic spread. This can explain the presence of endometriosis in very distant sites such as the pleura and brain. Increasing evidence exists for the role of epigenetics, oxidative stress, and immune dysfunction in the growth of ectopic endometrial tissue (20).

The clinical presentation of endometriosis is extremely variable and this leads to diagnostic and therapeutic challenges. Extrapelvic endometriosis is particularly difficult to diagnose due to its wide spectrum of presentations and ability to mimic several other conditions. Our patient presented to the general surgeons with an irregular abdominal mass that resembled an incisional hernia, but with associated weight loss and microcytic anemia. In view of her previous history of pulmonary TB, the two main differentials in her diagnosis at this stage were a gastro-intestinal malignancy and intra-abdominal TB. A transvaginal/transabdominal USS demonstrated an irregular, bulky uterus, and ascites. Uterine malignancy was then included in the differential diagnosis. It was only at laparotomy that the likely diagnosis of endometriosis became apparent. The abdominal wall protrusion that arose from her lower midline laparotomy scar and mimicked an incisional hernia, was confirmed as giant endometriosis. We identified two large ovarian endometriomas in the region of the adnexae, which confirmed concurrent pelvic endometriosis. The abdomen and pelvis were not explored in more detail at the time of her surgery, however the finding of bilateral ovarian endometriomas was highly suggestive of extensive pelvic endometriosis with a high chance of bowel involvement.

Endometriosis in a surgical scar is a rare entity, with an incidence of 0.03 to 0.15%. It is often misdiagnosed as a granuloma, abscess, or malignancy (10, 21, 22). We have found a few cases in the literature of abdominal wall endometriosis after surgical procedures. In a review of 445 cases of abdominal wall endometriosis, 57% occurred following cesarean sections, 11% after hysterectomy, and 12% have followed all other abdominal surgeries (23). Up to 80% of patients complain of scar pain which is often cyclical in nature. Interestingly, however, our case demonstrates that pain in cutaneous endometriosis is not always present. This patient's case supports the paradox often observed in endometriosis; small lesions can often be very painful, whereas marked disease is often not painful (24). Incisional endometriosis may, in part, be consistent with the metastatic theory of endometriosis, as endometrial tissue may displace to the wound during pelvic surgery. It could also be explained by metaplasia of stem cells during the healing process. It was unclear whether the uterine cavity was breeched during the original myomectomy that our patient underwent. The case we have described is most likely the result of metaplasia of undifferentiated cells located in the abdominal wall during the healing process following myomectomy.

Recurrent hemorrhagic ascites due to peritoneal or pelvic endometriosis is also rare; less than 50 cases have been described in the literature (25, 26). Endometrial tissue is highly dynamic and responds to cyclical hormonal changes. The development of hemorrhagic ascites in endometriosis is cyclical and most symptomatic during menstruation. Our patient sought medical attention because large volumes of brown fluid spontaneously discharged from her abdominal wall earlier that day. This had coincided with the onset of her menstrual period which was the day prior to this presentation. Pelvic TB and malignancy are common causes of hemorrhagic ascites (27, 28). Disseminated TB, in particular, was an important differential in the case of our patient in view of her previous history of pulmonary TB. However, endometriosis should always be considered in nulliparous women of childbearing age who present with hemorrhagic ascites.

Our patient received surgical treatment five years after the onset of her symptoms. Her complex social situation and failure to attend regular follow-up led to a delay in her diagnosis. Once extrapelvic endometriosis has been identified, surgical treatment appears to result in a cure in over 95% of cases (23). Following surgery, progressive reduction in hemorrhagic ascites has been observed, with complete remission within six months (27). The patient has recovered well post-operatively. At present, she remains asymptomatic. Her extensive pelvic endometriosis, however, was not treated at the time of laparotomy and excision of the abdominal wall mass. She is under follow-up by the gynecology team with the intent for further surgery.

Conclusion

Our case illustrates that, despite being a common dis-

ease, endometriosis can masquerade as several other conditions; therefore, it can be missed or diagnosed late. Delay in diagnosis will not only prolong symptoms but can also compromise reproductive lifespan. It is therefore crucial that endometriosis is considered early in the management of premenopausal women who present with an irregular pelvic mass or hemorrhagic ascites.

Acknowledgements

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Author's Contributions

C.P., S.A., T.O.; Background research, preparation of manuscript. All authors read and approved the final manuscript.

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Case Report

Uterus Dysplasia Associated with Cervico-Vaginal Agenesis

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Abstract

Müllerian ducts can form upper parts of normal female reproductive system and any failure in ductal fusion may result in to müllerian duct anomalies (MDA). We present a case of MDA and a uterus dysplasia with no evidence of cervical or upper vaginal tissue. This case showes the role of magnetic resonace imaging (MRI) on MDA diagnosis and urges the need for a unified reliable and practical classification more compatible with clinical practice.

Keywords: Amenorrhea, Magnetic Resonance Imaging, Müllerian Duct Hypoplasia

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Introduction

Recent studies have challenged the embryological origin of female genitourinary tract especially vagina as some rare types of anomalies were reported. According to one theory, the embryological development of the vagina proceeds from the mesonephric (Wolffian) ducts and the Müllerian tubercle instead of the Müllerian ducts and the urogenital sinus (1). Several Genetic mutations may lead to müllerian duct anomalies (MDAs). The most common classification system for MDAs was developed by the American Fertility Society (AFS) (2). It is important to know that this classification system is only a framework and that not all anomalies will fit completely into one of the categories (1). We present a patient with uterus dysplasia related to cervical and incomplete vaginal agenesis which is classified variably according to the present classifications. Informed consent was obtained from the adult participant. The Ethics of this report have been approved by Tehran University of Medical Sciences Institutional Review Board.

Case report

An 18-year-old girl referred to our institute for primary amenorrhea evaluation. She had normal growth pattern with no sign of periodic pelvic pain, pelvic discharge, or bleeding. The past history was negative for diethylstilbestrol exposure in her mother and no case of MDA was present in her first relatives. On a physical examination, her external genitalia was normal, she had hymen and permeable vagina and her secondary sexual characteristics (including breast budding and axillary and groin hair) appeared to be normal. The patient had incomplete vaginal agenesis: lack of vagina in the upper one-third with normal lower two-thirds. There were no signs of abdominal and pelvic mass, previous surgical scar, or any urological abnormality. Laboratory studies were within normal limits. She underwent a trans-abdominal ultrasound examination revealing separate divergent uterine horns with separate endometrial cavities, normal ovaries bilaterally and no evidence of cervical or upper vaginal tissue. Consequently, MRI was obtained for further evaluation. (Fig.1A, B). On the MRI, two small uteri were noted in the pelvic fossa with normal endometrium within them in axial T2 images without contrast administration (Fig.1A). In sagittal T2 images, cervix and one-third of upper vagina were not visible (Fig.1B). Mild to moderate fluid appeared within pelvic cavity but no signs of hydrometrocolpos or hematometrocolpus were seen. There was also no evidence of renal agenesis, abnormalities of ureters or skeleton. The patient was diagnosed with a MDA referred to gynecologist for treatment.

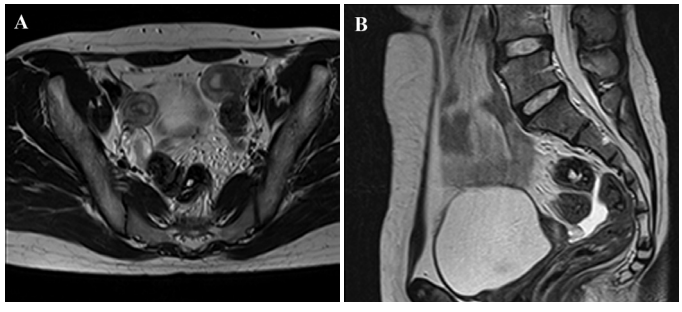

Fig.1: Magnetic resonance imaging study of the patient with cervico-vaginal agenesis. **A.** Axial T2 Weighted image shows two separated uterus with normal endometrial cavity and **B.** In Sagittal T2 Weighted image, cervix and 1/3 of upper vagina is not visible. Mild to moderate fluid is also present within pelvic cavity.

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Discussion

In this study, we have presented a case of uterus dysplasia with cervical and incomplete vaginal hypoplasia. MDAs are from the cause of interruption or dysregulation in Müllerian duct development at various stages of morphogenesis. Multiple etiologies such as genetic factors and teratogenic drugs (e.g. diethylstilbestrol, thalidomide) are related to these anomalies. The genetics of MDA are complex and not fully understood. Generally, they occurred sporadically but other modes of inheritance, such as autosomal dominant, autosomal recessive, and X-linked disorders have been reported. Also, it could be a component of a complex malformation syndrome (3). Uterus dysplasia is associated with premature birth, malpresentation, intrauterine growth retardation, spontaneous abortion and infertility.

In 1988, the AFS classified MDA into 7 major categories (2). Based on this method of classification, failure to go through developmental stages leads to a specific malformation. However, observed anomalies are not mutually exclusive and many MDAs often coexist. Also several cases of MDAs have been reported which fail to be classified as one of AFS classification categories (such as normal uterus with double cervix and vagina (4), septate uterus with cervical duplication (5), bicervical uterus with and without septate vagina (6), or double cervix and vagina with septate uterus (7). According to the European Society of Human Reproduction and Embryology (ESHRE) consensus, uterine anatomical abnormalities are described as following: U0, normal uterus; U1, dysmorphic uterus; U2, septate uterus; U3, bicorporeal uterus; U4, hemi-uterus; U5, aplastic uterus; U6, unclassified cases and cervical and vaginal anomalies are added subsequently. Our patient was in class I of the AFS and a U5a/Cervix4/Vagina4 ESHRE classification, group 3A of complex female genital malformations, IIIA1C of the embryological and clinical classification (2, 8-11). Applying MRI as the correct modality of choice for assessing of MDAs and sonohysterography are helpful to noninvasively examine the uterine anomalies (12).

Conclusion

Our observations together with recent discoveries in pathogenesis and genetics of MDAs may affect future investigations on more clinically practical classifications of MDAs. Indeed, more research projects on pathophysiological and genetic aspects of these anomalies are required for unifying the classification of MDAs.

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Author's Contributions

A.M., M.B., H.M., L.Y., Z.H.; Guarantor of integrity of the revised entire study. A.M., L.Y., M.B.; Study concepts and design. H.M., L.Y., Z.H.; Literature research. M.B.; H.M., A.M.; Manuscript preparation. M.B., L.Y., Z.H.; Manuscript editing. A.M., M.B., H.M., L.Y., Z.H.; Final Approval of the article and ensuring the integrity of the work.

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