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Contents

Review Article

Pregnancy, Preeclampsia, and COVID-19: Susceptibility and Mechanisms: A Review Study

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) enters cells through angiotensin converting enzyme 2 (ACE2), which expression of its gene increases during pregnancy that is resulted in an enhanced level of the ACE2 enzyme. It might enhance the risk of SARS-CoV-2 infection and its complications in the pregnant women. Although, pregnancy hypertensive disorders and severe infection with SARS-CoV-2 are correlated with high comorbidity, these two entities should be discriminated from each other. Also, there is a concern about the risk of preeclampsia and consequently severe coronavirus disease 2019 (COVID-19) development in the pregnant women. So, to answer these questions, in the present review the literature was surveyed. It seems there is higher severity of COVID-19 among pregnant women than non-pregnant women and more adverse pregnancy outcomes among pregnant women infected with SARS-CoV-2. In addition, an association between COVID-19 with preeclampsia and the role of preeclampsia and gestational hypertension as risk factors for SARS-CoV-2 infection and its complications is suggested. However, infection of the placenta and the SARS-CoV-2 vertical transmission is rare. Various mechanisms could explain the role of COVID-19 in the risk of preeclampsia and association between preeclampsia and COVID-19. Suggested mechanisms are included decreased ACE2 activity and imbalance between Ang II and Ang-(1-7) in preeclampsia, association of both of severe forms of COVID-19 and pregnancy hypertensive disorders with comorbidity, and interaction between immune system, inflammatory cytokines and the renin angiotensin aldosterone system and its contribution to the hypertension pathogenesis. It is concluded that preeclampsia and gestational hypertension might be risk factors for SARS-CoV-2 infection and its complications.

Keywords: Comorbidity, COVID-19, Preeclampsia, Pregnancy, Renin Angiotensin Aldosterone System

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Introduction

Severe acute respiratory syndrome caused by coronavirus 2 (SARS-CoV-2). The virus binding to angiotensin converting enzyme 2 (ACE2), a renin angiotensin aldosterone system (RAAS) counter-regulator, leads to its entrance to the cells and consequently, cells infection (1). Although, the ACE2 has 60% homology with the ACE1 enzyme, its different active site leads to its inhibition prevention by ACE inhibitors (2). ACE2 is the SARS-CoV-2 spike protein receptor (3). The cellular entry of SARS-CoV-2 through ACE2 that its expression increases during pregnancy might increase susceptibility to SARS-CoV-2 infection in the pregnant women and the risk of pregnancy complications including preeclampsia (4).

Pregnant women are at high risk of viral pneumonia compared to the general population, especially in the absence of antiviral therapy (5). The unfavorable impact of SARS-CoV-2 infection during pregnancy might be due to the RAAS dysregulation (4). The most common COVID-19 related adverse outcomes in the pregnant women include maternal sepsis, preeclampsia, premature rupture of membrane and post-partum hemorrhage (6). Also, hypertension is known as a strong risk factor for complicated COVID-19 (7).

Preeclampsia is presented with hypertension, proteinuria, edema, and a coagulation cascade activation (8). The RAAS dysregulation is involved in the pathogenesis of preeclampsia. Angiotensin (Ang)-(1-7) is produced through the action of ACE2 on angiotensin (Ang) I and the imbalance between angiotensin (Ang) II and Ang-(1-7) might be involved in the etiology of the preeclampsia (9).

There are two questions to be answered; whether pregnant women infected with SARS-CoV-2 are at greater

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risk of preeclampsia; and whether preeclampsia could be a risk factor for developing severe COVID-19. Therefore, the aims of this retrospective study were to review: i. The susceptibility of pregnant women to COVID-19, ii. The outcome of SARS-CoV-2 infection in pregnancy and preeclampsia as a pregnancy complication, and iii. The role of preeclampsia as a risk factor for infection with SARS-CoV-2 and its complications and its possible mechanisms.

The RAAS pathway

The RAAS pathway consists of renin, angiotensinogen (AGT), ACE/ACE1, ACE2, angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R) components. All these components exist in both systemic and local/tissue RAAS; however, the regulation of local RAAS is independent from the systemic RAAS that is found in many tissues such as kidneys, heart, lungs and blood vessels (10). This enzyme, renin, degrades AGT to inactive Ang I decapeptide. The ACE catalyzes the conversion of Ang I, a decapeptide, to Ang II, an active vasoconstrictor octapeptide. The Ang II elevates the aldosterone secretion and consequently increases blood pressure and inhibits the renin secretion. The ACE2 degrades Ang I into angiotensin (Ang)-(1-9) and also, Ang II to Ang-(1-7) peptides. The Ang-(1-7) exerts its vasodilatory, apoptotic and antiproliferative effects by binding to a G-protein coupled receptor, Mass receptor (11).

Preeclampsia

Preeclampsia, a complication of pregnancy with unknown etiology, is defined as the presence of hypertension and proteinuria after 20 weeks of gestation in a woman that has previously been normotensive. The preeclampsia diagnosis is a challenge, since non-specific signs detection basement. However, some biomolecules such as anti-angiogenic markers, renin angiotensin system related markers, immunological markers, metabolic markers, and endocrine markers have been suggested for early preeclampsia diagnosis (12). Dysfunction of the maternal vascular endothelium and abnormal placentation are risk factors for susceptibility to preeclampsia (13). Hypertension is the most serious clinical symptom of preeclampsia that affects mothers and fetus health. Thrombocytopenia, disseminated intravascular coagulation (DIC), and platelet aggregation are known to be associated with preeclampsia (14).

The criteria of preeclampsia diagnosis include high systolic and diastolic blood pressure (≥140 mmHg, and ≥90 mmHg, respectively), the protein excretion > 300 mg in 24 hours, the ratio of urine protein: creatinine of more than 0.3 and the presence of ≥30 mg/dl protein in a randomly provided urine sample (1+ reaction on a standard urine dipstick). Severe preeclampsia is defined as a blood pressure higher than 160/110 mmHg, more than 3+ urine protein, visual disturbances, headache, upper abdominal pain, increased serum creatinine and transaminases, thrombocytopenia and restriction of fetal-growth. Preeclampsia <34 weeks, gestation is defined as early-onset preeclampsia (8).

The RAAS dysregulation and preeclampsia

In a normal pregnancy the RAAS stimulation results in the increased plasma levels of renin and aldosterone. During pregnancy, pregnant women remain normotensive even in the presence of a two times increase in the Ang II level that could be due to the resistance to the Ang II presser effects and the AT1R down-regulation (15). The angiotensin peptides of Ang II and Ang IV act through the AT2R and angiotensin type 4 receptor (AT4R), respectively. The high expression of both AT2R and AT4R in the early stage of pregnancy contributes to normal placenta formation (16).

The renin angiotensin aldosterone system in the preeclamptic women is suppressed and vascular resistance increases, so, the hypervolemia, a physiologic condition in pregnancy, could not develop. In this pregnancy complication, the RAAS perturbation is correlated with an enhanced vascular responsiveness to Ang II (15). According to a hypothesis the presence of hypertension and proteinuria in the preeclampsia can be attributed to the imbalance in the RAAS and increased abdominal pressure, which may lead to functional or structural renal injuries (17).

The levels of Ang I, Ang II, and Ang-(1-7) peptides are increased during a normal pregnancy. In physiological pregnancy, increased activity of the RAAS is accompanied by reduced hypertensive Ang II function. So, in a normal pregnancy, the reduced hypertensive effect of Ang II along with increased aldosterone secretion allows the proper blood volume circulation in the vascular bed. The main mechanisms that maintain proper organ perfusion for the placenta during pregnancy are the enhanced circulating blood volume and cardiac output. However, in gestational hypertension, decreased the RAAS activity and enhanced sensitivity to Ang II effects have been detected. In preeclampsia, low activity of the RAAS results in the decrease of circulating blood volume and cardiac output reduction. Consequently, these alterations lead to the blood flow decrease in the kidney and placenta that will accompany with an abnormal placental development and followed by impaired intrauterine fetal growth (18).

In an advanced and severe form of preeclampsia, some clinical symptoms such as cerebral edema, hemolysis, renal failure, low platelets count, and elevated liver enzymes, are observed. The profile of RAAS components in the preeclamptic women is greatly different from healthy pregnant women; for example, the vasodilator Ang-(1-7) peptide is significantly decreased in the preeclampsia. The Ang-

(1-7) vasodilator peptide plays a significant role in human pregnancy and along with effect on the renal vascular resistance and renal function, might contribute in the physiologic vasodilation occurring during the pregnancy. The imbalance between levels of Ang II and Ang-(1-7) might be involved in the preeclampsia etiology. Although, dysregulated RAAS contributes to the preeclampsia risk, the triggering factor of RAAS imbalance is still unknown (9, 19).

Systemic inflammation activates the immune response in the brain. There is an interaction between the immune system and the RAAS in the central nervous system which is a contributing factor in the pathogenesis of hypertension. The RAAS and inflammatory factors act synergistically in blood pressure regulation in the brain (20).

Preeclampsia and COVID-19

A survey of 42 consecutive pregnancies indicated 34 cases with non-severe and 8 with severe COVID-19 (Table 1). A preeclampsia-like syndrome in the 6 out of 8 ICU admitted pregnant patients were reported of SARS-CoV-2 affected (21). Moreover, the symptoms of patients with severe COVID-19 were similar to those of preeclampsia. However, no preeclampsia-like symptoms were detected among 34 mildly COVID-19 pregnancies affected. Due to the small sample size and the possible effect of confounding factors, data interpretation needs more caution (22). Furthermore, in a population of 23 UK pregnant women, preeclampsia occurred in 10.5% of patients in the third trimester, one of which even developed liver dysfunction, HELLP and DIC (23). These patients that were included multi ethnic suffered from confirmed COVID-19 with mild symptoms. Since, proteinuria could be associated with severe COVID-19 infection, false positive diagnoses of preeclampsia, might be possible (24). In a report of 2184 pregnant women that 33.2% of them diagnosed with COVID-19 and among them there were 123 women with preeclampsia of which 8.1% had COVID-19, a strong association between COVID-19 with preeclampsia, especially in nulliparous women was detected. This study suggested preeclampsia and gestational hypertension are strong risk factors for SARS-CoV-2 infection and its complications (25). In a meta-analysis among pregnant women with SARS-CoV-2 infection, preeclampsia was one of the risk factors that were correlated with the severe COVID-19 complications such as admission to the intensive care unit (ICU), invasive ventilation and maternal death (26). A case of severe hypertension and heart failure in a woman whose delivery was complicated with both preeclampsia and SARS-CoV-2 was reported to occur after delivery and discharge from the hospital (27). Furthermore, a maternal death occurred in a delivery complicated by preeclampsia and the concomitant presence of postpartum COVID-19. Although, during the delivery patient was asymptomatic for COVID-19, she rapidly developed a severe respiratory distress and coagulopathy very soon after postpartum (28). Also, a pregnant patient concomitantly diagnosed with COVID-19 and preeclampsia has been reported with severe features and preterm birth (29). However, in a report of 9 pregnant women with COVID-19 from Wuhan, China, these women did not show a severe clinical characteristic of COVID-19 pneumonia and were similar to non-pregnant women with COVID-19 pneumonia and only one patient developed preeclampsia (30). Among 20 pregnant women from Peru with confirmed SARS-CoV-2 infection who developed preeclampsia, the severe respiratory symptoms of COVID-19 were absent and 80 % of patients were asymptomatic (31).

Table 1: Characteristics of studies reported pregnant women with and without preeclampsia infected with confirmed COVID-19

Reference	Pregnant women (n)	Pregnant women with COVID-19 (n)	Severity of COVID-19 in pregnant women (n)			Preeclamptic women with COVID-19 (n)	Severity of COVID-19 in preeclamptic women (n)		
			Non-severe	Severe	Death		Non-severe	Severe	Death
21	42	42	34	8	0	6	0	6	0
23	6779	23	15	8	1	2	0	2	0
25	2184	725	292[#]	NA	NA	59	22[##]	NA	NA
26	67,271	41,664	NA	NA	339	316	NA	316	NA
28	1	1	0	1	1	1	0	1	1
29	1	1	0	1	0	1	0	1	0
30	9	9	9	0	0	1	1	0	0
31	20	20	20	0	0	20	20	0	0
36	1	1	0	1	0	1	0	1[*]	0
37	1	1	0	1	0	1	0	1[*]	0

Reference number 26 was a meta-analysis. Severe disease includes Intensive Care Unit admission. *; Complicated with acute fatty liver of pregnancy and acute kidney injury, [#]; 433 women were symptomatic, [##]; 37 women were symptomatic, and NA; Not available.

In general, older age and the presence of greater number of comorbidities were associated with the COVID-19 severity (32). Gestational hypertension as a comorbidity factor is correlated to the severity of COVID-19. SARS-CoV-2 infection reduces the ACE2 availability on the surface of cells through targeted degradation of ACE2 via the clathrin-mediated endocytic pathway. In addition, SARS-CoV-2 affects other components of the RAAS including ADAM metallopeptidase domain 17, ADAM17. This enzyme cleaves the membrane-bound ACE2 receptors and downregulates them. On the other hand, RAAS dysregulation is associated with adverse pregnancy outcomes, especially preeclampsia and restriction of fetal growth. It has been suggested that the unfavorable impact of infection with SARS-CoV-2 on the pregnancy might be due the RAAS pathway disruption, which is the results of degradation and decreased availability of ACE2 by virus binding. Since, the SARS-CoV-2 enters the cells by binding to the ACE2, whose expression is enhanced during pregnancy; ACE2 might contribute to the increased susceptibility of pregnant women to SARS-CoV-2 infection. Moreover, COVID-19 could increase the risk of pregnancy adverse outcomes (4).

Association of preeclampsia with COVID-19 and its severity might be interpreted by the significantly lower levels of Ang-(1-7) peptide during complicated pregnancy, preeclampsia, and dysregulation of placental ACE2 by SARS-CoV-2 (33). Excess amount of ACE2 is expressed in the syncytiotrophoblast, the cytotrophoblast, the endothelium and the villi vascular smooth muscle, which mainly regulates blood pressure and fetus development. It has been suggested that intrauterine SARS-CoV-2 infection might alter the ACE2 expression and increased the Ang II level in the placental villi that lead to preeclampsia. COVID-19 is correlated with cytokine storm and hypercoagulability (34). Also, pro-inflammatory cytokines have been elevated in the both COVID-19 and preeclamptic patients. Furthermore, the hyperinflammatory state in preeclamptic women has been confirmed with maternal high serum ferritin levels (35).

Acute fatty liver of pregnancy (AFLP) is a rare variant of preeclampsia with an incidence rate of 1 in 13000 that could result in the mother and fetus mortality. There are two case reports of pregnant women in the late pregnancy who simultaneously had severe preeclampsia complicated by AFLP and acute kidney injury following infection with SARS-CoV-2. The women recovered and discharged from hospital. In pregnant women the SARS-CoV-2 infection can predispose them to hepatic dysfunction. The necessity of health care and awareness of complex synergistic interaction between COVID-19 and hypertensive disorders of pregnancy are considerable that in the patient with AFLP and multi-organ dysfunction are remarkable and more important (36, 37).

Pregnancy and the severity of COVID-19

Pregnant patients infected with SARS-CoV, and Middle East Respiratory Syndrome (MERS)- CoV had more adverse outcomes of pregnancy, including intrauterine growth restriction, spontaneous miscarriage, and premature delivery. And also, the rate of mortality among infected pregnant patients was more than the general infected population (25 vs. 10%, respectively) (23). Among French pregnant women, mostly symptomatic it was revealed that COVID-19 can be serious and acute in pregnant women. Although, the most serious COVID-19 was observed in women with the highest rates of comorbidities such as obesity, diabetes mellitus, hypertension, and advanced age (33). A retrospective study was conducted to compare clinical outcomes and the results of laboratory tests of COVID-19 infected pregnant women of $\geq$ 20 weeks gestational age with non-pregnant women from four large hospitals of France and Belgium. This study found a significant high risk of ICU admission among pregnant women in comparison with non- pregnant women. Also, they did not report mortality in the groups (38). This study along with a report from Iran with 7 out of 9 deaths due to severe COVID-19 in their pregnant women (39) and one study from Sweden with higher risk of ICU admission in the pregnant and postpartum women with COVID-19 in comparison with non-pregnant women (40) indicated more severe outcomes of COVID-19 in pregnant women than their non-pregnant women. However, Blitz et al. (41) evaluated the data from a large hospital system in the New York State, USA among hospitalized women with COVID-19 and reported that the risk of ICU admission in the pregnant women was not higher than the non-pregnant women. In a retrospective study of Wuhan, China patients (n=82), an increase in the COVID-19 susceptibility and its severity were not observed in the pregnant women (n=28) in comparison with the non-pregnant women (n=54) (42).

Maternal COVID-19 and placental infection

The infection of the placenta and SARS-CoV-2 vertical transmission is rare. In a meta-analysis including 1316 pregnant women infected with SARS-CoV, MARS-CoV, and SARS-CoV-2, no transmission of CoVs from the mother to the fetus was detected (43). In the Di Mascio et al. study, analysis of a small number of cases (n=41) suggested that COVID-19 was associated with a higher preterm birth rate, preeclampsia, cesarean delivery, and prenatal death, with no clinical evidence of vertical transmission (27). Also, no evidence of vertical transmission through intrauterine infection in the COVID-19 pneumonia affected woman in the late pregnancy was found (30). However, significant abnormalities in the placenta morphology have been detected among COVID-19 infected patients (44). Examination of 16 placentas from mothers with COVID-19, including 15 live births and one intrauterine fetal death that demonstrated an increased prevalence of maternal vascular malperfusion in the COVID-19 patients, placentas compared with controls, a pattern indicative of injury of the placenta that is associated with adverse perinatal outcomes (45). Although, one COVID-19 infected pregnant woman in the

second trimester was reported that placental infection and severe early-onset preeclampsia were detected. This study suggested that COVID-19 might contribute to placental inflammation and consequently result in early-onset preeclampsia. Both infections with SARS-CoV-2 and hypertensive disorders of pregnancy decrease the ACE2 activity which leads to enhanced tissue levels of Ang II. It seems that RAAS dysregulation in the COVID-19 patients might contribute to hypertensive complications of pregnancy such as preeclampsia; and COVID-19 may increase the severity of these complications by infecting the placenta (46). The ACE2 is involved in the placentation and also plays a role in the multiple pregnancy adverse outcomes, including miscarriage, ectopic pregnancy, and preeclampsia. Regarding its presence in the placenta, it is likely SARS-CoV-2 might bind to ACE2, which leads to infection and affecting placental function and fetal development; this entity should be elucidated (44).

Diagnostic and therapeutic roles of exosomes in COVID-19 and preeclampsia

Exosomes are nano-sized extracellular vesicles that are secreted by all cell types and have structural similarities with viruses. Exosomes are secreted by cells infected with viruses and mediated in communication between infected and un-infected cells. Also, the contents of exosomes induce the inflammation through activation of the receptors of the cells. So, it seems exosomes be involved in the propagation of the SARS-CoV-2 and inflammation induction contributing to organ dysfunction in the severe COVID-19. Also, theses extracellular vesicles are involved in the pathogenesis of pregnancy complications such as preeclampsia. Due to modulation of the production and composition of exosomes by SARS-CoV-2, they can be used for COVID-19 diagnosis and also could have therapeutic benefits in the COVID-19. The engineered exosomes can be used as a therapeutic tool for different, diseases (47, 48). The exosomal contents can be released into the circulation. This various content can be acted as potential diagnostic biomarkers. Exosomes can be used in the early detection of complications of pregnancy including preeclampsia. In preeclampsia, exosomes contain specific miRNA, DNA and proteins that secreted by trophoblasts and could help in the preeclampsia onset prediction much earlier than blood protein markers (48). Also, the engineered exosomes can be used as different antiviral therapeutics types including COVID-19 treatment.

Conclusion

According to the literature, many studies reported higher severity of COVID-19 among pregnant women in comparison with non-pregnant women. And also, there are more adverse pregnancy outcomes such as intrauterine growth restriction, premature delivery, and spontaneous miscarriage in the pregnant women infected with SARS-CoV, and MERS-CoV. Also, an association between COVID-19 with preeclampsia and the role of preeclampsia and gestational hypertension as risk factors for infection with SARS-CoV-2 and its complications is suggested. There were rare reports of placenta infection and SARS-CoV-2 vertical transmission. Although, many studies reported contribution of COVID-19 to placental inflammation and pregnancy complications such as preeclampsia, there are still some studies that indicated no increased in the COVID-19 severity among pregnant women. Discrepancies in the studies could be due to differences in the characteristics and prevalence of risk factors among populations or ICU admission threshold. More studies are necessary to confirm the greater COVID-19 severity in the pregnant women and its role in development of pregnancy complications. However, until then, COVID-19 is supposed to influence the pregnancy outcomes or susceptibility of pregnant women to severe forms of COVID-19 by the following mechanisms: i. In both SARS-CoV-2 infection and hypertensive disorders of pregnancy, including preeclampsia, the activity of ACE2 decreases, which results in increased Ang II, decreased Ang-(1-7) and imbalance between the levels of Ang II and Ang-(1-7). Therefore, COVID-19 might contribute to the pathogenesis of hypertensive disorders of pregnancy such as preeclampsia through RAAS dysregulation, which is itself a major mechanism of pregnancy hypertension. Ii. Severe forms of COVID-19 are associated with the presence of comorbidities; furthermore, hypertensive disorders of pregnancy are also related to comorbidities, which might explain the severity of COVID-19 in these patients. Iii. There is an interaction among the immune system, inflammatory cytokines and the RAAS and its contribution to the pathogenesis of hypertension. Among patients with COVID-19 and also in preeclamptic patients, there are increased pro-inflammatory cytokines and hyper-inflammatory state that could be considered in the evaluation of the disease severity in these patients.

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Authors' Contributions

B.S., Z.R.: Contributed to study conception and design, preparation of final manuscript, and writing of the manuscript. M.S., Z.M.A., F.M., M.Sh., R.M., S.R.; Contributed to the design, and writing the initial draft. Z.R., Z.M.A.; Editing and revising. All authors read and approved the final manuscript.

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Review Article

Effects of The Mitochondrial Genome on Germ Cell Fertility: A Review of The Literature

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Abstract

Infertility is one of the major problems faced in medicine. There are numerous factors that play a role in infertility. For example, numerous studies mention the impact of the quantity and quality of mitochondria in sexual gametes. This is a narrative review of the effects of the mitochondrial genome on fertility. We searched the PubMed, Science Direct, SID, Google Scholar, and Scopus databases for articles related to "Fertility, Infertility, Miscarriage, Mitochondria, Sperm, mtDNA, Oocytes" and other synonymous keywords from 2000 to 2020. The mitochondrial genome affects infertility in both male and female gametes; in sperm, it mainly releases free radicals. In the oocyte, a mutation in this genome can affect the amount of energy required after fertilisation, leading to gestation failure. In both cases, infertile cells have substantially less mitochondrial DNA (mtDNA) copies. The effects of mtDNA on gamete fertility occur via changes in oxidative phosphorylation and cellular energy production. Also, a reduction in the number of mtDNA copies is directly associated with sex cell infertility. Therefore, evaluation of the mitochondrial genome can be an excellent diagnostic option for couples who have children with neonatal disorders, infertile couples who seek assisted reproductive treatment, and those in whom assisted reproductive techniques have failed.

Keywords: Infertility, Mitochondria, Mitochondrial Genome, Oocyte, Sperm

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Introduction

The mitochondrion has emerged from prokaryotic ancestors, the Proteobacteria species, and continues to survive as endosymbionts in eukaryotic cells (1). Mitochondria is a double-membrane organelle found in most eukaryotic cells that is primarily responsible for cellular energy production. In addition to providing energy to cells, mitochondria are involved in several other tasks, including signal transduction, cell differentiation, death, and cell cycle control and growth (2).

In somatic tissues, mitophagy is a part of the cellular cycle mechanism responsible for destroying damaged mitochondria. Any deviation from this process can lead to mitochondrial dysfunction and the accumulation of defective organelles, which plays a major role in the aging process (3, 4).

Mitochondria, unlike other organelles of animal cells, tend to rely on their DNA. Hence, their work depends not only on mitochondrial DNA (mtDNA) itself, but also on proteins transcribed from nuclear DNA (5).

One of this organelle's unique properties is that a specific genome is conserved and has many copies. Most prokaryotic ancestral genes may have been lost during endosymbiont evolution or transferred to the eukaryotic host nuclear genome (4).

mtDNA is a small genome inherited from the mother. A mature oocyte contains more than 150,000 copies of mtDNA, while sperm only contain about 100 copies. Mitochondrial oxidative phosphorylation is suggested to be an essential determinant of oocyte quality and sperm motility (6).

There are two chains of human mitochondrial DNA - heavy and light. Heavy chains have extensive amounts of guanine and encode 12 subunits of the oxidative phosphorylation process and two ribosomal RNAs (12s and 16s tRNA). On the other hand, the light strand encodes one subunit and eight tRNAs. As a result, mtDNA generally contributes to oxidative phosphorylation, encoding two rRNAs, 22 tRNAs, and 13 protein subunits (7).

Conservation of the mitochondrial genome is presumed to be due to the noticeably hydrophobic nature of these 13 proteins encoded by this genome (8). Transportation of these hydrophobic proteins between the cytoplasm and

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the mitochondrial matrix via the internal and outer membranes is considered problematic. Although mtDNA is noticeably polymorphic, it appears to have an adequately conserved gene content and structure as evidenced by the results of animal studies to date, which have shown similar sets of mitochondrial genes (9, 10).

The mitochondria pleiotropic elucidates the main position of these organelles in the aging process, and in numerous genetic and neurodegenerative illnesses, cancer, weight problems, and diabetes (11). There is growing evidence that mitochondria additionally contribute to fertility, and mitochondrial defects may be at least partly responsible for male and female infertility. Mitochondrial defects seem to affect, not just the gametes, but also the fertilisation process and early embryonic development (12).

Various mitochondrial DNA and, consequently, mitochondrial functional defects can decrease fertility of the male and female gametes, deteriorate foetal health, and lead to foetal loss (13, 14). Although some mtDNA defects do not directly affect fertility, they can be transmitted to the foetus through various diseases. Mitochondrial DNA defects in oocytes are transmitted directly to the foetus; thus, they can be transmitted more effectively than sperm mitochondrial defects. Unlike oocytes, the mitochondrial genome in sperm has an indirect effect on fertility (15). Therefore, in this narrative review study, we intend to evaluate and report the impact of the mitochondrial genome on pregnancy and infant health.

In this narrative review, we selected a set of related articles from 2000 to 2020 obtained after a search result of the terms "Fertility, Infertility, Miscarriage, Mitochondria, Sperm, mtDNA, Oocyte", and other synonymous keywords in the PubMed, Science Direct, SID, Google Scholar, and Scopus databases. A variety of studies from different categories were included in this paper. Then, a subset of documents was evaluated and selected based on their titles and abstracts. After the search and evaluation phases, the papers were carefully studied, and the causes of mtDNA defects and their associated disease characteristics were registered and categorized. The data obtained were recapitulated, then organized and presented in this study. The Ethics Committee of Kurdistan University of Medical Sciences, Sanandaj, Iran approved this study (IR.MUK.REC.1399.184).

The role of the mitochondrial genome on sperm properties

First, we examined the effects of the mitochondrial genome on sperm fertility. Energy production along with apoptosis are two primary roles of mitochondria in spermatogenesis. In addition, mitochondria are involved in several effective processes in spermatogenesis and fertility. The sperm mitochondria are located on the margins of the tail microtubules in order to provide the necessary energy for motility. The mitochondrial volume is related to the flagella's period and frequency (16). The effects of mitochondria in germ cell proliferation, mitotic regulation, and cell destruction via apoptosis are well-known (17).

Sperm DNA integrity is one of the vital determinants of fertilisation and sperm fertility; both endogenous and exogenous factors can damage and endanger foetal health. Clinical studies suggest that men with idiopathic infertility appear to have moderate to severe sperm DNA damage. The most crucial source of this DNA damage appears to be oxidative stress, which is caused by free radicals that are primarily produced in the mitochondria. Due to the high level of unsaturated fatty acids in the membrane and the small volume of cytoplasmic space, sperm cells are more susceptible to oxidative attacks than other cells. Structural defects in the mitochondria, often due to mitochondrial DNA changes, facilitate the release of free radicals into the sperm cytosol, and result in nuclear damage and infertility (13, 18).

Sperm parameters that include motility, capacity, acrosomal reaction, and oocyte interaction depend on mitochondrial regulation of reactive oxygen species (ROS) levels. If the mitochondria fail to supply sperm with motor energy, the sperm physically loses their fertility (2).

Studies show that the proportion of mtDNA deletion in sperms with poor motility and reduced fertility is significantly higher than in those with high motility and good fertility. Male infertility can be defined as low sperm motility (asthenozoospermia) and/or low sperm counts (oligospermia). Some evidence shows that there are mitochondrial genome (mtDNA) mutations in patients with fertility problems; therefore, it is assumed that mitochondrial respiratory chain defects might contribute to male infertility. In addition, the evidence suggests that the frequency of three major 4977, 7345, and 7599 base pair mitochondrial genome deletions in azoospermic and oligospermic patients are higher than in fertile men (19).

Mitophagy is the basis of paternal mitochondrial elimination shortly after fertilisation (20). The paternal mitochondria lose mitochondrial membrane potential ($\Delta\Psi m$) after entering the oocyte, which stimulates mitochondrial destruction (20-23).

The mitochondrial genome is processed and repaired by identical enzymes and mechanisms present in the nucleus. However, mitochondria lack some of these nuclear enzymes; therefore, the mitochondrial DNA repair system appears to be weaker than the nucleus (24). Along with ROS generation and the lack of histone-protective proteins, this poor repair system may be responsible for the high rate of mutations in mtDNA (25, 26).

mtDNA mutations are involved in mitochondrial and age-related diseases. Pathogenic mtDNA mutations are always present in fragments of mtDNA copies. One study has examined sterility in male mice from mutations in DNA. The results showed that a reduction in the copy number of mtDNA was associated with mitochondrial damage to spermatocytes and spermatids in the testes, which resulted in infertility. In contrast, increasing the copy number of mtDNA increases fertility, and it is common for morphology and spermatocyte proteome tests to be performed. Thus, increasing the copy number of mtD-

NA can positively improve the malignant disease caused by mtDNA mutations, which significantly influences the development of future therapies to treat mitochondrial dysfunction (27).

The large 8.7 k-b fragment of mtDNA contains complex III (cytochrome B), complex IV (cytochrome c oxidase, COXIII), complex V genes, and ATPase 6 and 8 synthase genes. A study that assessed the 8.7k-b fragment by long-range PCR showed several deletions in this fragment. The results of this study indicated that the 8.7 k-b fragment deletion frequency in infertile groups was higher than in the control group. In addition, a comparison of different types of infertility showed that the deletion rate was higher in oligoasthenoteratozoospermia (OAT) patients. As a result, they concluded that a significant relationship existed between deletion of the 8.7 k-b fragment and male infertility, which was particularly more effective in OAT subgroups (28).

Examination of sperm mitochondrial parameters is performed to determine semen quality. According to Table 1, these parameters include mitochondrial membrane potential (MMP), mtDNA copy number (mtDNAcn), mtDNA integrity, and apoptotic indexes. These mitochondrial biomarkers are strongly influenced by the individual's lifestyle and predict the quality of semen in the population. The relationship between these mitochondrial biomarkers and semen characteristics is shown in Table 1.

Studies have shown that current drinkers have higher MMPs, and mtDNAcn increases with age (29).

Table 1: Mitochondrial biomarkers and semen characteristics

	Semen volume	Semen concentration	Total sperm count	Sperm motility
MMP	Direct	Direct	Direct	Direct
mtDNAcn	_	Indirect	Indirect	Indirect
mtDNA integrity	_	Direct	Direct	Direct

MMP; Matrix metalloproteinase and mtDNAcn; Mitochondrial DNA copy number.

The role of the mitochondrial genome in oocyte properties

In all mammals, the mitochondria of metaphase II oocytes are tiny organs surrounded by numerous cristae near the dense electron matrix (12). This phenomenon shows weak energy activity and low ATP production (30). Mitochondria then appear to spread to the ooplasm at this stage (31). The internal structure of mitochondria does not change after fertilisation, but these organs undergo nuclear rearrangement in response to energy requirements. Nuclear localisation in blastomeres appears to have been preserved during the first division of embryonic cells (32, 33). Inadequate mitochondrial redistribution may lead to poor oocyte fertilisation and embryonic development (31).

After fertilisation, the oocyte needs an extensive amount of energy to supply important events such as spindle formation, chromatid separation, and cell division. Before the blastocyst is implanted, the developing zygote is mainly dependent on mitochondria for its energy demand. The number of mitochondria tends to decrease with each cell division, and the foetus receives its mitochondria only from the oocyte. Mitochondrial DNA mutations diversify the efficiency of the oxidative phosphorylation pathway and thus the production of cellular energy. Mild destructive mutations may significantly reduce sperm cell function due to the high energy demand and low mitochondrial numbers, but have little effect on somatic cells or oocytes (34).

Mitochondria of the oocytes of infertile women have numerous DNA deletions and nucleotide changes that may hinder their function. A combination of the fewer mtDNA copies and increased number of mutations and deletions in mtDNA can lead to inadequate mitochondrial activity for foetal growth and prompt pregnancy failure (35). The mtDNA copies in patients who suffer from fertilisation failure are significantly lower than those with regular fertilisation. For unknown reasons, the number of mitochondrial oocyte copies in preterm infants was significantly lower compared to patients with *in vitro* fertilization (IVF) failure due to severe sperm abnormalities. Small amounts of mtDNA may be due to cytoplasmic oocyte maturation (36). Compared to younger women, more mtDNA deletions have been observed in oocytes of older women (37). Previous reports on the relationship between oocyte quality and mitochondrial content suggest that low mtDNA content can prevent fertilisation (38).

Studies on mtDNA segregation in pedigrees indicate that in the event of a point mutation, there can be a thorough change in the set of mutations in offspring compared to parents in a single generation (39-41), considering the large number of mitochondrial genome copies in oocytes, assuming that very few mtDNAs can fill the oocyte and, thus the organism. This idea led to the bottleneck theory (42). According to this theory, there can be a significant reduction in mitochondrial counts before they are greatly amplified during ovulation.

Primitive gametes recruit less than ten copies of the original mitochondrial genome to fit the organism. Because the number of mitochondria per cell is low, cells with the best mitochondrial profile can be selected and those with mitochondrial defects can be eliminated. In most cases, this mechanism disrupts the mutant mitochondrial genome and homogenizes the mtDNA population. In order to maintain mitochondrial integrity across generations, deleterious mutations, such as deletions, are usually eliminated and not passed from the mother to the ovaries. This mechanism also significantly reduces the rate of maternal transition point mutations or can inadvertently amplify them (40).

Thus, the bottleneck theory explains how mtDNA is renewed and purified from descendant to descendant through a "narrow neck". Following a sharp decrease in mtDNA content on bottleneck removal, germ cells increase the selected range of mitochondria by clonal selec-

tion and allow individuals to have a homogeneous group of mtDNAs (43).

mtDNA is highly polymorphic in single nucleotide polymorphism (SNPs) haplogroups and subhaplogroups, which reflects species migration and phylogenetic evolution (44). An increasing number of reports suggest that various mtDNA haplogroups that contribute to various mitochondrial respiratory chain activities may be associated with specific human diseases. The effect of specific haplogroups on mtDNA on human fertility has been shown, and a link has been found between different haplogroups and sperm quality. For example, high intake of haplogroup T is associated with a decrease in sperm motility and patients with complex I and IV respiratory chain dysfunction (45). Similarly, high presentation of haplogroup U is associated with reduced sperm motility and vitality (46). The consequences of mtDNA polymorphisms at the molecular level can prompt biochemical abnormalities in specific cells if associated with environmental factors. The possibility of mtDNA haplogroups that affect oocytes and embryonic well-being is an ongoing area of research. Animal studies suggest that specific polymorphisms in the mtDNA control region can affect the proportion of high developmental ability oocytes retrieved by the ovum pick-up (OPU) procedure and the rate of transferable nuclear transfer embryos (47). In addition, the results of animal studies showed that haplogroups could affect the fertility rate. In this study, further analysis through pairwise polymorphism comparison, which resulted in two significant haplogroups, showed that the blastocyst formation rate also varied significantly between these two groups. These results suggest that foetal growth ability might be affected by mitochondrial haplogroups; however, this hypothesis is still being tested at a large scale (48).

Mitochondria undergo constant destruction and renewal in cells, as a quality control mechanism to ensure efficacy. Mitochondrial destruction involves a selective form of autophagy (referred to as mitophagy) through which the entire organelle, including its mtDNA molecules, is destroyed. Significantly, this intrinsic pathway is associated with mitochondrial dynamics. On the one hand, the fusion of mitochondria is made possible by mixing the defective mitochondrial content with functional content. On the other hand, mitochondrial fission causes the dysfunctional organelles of the mitochondrial network to be deleted by preventing their further fusion and completion, prompting their destruction through mitophagy.

The rate of mitochondrial demolition specifies whether oocyte oncotic cell death (after significant demolition) or apoptotic cell death (after milder demolition) or early foetal growth continues before foetal cell death (49, 50). This variation in type or cell death time is likely due to a decrease in ATP levels. Oncosis, a process that does not require ATP, occurs in oocytes that are greatly reduced by ATP, while apoptosis (a process that requires ATP) (51) occurs in oocytes or early embryos that are damaged but can still be preserved. The low ATP levels in these observations can be easily explained by impaired mitochondrial ATP production.

Interestingly, injection of approximately 5000 mitochondria into the oocytes in rats with high ovarian atresia resulted in reduce the severity of apoptosis to a significant 70% in the control oocytes (without medication mitochondrial or no buffer injection only) compared to 36% injected eggs with mitochondria .Oocyte mitochondrial DNA defects can be associated with various diseases (52). Diseases of mitochondrial DNA comprise a series of diseases that are either inherited or caused by mutations. People with mitochondrial DNA diseases may have a combination of healthy and mutated mitochondrial DNA in their cells, called heteroplasmy, which could affect the severity of their disease. Despite the effect of sperm on the quality and the result of fertilisation, the foetus acquires the mitochondrial diseases from oocytes; sometimes, mitochondrial replacement therapy is the only way to prevent transmission of these diseases (53).

Previous reports have shown that the average mtDNA copy number did not significantly differ in dystrophic ovary patients compared to their control groups, but it was dramatically lower in the oocytes of women with ovarian failure ($P<0.0001$). The two ends of the mtDNA copy number spectrum were distinct, and there was no interference between patients with ovarian failure and those with typical characteristics. These results showed that a decrease in mtDNA content was associated with the poor oocyte quality observed in ovarian failure.

These findings are indicative of abnormal mitochondrial biosynthesis and possibly cytoplasmic immaturity during oocyte development. In case of ovarian dystrophy, impaired oocyte maturation may not be associated with mitochondrial biosynthesis. These changes in egg maturation may explain the clinical outcomes observed in these two syndromes. A diffuse deficiency of the oocyte group indicates ovarian dystrophy, but a subset of mature oocytes may still develop into quality embryos after fertilisation. This vastly different from ovarian incompetence, which is characterized by poor quality oocytes and embryos. According to recent reports, a decrease in mtDNA in immature oocytes associated with ovarian failure may be due to a mutation in a specific mtDNA polymerase (POLG1), which is responsible for the onset of menopause (54, 55). Since POLG1 and TFAM play a crucial effect in mtDNA replication and there are strong links between ovarian failure and early menopause, it has been hypothesized that insufficient replication capacity of mtDNA due to POLG1 mutations may lead to the same mtDNA removal that we studied (56, 57).Contributions to the study of the reduced expressions of the mitochondrial genes *ND2*, *COI*, *COII*, *ATPase 6*, *COIII*, *ND3*, *ND6*, and *Cyt b* in infertile human oocytes support the idea that mitochondrial quality is closely related to the viability and growth of oocytes. Foetal growth is highly dependent on ATP content (58).

Although paternal mitochondria are not directly inherited, they do affect the results of fertilisation and fertility. In some cases, damage to mitochondrial DNA can also damage the mitochondrial structure and lead to free radical release. Because of the characteristics related to sperm structure, these free radicals mainly affect the sperm and cause infertility (59). Deleting mtDNA copies in sperm leads to reductions in fertility, asthenozoospermia and oligospermia, and respiratory chain disorders; these deletions increase with age. A decrease in the number of mtDNA can distort both normal differentiation and growth of spermatocytes and spermatids in the testes. In contrast, by increasing the number of mtDNA copies, fertility can be improved, which could act as a basis for developing treatments for various types of infertility. Mitochondrial parameters such as MMP, mtDNAcn, mtDNA integrity, and apoptotic parameters for determining the quality of semen can be used to determine the causes of infertility.

Conclusion

Most mitochondrial genetic defects are inherited from the mother through the oocyte; thus, the maternal mitochondrial genome is critical. The foetus needs an extensive amount of energy for the post-fertilisation events. Still, harmful mutations in mtDNA impair the oxidative phosphorylation pathway and cellular energy production, which lead to miscarriage. Like sperm, the number of mtDNA copies in the oocyte is directly related to cell fertility, such that infertile cells have substantially fewer copies than fertile cells. As a result, low mitochondrial DNA content due to insufficient mitochondrial biogenesis or cytoplasmic maturation may adversely affect oocyte fertility. Low mtDNA counts constitute a significant cause of infertility in older women.

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Authors' Contributions

A.A.; Contributed to the concept, design, and draft of the manuscript. R.R.D., B.K.; Design, performed the literature search and screening, data extraction, manuscript draft, and obtained Ethical Committee approval. M.H.; Drafted the manuscript. All authors read and approved the final version of the manuscript.

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Review Article

Impact of Various Parameters as Predictors of The Success Rate of *In Vitro* Fertilization

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Abstract

A woman who is infertile is defined as a woman who is unable to conceive after having unprotected sex for more than one year. 20-25% of couples worldwide suffer from infertility each year (60 to 80 million couples). *In vitro* fertilization (IVF) plays a significant role in the treatment of various types of infertility, including fallopian tube defects, endometriosis, immunity, and male causes. IVF is a complex procedure that can be used to aid fertility or prevent genetic problems in the fetus. The objective of this review is to investigate factors that affect IVF failure and success rates. Need for this review is predicated on the different results obtained from previous studies, the high prevalence of infertility, and the lack of a similar study in this field. Articles were regarded as suitable if they evaluated the association between any factor and IVF outcome. A comprehensive search of databases was completed from their inception until March 2021. Our search resulted in 1278 articles. After assessing the titles and abstracts, we selected 70 articles for further reading. Our review shows that the effectiveness of IVF treatment depends on many factors. These include the physical environment, genetics, psychological factors, serum levels of certain hormones, sperm characteristics, as well as the age and body mass index of couples. Based on the results of our study, nutrient supplementation maybe beneficial for the enhancement of semen quality. For better IVF outcome, it is better for obese women to balance their body mass index (BMI) before IVF procedure initiation.

Keywords: Infertility, *In vitro* Fertilization, Hormones, Genetics

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Introduction

Infertility, which is defined as being unable to conceive after having unprotected sex for more than one year, affects approximately 20% of couples worldwide and 25% of couples in developing countries. It affects diverse aspects of infertile couples' lives (mental, physical, sexual, and social aspects) and thus requires appropriate interventions (1, 2). Accordingly, understanding the main cause of infertility and choosing the right treatment method within the patient's affordability and availability is very important for designing treatment methods and programs (2, 3).

Assisted reproductive technologies (ART) are usually used as strategies to manage infertility with multifactorial causes (including genetically predisposed diseases). ART has evolved rapidly since 1976, and scientists have been trying to establish the proper approach option for each infertile couple. Among assisted reproductive technologies, *in vitro* fertilization (IVF) is the most popular (4).

IVF is a multi-step procedure meant to help conception or counteract genetic issues. In the first step of IVF, mature eggs are obtained from ovaries. These are fertilized by sperm in the laboratory. The next step is transference of the fertilized egg (embryo) to the uterus. In general, the full IVF course requires about three weeks depending on stimulation protocols, and sometimes these levels are divided into different sublevels, and the process may take more time (5).

Despite continuous advances in IVF and increasing success, only about one-third of women undergoing IVF will become pregnant, and about 60% of cases fail (6). Various factors are considered as causes of infertility, such as infertility duration, lifestyle, recovered eggs, endometrial thickness, number of transferred embryos, and quality of blastocyst, as well as demographic factors, like ethnicity (7).

The efficacy of IVF depends on the treating clinic's overall success rate and the infertile couple's characteristics. Accordingly, among the factors thought to affect IVF success rate are the physical environment, genetics, psychological factors, serum levels of some hormones, sperm and egg characteristics, as well as age and body mass index (BMI) of couples (8-10).

The objective of this review is to investigate the factors that affect IVF failure and success rates.

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This review is focused on studies of multiple factors and their influence on the outcome of IVF technology. Articles were eligible if they evaluated the association between any factor and IVF outcome. Articles were selected if the target population consisted of patients undergoing IVF and intra-cytoplasmic sperm injection (ICSI) procedures.

A thorough search of four databases (PubMed, Embase, Cinahl, and Cochrane) was conducted from their inception until March 2021. The reference lists of review articles and relevant studies were hand-searched to identify other potentially eligible studies. Abstracts from conference proceedings were also considered. No language filters or any other restrictions were applied. Keywords used for the searchers were: IVF, age, BMI, psychological factors, sperm features, ovarian stimulation, hormonal profile, ICSI, IVF, single-nucleotide polymorphism (SNP), and genetic. We downloaded all references identified into EndNote software (version X7). Two authors (Radin Dabbagh Rezaeiyeh, and Arian Mehrara) agreed on the inclusion criteria. Articles were incorporated if they were: original, review, or peer-reviewed research. One author (Amin Mohammad Ali Pour) conducted the initial screening analysis. After removing duplicates and screening the titles and abstracts of the articles, those meeting the inclusion criteria were reviewed. The reference list of every selected article was carefully checked to identify other potentially eligible studies.

Our search resulted in 1278 articles (excluding duplicates). The article selection process was such that articles that did not qualify and did not meet the inclusion criteria were removed at the title screening. Three hundred twenty-one articles met inclusion criteria and were chosen for abstract screening. Out of those 321 articles, 180 were found eligible for full text screening. After assessing the full text of these 180 papers, we selected 70 articles for further reading (Fig.1).

Factors affecting the success or failure of *in vitro* fertilization

In Table 1, we present a summary of factors that affect the failure and success of IVF. Each factor is explained and discussed at the end of the Table.

Table 1: Summary of the factors that have been identified as influencing IVF outcomes and their effects on fertility

Factors	IVF outcomes			Role
	Fertilization rate	Pregnancy rate	Life birth rate	
Demographic				
Age (higher)				
BMI (higher)			↓	
Psychologic		↓	↓	
Depression				
Anxiety			↓	
Distress			↓	Most women undergoing ART are anxious and depressed due to infertility.
Hormonal		↓		Some studies show that the pregnancy rate is lower among distressed women before and during treatment.
LH				
FSH (high level on day 2 or 3 of cycle)				LH prepares the uterine environment for the fertilized egg to grow.
Estradiol (elevated abnormal levels)		↓		Women with higher blood FSH concentration have a lower chance of pregnancy in contrast to women of the same age with lower FSH levels.
Progesterone		↓		Increased abnormal levels of Estradiol are associated with decreased response to ovulation-inducing drugs resulting in reduced IVF success.
AMH				Progesterone prepares the uterus for the arrival of a fertilized egg.
Sperm characteristic (abnormalities)				AMH levels shows a good correlation with ART outcomes, thus it is considered to be the most accurate biomarker for ovarian storage.
Motility				
Number	↑	↑		Higher sperm motility results in higher chance of pregnancy and fertilization
Morphological	↑	↑		Higher Sperm total number increases the chance of oocyte-sperm fusion.
Genetic factors	↓	↓	↓	Sperm deformity is a reliable predictor of fertility success in patients undergoing IVF.
PGD				
Analysis of genetic variants		↑		PGD methods are designed to minimize the possibility of transmitting genetically abnormal embryos after IVF. Theoretically, choosing genetically normal embryos for transmission leads to more successful pregnancies and fewer miscarriages.
				Genetic analysis involved in ART can be based on methods of PGD and analysis of genetic variants affecting the success rate of IVF.

BMI; Body mass index, LH; Luteinizing hormone, FSH; Follicle-stimulating hormone, AMH; Anti-müllerian hormone, PGD; Preimplantation genetic testing, ART; Assisted reproductive technology, and IVF; *In vitro* fertilization.

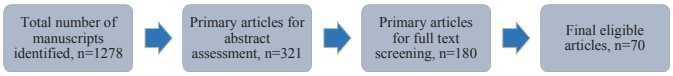

Fig.1: Initial to final search steps for selecting manuscripts.

Age

Women's age is considered among the most important elements affecting the likelihood of achieving pregnancy in ART programs. As age increases, women's fertility and live birth rates decrease significantly (especially after 35 years). Increasing maternal age leads to a decrease in pregnancy rate (although this is similar between IVF and ICSI), fertilization rate, and number of recovered eggs (11, 12). Accordingly, decreased female reproductive capacity increases with age due to the gradual reduction of eggs from the ovaries and a significant reduction in egg quality. Morphological evaluation methods for human embryos have proven that maternal age has a significant effect on the quality of human embryos. In addition, the rate of aneuploidy in human embryos increases with age (13, 14).

The results of the study by Yan et al. (15) showed that older women had a weaker response during controlled ovarian hyper-stimulation (COH), fewer retrieved eggs, low oocyte fertilization rate, low-quality embryo rates, low embryo implantation rate, low delivery, high abortion, and high preterm delivery. Goldman et al. (16) stated that age-related fertility decline had much more effect on the live birth rate at older ages than BMI. As age can't be altered, it can be concluded that spending more time on lowering BMI before IVF is of significant benefit in older women.

Body mass index

In addition to age, the obesity factor is also very important in reproductive support programs. The harmful consequences of obesity on the reproductive system include maternal complications, infertility, and menstrual disorders. Decreased fertility is attributed to various parameters in obese women, such as endocrine and metabolic dysfunctions, which sequentially may affect follicular proliferation, implantation, and the growth of clinical pregnancy (17). Hence, monitoring the effects of body weight during IVF is of paramount importance. Many studies have demonstrated that pregnancies and live birth rates in overweight and obese individuals are reduced in comparison to women of normal weight (18, 19). Possible causes of these discrepancies include increased gonadotropin requirements during ovarian stimulation, fewer recovered oocytes, decreased serum estradiol concentrations, and low fertilization rates. In contrast, other studies have shown that obesity has no effect on gonadotropin requirement, and the number of ovarian stimulation days does not affect estradiol levels (20, 21).

It has been found that obese women who become pregnant after IVF are at risk of miscarriage and obstetric difficulties generally. But it must be taken into consideration that whether achieving a specific BMI and spending a lot of time losing weight before the beginning of the IVF cycle is detrimental to the possibility of live birth, given that the woman is constantly ageing. Obesity increases ovulation induction time, decrease edtradiol peak, and decrease the number of mature follicles. In addition, obesity may adversely affect the quality of eggs and embryos (22, 23).

Dokras et al. (22) observed approximately 1,300 patients in one study, the IVF failure rate was 25% in obese women in comparison to 10.9% in normal weight women. In addition, IVF failure in obese women with polycystic ovary syndrome (PCOS) is more probable than in normal women. Contrary to these findings, many studies, including the study by Kim et al. (24), did not find any considerable gap in clinical pregnancy rates between obese and normal weight women. In a recent study Maged et al. (25) found that implantation, chemical pregnancy, and clinical pregnancy rates were inversely related to increasing BMI. From January 2013 to February 2018, Hallisey et al. (26) performed a cohort study. Their population included women aged over 45 years who underwent IVF with PGT (preimplantation genetic testing). Five hundred thirty-three cycles were separated into 3 groups of women categorized by BMI as normal, overweight, and obese. Euploidy rate was the primary outcome. Their study showed that a higher miscarriage possibility in women with higher BMI and a lower probability of having a live baby after IVF. They stated that the root cause of these discrepancies is currently unknown and it is accepted that obesity may be associated with higher rates of aneuploidy, which can lead to worsening pregnancy outcomes. The dosage of gonadotrophin required for ovarian stimulation is higher in women with BMI more than 25 kg/m^2 is also but not the ovarian stimulation duration (27).

Psychological factors

Despite the prevalence of infertility, most infertile women do not share their stories with family or friends, thus increasing their psychological vulnerability. Inability to reproduce normally can result in low self-esteem and feelings of shame and guilt. These negative emotions can lead to different levels of depression, nervousness, distress, and poor quality of life. Most women undergoing ART are frequently anxious and depressed due to infertility. Nearly 32% of women in the early stages of infertility treatment are at risk of mental disorders (28-30). Several studies have examined the relationship between psychological symptoms before and during the ART cycle and subsequent pregnancy. These have provided conflicting results. Some have shown that the pregnancy rate is lower among distressed women before and during treatment, while other studies have not found such a result (31). Several plausible psychological pathways play a role in the likelihood that a woman's distress will affect her fertility or may disrupt infertility treatment success. These pathways include the hypothalamic-pituitary adrenal (HPA) axis, which plays a role in stress response regulation, and the hypothalamic-pituitary gonadal axis

(HPG), which regulates reproduction (32). The physiological pathways that influence psychological factors involved in pregnancy are still generally unknown, but these factors can be associated with incomplete ovulation, secondary amenorrhea, and irregular menstrual periods. Various mechanisms have been proposed for the negative effect of psychological factors on infertility, including impaired gonadotropin secretion, local effect of catecholamine on the uterus and fallopian tubes, and impaired immune processes involved in maintaining fertility (33). Cesta et al. (34) studied women who received infertility treatment from September 2011 to December 2013 and followed them until December 2014. Before IVF initiation, data was gathered through an online questionnaire as well as clinical charts. Cortisol from saliva samples was measured and the correlation between stress and cycle outcomes (embryo and oocyte quality parameters and clinical pregnancy) was examined. Unexpectedly, it was revealed that women with higher salivary cortisol concentration had no different IVF outcome than women with normal cortisol levels. Psychosocial care could be helpful for couples experiencing infertility treatment. It has been established that psychosocial care can be effective in decreasing plasma cortisol levels and psychological distress and can improve the clinical rate of pregnancy significantly. Another study by Cui et al. (35) showed that depression during IVF has adverse effects on pregnancy outcomes. Accordingly, measurement of angiotensin II and salivary amylase may be a reference indicator for patients' psychological status during IVF.

Hormonal profiles

Simultaneous combined endocrine events involving the anterior pituitary, hypothalamus, and ovaries are a reflection of the menstrual cycle. These events are important for successful ovulation, egg growth, implantation, and fertilization. In general, the levels of follicle-stimulating hormone, estradiol, luteinizing hormone, and anti-mullerian hormone affect the success or failure of IVF, so it is necessary to check these hormones before performing any ART. Gonadotrophins [follicle-stimulating hormone (FSH) and luteinizing hormone (LH)] are used for ovulation during IVF procedure (36). The functions of some of these hormones are listed below:

Follicle-stimulating hormone

FSH helps in regulating the menstrual cycle and producing eggs. On day 2 or 3 of the menstrual cycle, FSH levels are tested to determine ovarian function and assess egg quality. Generally, women with high levels of blood FSH on day 2 or 3 of the menstrual cycle have a smaller chance of having a live baby than other women of the same age, even with ovulation induction and IVF (37). Abdalla and Thum (38) studied all patients who were candidates for IVF/ICSI treatment between January 1997 and December 2001 in Lister hospital, London. Patients were divided into four groups by FSH level. Follicle maturity, miscarriage rate, pregnancy rate, live birth rate, were defined as outcome measures. The authors concluded that an increase in basal FSH levels did not indicate a deterioration in egg and embryo quality and did not lead to a decrease in fertilization or an increase in abortion. The findings of this study showed that the decrease in pregnancy rate is due to the reduction in the number of eggs collected and consequently the limited selection of available embryos for transfer.

Estradiol

Besides the quantification of FSH, ovarian function and egg condition can be evaluated by measuring estradiol (an important form of estrogen). Estradiol is also examined on day 2 or 3 of the menstrual cycle. These test results are not definite indicators of infertility, but increased abnormal levels are associated with decreased response to ovulation-inducing drugs resulting in reduced IVF success. The human corpus luteum (CL) produces significant amounts of progesterone (P4), estradiol (E2), androgens, growth factors, and nonsteroidal hormones. The overall maintenance of CL function depends entirely on the regular stimulation of pituitary luteinizing hormone (LH) or human placental gonadotropin (hCG) to maintain steroidogenesis in granulosa cells (39). Drakakis et al. (40) assessed the effect of estradiol support on IVF success. They performed their prospective study in the assisted reproduction unit of the First Department of Obstetrics and Gynecology of the Athens University Medical School, from August 2004 to February 2005. They examined patients who were under IVF/ICSI treatment. Implantation and pregnancy rates assessed in the two groups were considered as major outcome measures. They found a steep elevation in implantation rate and pregnancy rate in women who received luteal phase estradiol support compared to women who did not. However, the mean number of fertilized oocytes, transferred embryos, and retrieved oocytes approximately remained the same.

Luteinizing hormone

LH stimulates the ovaries to release eggs and begin to produce progesterone (a hormone that prepares the uterine environment for the fertilized egg to grow). LH can be detected in a woman’s urine just before ovulation. Urine LH tests are frequently conducted to help with the timing of intercourse to raise the chance of pregnancy (41).

Abbara et al. (42) showed that there was an unexpected negative association between increased progesterone and LH levels during egg maturation. In addition, elevated progesterone appears to be the most reliable biochemical predictor of oocyte maturation following all stimulation factors.

Progesterone

Right after ovulation, the ovaries produce progesterone. Progesterone prepares the uterus for the arrival of a fertilized egg approximately in the middle of the cycle - 12

to 16 days after the first day of the menstrual cycle. Progesterone concentration generally peaks within 7 days of ovulation, and the amount of blood progesterone can be measured through blood tests. When a basal level of blood progesterone is established, the doctor will order a mid-luteal serum progesterone test around day 21 of the menstrual cycle (43). Tulic et al. (44) at the Gynecology and Obstetrics Clinic Center of Serbia conducted a cohort study. The study included all patients who met the criteria of inclusion (infertility diagnosed, 18-40 years, regular menstrual cycle, 18-30 kg/m^2 BMI, 18-40 years, without a legal guardian), enrolled in the ART procedure during the study period (January 2015 to December 2015). Embryos were classified into four classes: class A (perfect symmetry), class B (moderate asymmetry), class C (pronounced asymmetry). Main outcome measures included pregnancy outcome and procedure success. They stated that low levels of progesterone on oocyte retrieval day (<2.0 ng) in an ART procedure is associated with high levels of FSH and low levels of AMH and lead to the delivery of healthy infants in more than 50% of cases. However, many researchers have found no significant difference between high levels of progesterone and a reduced pregnancy rate due to different data assessment protocols (45).

Anti-mullerian hormone

AMH is a glycoprotein and known as a member of the growth factors of the β family. This hormone is produced by the antral and small antral follicles in the ovary and plays a significant part in folliculogenesis and determining the number of primary follicles. In addition, AMH levels shows a good correlation with ART outcomes and are thus considered as the most accurate biomarkers for ovarian storage (46). In general, current ovarian stimulation protocols are performed during IVF treatments to personalize protocols based on female AMH levels (47). Although there is no definitive value for normal and abnormal AMH, it is generally accepted that AMH >0.8-1.0 ng/ml indicates normal ovarian reserve (48). It is well accepted that young women with high AMH levels have significant fertility performance, while older women with low AMH levels have poor IVF outcomes. However, due to high individual heterogeneity, there are differences in some patients (49). Güngör and Gürbüz (50) carried out a retrospective study between November 2014 and September 2019 at the Gynecology and IVF Department. They chose a logistic regression model rather than linear regression and negative binomial regression due to better dataset fit. Patients were separated into three groups (15 oocytes or more=excessive ovarian response, 6 to 15 oocytes=normal ovarian response, and 5 oocytes or less=weak ovarian response). They considered the number and the quality of the retrieved oocytes as a means of ovarian response quality. They found that higher levels of serum AMH is associated with higher quality of ovarian response (especially the number and quality of eggs).

Sperm characteristics

In the study of infertile couples, sperm testing is the most important and basic method to assess the cause of infertility and choose the treatment method. Evaluation of sperm characteristics such as motility, total number, and morphological abnormalities of sperm seems to be very important to predict successful fertilization, implantation, fetal growth, and continuous pregnancy. Various studies have been performed on the role of different semen parameters on fertilization and pregnancies after IVF. Higher sperm motility and sperm total number are associated with higher chance of pregnancy and fertilization (51).

Sperm morphology is one of the most important parameters. Sperm deformity is a reliable predictor of fertility success in patients undergoing IVF (52). According to this criterion, when less than 14% of sperm are of normal morphology the pregnancy rate decreases. In cases where normal morphology is less than 4%, the treatment result may be very poor (53).

Another parameter that has been investigated for its effect on reproductive fertility techniques is the age of men. Older age is significantly associated with decreased semen volume, sperm count, motility, and normal morphology (54). Male genital infection is one of the leading causes of male infertility worldwide. Bacterial invasion of the reproductive system has often been shown to be associated with decreased sperm function and lead to infertility (55). The quality of semen is so important for the IVF outcome and nutrients are among the most important factors, affecting the quality of semen (56).

Silea et al. (54) examined 500 semen samples from of patients who sought infertility treatment over 5 years (April 2013-April 2017). They evaluated semen samples both macroscopically and microscopically using WHO criteria published in 2010 as the standard threshold. Their findings showed that sperm volume and pH were not affected by age but that sperm viability and progressive motility decrease with age. According to the results of the study by Morin et al. (57), total motile sperm count (TMSC) is the most significant of all the parameters used to assess sperm quality. In addition, this study, along with other studies, has shown that TMSC counts are superior to WHO criteria in predicting the success of IVF cycles. There is a significant difference between the samples of semen contaminated with bacteria compared to the control group in terms of reduced sperm concentration and a significant reduction in sperm motility (55).

Genetic factors

In recent years, seemingly ineffective genetic differences, known as genetic polymorphisms among healthy people in the community, have been the focus of studies in many multifactorial diseases, including miscarriage and implant failure. In general, the genetic analyses involved in ART are based on methods of pre-implantation genetic

diagnosis (PGD) and analysis of genetic variants affecting the success or failure of IVF.

Genetic analysis to increase the success rate of *in vitro* gertilization outcome

Since IVF technology has emerged, multiple attempts have been made to increase efficiency and success. Accordingly, selecting a healthy fetus is considered one of the most important strategies, every aspect of which has been evaluated. Staessen et al. (58) reported that in women over the age of 37, just 35% of day 3 embryos with over eight cells and 65% of proliferating blastocysts were normal. PGD methods are designed to minimize the possibility of transmitting genetically abnormal embryos after IVF. Theoretically, choosing genetically normal embryos for transmission leads to more successful pregnancies and fewer miscarriages (59). Genetic techniques used to select genetically normal embryos include fluorescence in situ hybridization (FISH), comparative genomic hybridization (CGH), whole genome amplification (WGA), array-CGH, next-generation sequencing, real-time quantitative polymerase chain reaction (RT-qPCR), and SNP arrays.

Single-nucleotide polymorphism affecting *in vitro* fertilization outcome

So far, several studies have been performed on the effects of different SNPs on various aspects of human reproduction, including recurrent miscarriage, infertility, and fetal implant failure. The association of SNPs in various genes, including *MTHFR*, Leiden factor V, progesterone receptor, FSH receptor, plasminogen activating factor (*PAI-1*), prothrombin, and estrogen receptor gene, with different aspects of fertility has been observed.

Several studies show that thrombophilia leads to repeated implantation failure. Leiden factor V genetic mutations and prothrombin G20210A mutations have generally been shown to lead to failure of ART (60).

The *P53* gene is considered to be one of the genes most integral to the efficient regulation of different physiological processes, including fertility. This gene interacts with the *LIF-1* gene and plays a crucial role in controlling and regulating the implantation process. LIF levels are seriously reduced in most females with infertility of unknown cause. Some studies suggest that the prevalence of the codon 72 polymorphism in the *P53* gene has a significant effect on implantation rejection rate in IVF cycles (61).

Growing evidence suggests that vitamin B status may modulate infertility treatment outcomes. Water-soluble B vitamin folate is believed to be vital for biosynthetic and epigenetic processes and furthermore, regulate the synthesis and methylation of nucleic acids and proteins. As a result, folate has been proven to be essential during follicular and embryonic developmental periods (62). The *MTHFR* gene is one of the key genes in the folate pathway. The *MTHFR*: c.677C>T polymorphism results in a significant change in folate concentration. Because enzyme activity and serum folate concentrations are highest in people with CC wild genotype, this is considered to be the most effective genotype for health. However, current discoveries suggest that, according to the IVF treatment outcome, the heterozygous CT genotype of *MTHFR* in nucleotide 677 of the mother results in a higher percentage of good quality embryos and a major chance of clinical pregnancy compared to the homozygous CC and TT genotypes. Consistent with these outcomes, it has been shown that the CT genotype, instead of the CC genotype in the woman significantly increases the chances of getting pregnant with IVF treatment (63). Another polymorphism studied is the *MTHFR*: c.1298A>C, which is associated with higher concentrations of basal FSH and a reduced reaction to ovarian stimulation. The study by Rosen et al. showed that the CC genotype reduced the ovarian response to FSH stimulation compared to the AA and AC genotypes (64).

The solute-carrier gene (SLC) superfamily encodes membrane-bound transporters. A prospective study by Haggarty et al. (63) carried out from October 2000 to September 2004 included 602 women undergoing fertility treatment. Plasma and red-blood-cell concentrations were measured by radioimmunoassay and the absorbed amount of vitamin B12 and folate were evaluated through a questionnaire. Five B-vitamin-associated-gene variants were measured in women who were treated, as well as 932 women who conceived naturally. They found that the *SLC19A1* c.80G>A polymorphism increased homocysteine (Hcy) concentration in heterozygous GA people compared to patients with wild-type genotype. Higher concentrations of Hcy usually lead to detrimental effects on IVF outcomes.

In addition, other variants involved in the success rate of IVF include MTHFR: c.677C>T and CTH (cystathionine gamma-lyase) c.1208G>T. Accordingly, heterozygous individuals have favorable IVF results for these variants compared to wild-type homozygous individuals (65).

The *LHB* gene (Luteinizing hormone beta) is located in the 11p13 chromosome region and has three exons. Trp8Arg, Ile15Thr, and Gly102Ser polymorphisms lead to menstrual irregularities, infertility, and recurrent miscarriages. Furthermore, in women undergoing IVF treatment, these variants have been shown to play a marked role in the IVF success rate (66).

Growth differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (BMP15) gene are expressed in oocytes from primary phase follicles. Both proteins play a key role in specifying follicle growth and ovulation rate. Accordingly, polymorphisms in these genes (*GDF9*: c.546G>A, *BMP15*: c.2673C>T, c.29C>G, IVS1+905A>G) are also associated with fertility success rates and increased occurrence of dizygotic twins (67).

One of the important genes in the pathway of ovarian metabolism is the aromatase gene (*CYP19A1*). Aromatase is viewed as one of the main enzymes in ovarian ster-

oidogenesis, which catalyzes the ultimate stage of conversion of testosterone and androstenedione androgens to estradiol and estrone. Tetranucleotide repeat polymorphism (TTTA) n in intron 4 of the *CYP19A1* gene leads to aromatase hyperactivity. In general, women with fewer (TTTA) repeats in this gene show lower estrogen concentrations which results in susceptibility to unexplained infertility (68).

Conclusion

The purpose of this review was to summarize the factors that have been identified as influencing IVF outcomes. We have reviewed 70 studies that have reported multiple factors involved in IVF treatment. Prior research has focused on a few factors involved in the IVF success rate.

Predicting the likelihood of pregnancy after IVF can help stop over-treatment and equilibrate the chances of IVF success. In this review, we evaluate 6 predictors, especially genetic factors that can help predict the success of IVF. Based on the available literature, we conclude that female age, BMI, psychological factors, hormonal profiles, sperm characteristics, and genetic factors are predictive factors of IVF success.

In this regard, a wide range of factors could be taken into consideration on which a particular emphasis should be placed. As a matter of fact, nutrients are viewed as one of the essential factors in IVF success rate. With regard to semen quality which is believed to be a vital factor, it proves efficient in IVF outcomes. As nutrients play a massive role in the quality of semen, it is of major significance. Therefore, the consumption of nutritional supplements could have innumerable constructive impacts on IVF. What is more, Females' BMI is considered to be an integral element in the serum level of hormones. Consequently, once BMI decreases and takes place in a normal range, the IVF process could have more productivity and efficiency in obese women. Due to the fact that sperm characteristics are regarded as one of the most basic ways in order to evaluate the cause of infertility among couples. Hence, when visiting an infertility center, this factor is of more importance compared to other elements, and it is better to check sperm-related characteristics first. Moreover, due to unique genetic sequences that vary from person to person, based on their mutations, a unique personal approach could be taken into account. This process could give a tremendous boost to the level of IVF success rate.

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Authors' Contributions

R.D.R., A.M.; Performed the research. A.M.A.P., J.F., S.F.; Designed the research study. S.F., R.D.R, A.M.A.P.; Analyzed the data. R.D.R, A.M., A.M.A.P., J.F.; Wrote the manuscript. All authors read and approved the final manuscript.

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Original Article

Germany Endometriosis Pattern Changes; Prevalence and Therapy over 2010 and 2019 Years: A Retrospective Cross-Sectional Study

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Abstract

Background: The aim of this study was to investigate whether the prevalence and the therapy patterns of endometriosis differ in 2010 and 2019.

Materials and Methods: This retrospective cross-sectional study was based on the data from the IQVIA Disease Analyzer database and included women with at least one visit to one of the 136 private gynecologist practices in Germany in 2010 or 2019. The prevalence of endometriosis as well as prevalence of each endometriosis therapy such as Dienogest, other Progestins than Dienogest, and Gonadotropin-Releasing Hormones, was calculated in both years.

Results: The present study included 346,249 women documented in 2010 and 343,486 women documented in 2019. The prevalence of endometriosis increased from 0.53% in 2010 to 0.66% in 2019 (P<0.001). The proportion of endometriosis patients treated with Dienogest increased significantly between 2010 and 2019 (18.1 vs. 35.0%). The proportion of women prescribed other Progestins than Dienogest has not significantly changed between 2010 and 2019 (8.4 vs. 8.3%). Gonadotropin-releasing hormones were prescribed only rarely in both 2010 and 2019, with a significant decrease in prescriptions between these two years (3.7 vs. 2.0%).

Conclusion: There were significant changes in the prevalence and medical therapeutic patterns of endometriosis in 2010 versus 2019 reflecting changes in therapy guidelines and possibly in diagnostic methods.

Keywords: Dienogest, Endometriosis, Gonadotropin-Releasing Hormones, Prevalence, Progestin

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Introduction

Endometriosis, a chronic disease, is defined as the presence of functional endometrial mucosa outside the uterine cavity, often in the pelvis, but also more rarely in locations such as the pericardium, pleura cavity and even in the brain tissue. It is one of the most common benign gynecological diseases in pre-menopausal women. Symptoms of endometriosis re included pelvic pain, dysmenorrhea, periovulatory pain, dyspareunia, dyschezia, dysuria and infertility. Endometriosis affects approximately 5-10% of reproductive age woman, which equates to around 190 million women worldwide, with prevalence peaking between 25 and 35 years of age (1-3).

Despite of benign proliferative nature, endometriosis shares certain characteristics with neoplastic processes, including inflammatory state, invasion of adjacent tissues, induction of angiogenesis, and resistance to apoptosis (4).

Further research, new therapeutic options have come to the market. Subsequent of this improved knowledge, awareness of endometriosis has increased in the medical communities and the general population. Although therapeutic options have improved, one of the main problems has been remained : correct diagnosis. According to the European Society of Human Reproduction and Embryology (ESHRE) guidelines (2014), the gold standard endometriosis diagnosis is a combination of laparoscopy visualization and histological confirmation subsequent: endometrial glands and/or stroma existence (5).

The laparoscopic surgery is defined as a gold standard for diagnosis, while it is invasive and costly, and endometriosis remains undiagnosed (6).

Despite of various therapeutic options for this condition, medical therapy is placed in the secondary stage after surgical treatment , a gold standard (7-9).

Several medical therapies aim to create a hypoestrogen-

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ic state in the endometriosis affected women Examples of these treatments include gonadotropin-releasing hormone agonist (GnRHa), GnRH antagonist (GnRH-ant) and synthetic androgens (10).

Since laparoscopy is often perceived as excessively invasive by patients, especially young women, it seems impractical as a first-line diagnostic tool. As a result, investigators have sought to identify non-invasive tools such as biomarkers for early diagnosis that might prevent or delay the progression of endometriosis (7). However, a study in 2016 that tested various biomarkers for clinical reliability showed that none of the tested biomarkers had a clinical reliability that was comparable to the current gold standard (8).

Overall, the therapeutic approach for endometriosis must be adapted individually for each patient, as age, fertility, desire to have children, family planning measures, degree of pain and personal impairment and the mode of action and side effects of the medications vary from patient to patient. Above all, it is essential to weigh up the side effects of the preparations.

The aim of this study is to investigate whether the prevalence and the therapy patterns of endometriosis was different in 2010 and 2019.

Materials and Methods

This retrospective cross-sectional study was based on data from the Disease Analyzer database (IQVIA), which includes diagnoses, drug prescriptions, and basic demographic data obtained directly and in anonymous format from computer systems used in the practices of general practitioners and specialists. The database covers approximately 3% of all outpatient practices in Germany. Diagnoses, prescriptions, and the quality of reported data are monitored by IQVIA on an ongoing basis. IQVIA uses summary statistics from all doctors in Germany published yearly by the German Medical Association to determine the panel design according to specialist group, German federal state, community size category, and physician age. This sampling method is appropriate for obtaining a representative database of general and specialized practices (11).

This study included girls and women (14 years or older) with at least one visit to one of the 136 private gynecologist practices in Germany in 2010 or 2019. The selection of the study samples from the database is shown in the Figure 1. The first outcome of the study was the change in the prevalence of endometriosis diagnosis (ICD 10: N80) in the year 2019 compared to 2010. The prevalence was defined as the number of women diagnosed with endometriosis in the selected year divided by the total number of women with at least one visit in the same year. The second outcome was the change in the endometriosis therapy prevalence as estimated for three treatments: Dienogest (ATC: G03DB08), other Progestins than Dienogest (ATC: G03DA04), and Gonadotropin-releasing hormones (ATC: L02AE). The therapy prevalence was defined as the number of women with at least one prescription of a defined drug in the selected year divided by the total number of women with diagnosed with endometriosis in the same year. Both prevalence analyses were also calculated by age group (age 14-20 years, age 21-30 years, age 31-40 years, age 41-50 years, age >50 years).

Totally, all data were analyzed using chi-squared tests for categorical variables and t tests for continuous variables. Chi-squared tests were used to compare endometriosis prevalence and the prevalence of defined treatments. Analyses were carried out using SAS version 9.4 (SAS Institute, Cary, USA).

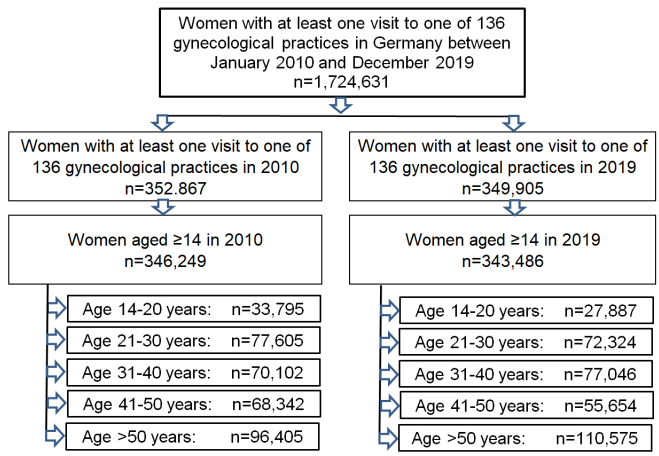

Fig.1: Selection of study sample.

Ethical statement

German law allows the use of anonymous electronic medical records for research purposes under certain conditions. According to this legislation, it is not necessary to obtain informed consent from patients or approval from a medical Ethics Committee for this type of observational study that contains no directly identifiable data. Because patients were only queried as aggregates and no protected health information was available for queries, no institutional review board approval was required for the use of this database or the completion of this study.

Results

Prevalence of endometriosis

The present study included 346,249 girls and women documented in 2010 and 343,486 women documented in 2019 who visited one of 136 gynecologist practices. In total, 1,830 women had a documented diagnosis of endometriosis in 2010 and 2,272 in 2019, resulting in a prevalence of 0.53% in 2010 versus 0.66% in 2019 (P<0.001). The prevalence significantly increased from 2010 to 2019 in all age groups investigated with the exception of the group aged 14-20 years (Fig.2).

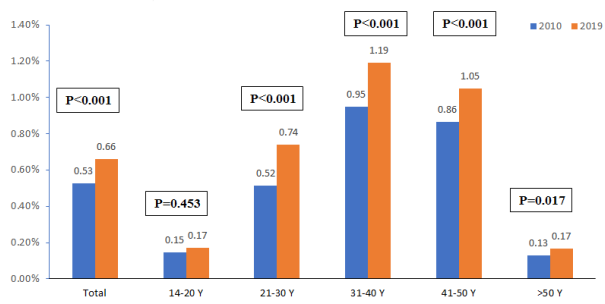

Fig.2: Prevalence of endometriosis diagnosis in the gynecological practices in the Germany in 2010 and 2019.

Baseline characteristics of study patients

The basic characteristics of our patients are displayed in the Table 1. There were no significant differences between 2010 and 2019 in terms of mean age (37.6 years vs. 37.1 years), history of hysterectomy (6.8 vs. 7.8%) and history of other surgical treatment for endometriosis (21.0 vs. 21.3%), respectively. The most common endometriosis diagnosis was unspecified endometriosis (ICD-10: N80.9) with 59.5 in 2010 vs. 61.3% in 2019, followed by endometriosis of the uterus (ICD-10: N80.0), which occurred slightly less frequently in 2019 (13.3%) than in 2010 (16.9%).

Treatment prevalence

Table 2 shows the results of the treatment prevalence analysis. The proportion of women with other Progestins than Dienogest prescription has not changed significantly between 2010 and 2019 (8.4 vs. 8.3%, P=0.912). The proportion of endometriosis patients treated with Dienogest increased significantly between 2010 and 2019 (18.1 vs. 35.0%, P<0.001). Although this increase was observed in all age groups, the proportion of patients treated with Dienogest was highest in the youngest age group (60.4%) and lowest in women >50 years (15.6%). Gonadotropin-releasing hormones were prescribed rarely in both 2010 and 2019, with a significant decrease in prescriptions by 2019 (3.7 vs. 2.0%, P<0.001).

Table 1: Baseline characteristics of our patients

Variable	2010 (n=1, 830)	2019 (n=2, 272)	P value
Mean age in years (standard deviation)	37.6 (SD: 9.3)	37.1 (SD: 9.8)	0.183
Age 14-20 Y	50 (2.7)	48 (2.1)	<0.001
Age 21-30 Y	400 (21.9)	536 (23.6)	
Age 31-40 Y	666 (36.4)	917 (40.4)	
Age 41-50 Y	591 (32.3)	585 (25.8)	
Age >50 Y	123 (6.7)	186 (8.1)	
Endometriosis diagnosis code			
Endometriosis of uterus (N80.0)	309 (16.9)	302 (13.3)	0.001
Endometriosis of ovary (N80.1)	117 (6.4)	174 (7.7)	0.117
Endometriosis of pelvic peritoneum (N80.3)	164 (9.0)	244 (10.7)	0.059
Other endometriosis (N80.2, N80.4, N80.8, N80.5, N80.6)	152 (8.3)	159 (7.0)	0.116
Endometriosis, unspecified (N80.9)	1,088 (59.5)	1,393 (61.3)	0.226
History of hysterectomy	124 (6.8)	177 (7.8)	0.216
History of other surgical treatment for endometriosis	385 (21.0)	484 (21.3)	0.837
Symptoms documented in the year of endometriosis diagnosis			
Abnormal uterine and vaginal bleeding (N91-N93)	432 (22.3)	537 (23.6)	0.232
Pain and other conditions associated with female genital organs and menstrual cycle (N94)	760 (41.5)	843 (37.1)	0.004

Data are presented as mean (SD) or n (%).

Table 2: Medication treatment of women diagnosed with endometriosis diagnosis in gynecological practices in the Germany in 2010 and 2019

Age group	Progestins others than Dienogest			Dienogest			Gonadotropin-releasing hormone		
	2010	2019	P value	2010	2019	P value	2010	2019	P value
All women	154 (8.4)	189 (8.3)	0.912	332 (18.1)	796 (35.0)	<0.001	68 (3.7)	45 (2.0)	<0.001
Age 14-20 Y	4 (8.0)	1 (2.1)	0.183	19 (38.0)	29 (60.4)	0.027	3 (6.0)	0 (0.0)	0.085
Age 21-30 Y	48 (12.0)	47 (8.8)	0.105	93 (23.3)	221 (41.2)	<0.001	28 (7.0)	7 (1.3)	<0.001
Age 31-40 Y	59 (8.9)	96 (10.5)	0.243	123 (18.5)	320 (34.9)	<0.001	25 (3.5)	32 (3.5)	0.969
Age 41-50 Y	41 (6.9)	36 (6.2)	0.587	79 (13.4)	197 (33.7)	<0.001	13 (2.2)	6 (1.0)	0.110
Age >50 Y	3 (2.4)	9 (4.8)	0.285	8 (6.5)	29 (15.6)	0.016	1 (0.8)	0 (0.0)	0.218

Data are presented as n (%).

Discussion

This retrospective study shows that the prevalence of endometriosis increased significantly between 2010 and 2019. Furthermore, the proportion of endometriosis patients treated with Dienogest increased substantially between 2010 and 2019, while the proportion of women prescribed other Progestins than Dienogest and Gonadotropin-releasing hormones has not changed significantly.

The prevalence of women with endometriosis and those receiving endometriosis therapy found in this study is lower than other studies, 0.53% and 0.66% vs. up to 10%, respectively (12, 13). This significant difference in the prevalence is most likely due to the different layout of these studies. Some studies include the general population, whereas others focus on women in a high-risk population, for example infertile women (13, 14). The prevalence given in the ESHRE guideline falls within the range of 2 to 10% (5), whereas the guideline of the Association of the Scientific Medical Societies in German (15) shows a prevalence of 0.8% to 2%. Although prevalence differs among studies, most studies have two findings in common (5, 15). First, there are a large number of patients with undetected endometriosis, which may result from misdiagnosis, non-diagnosis or incorrect coding of endometriosis. Second, there is a general trend indicating that the overall prevalence of the disease is increasing over the years. This trend may be influenced by new diagnosis standards and guidelines for the ICD-10 classification of endometriosis or the increased relevance and awareness of endometriosis over the last decade. The growing prevalence of endometriosis is a positive trend. Period pain is not seen solely as an incidental secondary symptom of menstruation, but may be increasingly understood as a part of the pathology of endometriosis and used for diagnosis. In the majority of patients, the suspected diagnosis is made on the basis of a thorough history supported by clinical examination, including vaginal ultrasound and, less frequently, accompanied by MRI or laparoscopy (16).

Drug therapy is not claimed to cure the condition, although is rather considered as a symptomatic and suppressive approach. The therapy need to be tailored individually to each patient in terms of its duration and side effects. In addition, a rapid recurrence of symptoms and disease is observed when therapy is discontinued (9).

The essential principle of hormonal therapy for endometriosis is the induction of therapeutic amenorrhea (15). As seen in the study, Dienogest seem to be the treatment of choice for most patients (17). A narrative literature review and expert commentary by Murji et al. (17) stated that Dienogest in a 2 mg doses presents an effective and tolerable alternative to surgical intervention for the long-term management of endometriosis, offering several important advantages over combined oral contraceptive pills. Studies have provided evidence of the effectiveness of Dienogest in several respects. These effects showed that a 2 mg/day dose of Dienogest inhibits ovulation and downregulates proinflammatory cytokines, including IL-6 and IL-8, and monocyte chemoattractant protein-1 (17, 18). Another study showed that Dienogest has a direct inhibitory effect on aromatase expression in endometrial cells (19), while a further paper found that Dienogest may have the ability to overcome Progestins resistance by directly increasing the Progestin receptor-B (20). The guideline for the treatment of endometriosis by the AWMF states that Dienogest reduces the pain associated with endometriosis by inducing decidualization and atrophy of endometriosis lesions, suppressing growth mediated by matrix metalloproteinases and inhibiting angiogenesis. To date, only Dienogest and Gonadotropin-releasing hormones have been approved for hormonal therapy, in German-speaking countries (DGGG, OEGGG, SGGG) (14). The consensus-based recommendations for the treatment of endometriosis by the AWMF suggest Dienogest as a first-line substance only. Treatment with GnRH or other Progestins than Dienogest is only suggested as a second-line therapy, which could explain the following: Comparing guidelines from 2010 and 2019, the recommendation for GnRH has changed so that other substances such as Dienogest are preferred. This shift is due to the negative side effects of GnRH, which include hot flashes or metabolic abnormalities (15, 21). This study shows that the total number of patients treated with GnRH in 2010 and in 2019 was far below the number treated with Dienogest or other Progestins, at 3.7 and 2%, respectively, compared to 8.4 and 8.3% respectively for other Progestins and 18.1 and 35% respectively for Dienogest. GnRH caused a 4-6% decrease in the bone mineral density (BMD) after 24 weeks of treatment in comparison with a decrease of just 0.5-2.7% in the BMD in the women who were treated with Dienogest without add-back therapy. While both drugs induce a hypoestrogenic state that falls outside the recommended therapeutic window of 20-60 pg/mg this state is more moderate level with Dienogest than with GnRH or even other Progestins (22).

This study has several limitations. Since endometriosis diagnosis was based on the ICD codes and not on the biological data, the prevalence of endometriosis might have been underestimated. Furthermore, no information was available on how endometriosis diagnoses were made, the symptoms exhibited by patients, and how treatment responses were evaluated by gynecologists. Moreover, even if women included in this study were also treated in hospitals, we did not have access to the related data. The next limitation is a lack of several variables which were not documented in the database used, which include smoking behavior, alcohol use, family status, family history of endometriosis and other risk factors. Finally, we were able to analyze prescriptions for different drugs, but not laparoscopy, which as already mentioned is the gold standard in the treatment of endometriosis. The main strength of this work was the number of patients and gynecologists included.

Conclusion

There were significant changes in the prevalence and

medical therapeutic patterns of endometriosis between 2010 and 2019, reflecting changes in therapy guidelines and possibly in diagnostic methods.

Acknowledgements

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Authors' Contributions

J.G., K.K., M.D., M.K.; Study conception and design. J.G., K.K.; Analysis performance. J.G.; Writing first draft of the manuscript. All authors commented on previous versions of the manuscript, read, and approved the final manuscript.

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Original Article

Intraovarian Injection of Autologous Platelet-Rich Plasma Improves Therapeutic Approaches in The Patients with Poor Ovarian Response: A Before-After Study

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Abstract

Background: Advanced age is associated with a decline in the natural oocytes, low oocyte yield, and also increases the assisted reproductive technology (ART) failure rate, and consequently resulted in a pregnancy rate decrease. Platelet-rich plasma (PRP) is one of the proposed therapeutic strategies for women with poor ovarian response (POR). Because of the autologous source of PRP, the lowest risks of disease transmission, immunogenic and allergic reactions have been expected. This study aimed to evaluate the single-dose intraovarian injection of autologous PRP in poor ovarian reserve.

Materials and Methods: We conducted a clinical trial study in the Al-Zahra hospital and Milad Infertility Clinic, Tabriz, Iran (April and May, 2021). A total of thirty-five women with a POR and mean age 40.68 ± 0.34 enrolled in this study. After injection of autologous PRP into the ovaries, the number of oocytes, antral follicles, and level of estradiol, anti-Müllerian hormone (AMH), follicle-stimulating hormone (FSH), luteal hormone (LH), FSH/LH ratio also were evaluated while, these parameters were evaluated before PRP administration.

Results: At the 2-month follow-up, women treated with PRP showed a significant elevation in the number of oocytes (3.68 ± 0.24, P=0.0043) and embryos (3.17 ± 0.14, P=0.0001), as well as in the estradiol levels (404.1 ± 16.76 vs. 237.7 ± 13.14, P=0.0003).

Conclusion: Single PRP injection is effective and might be a promising therapeutic approach in the patients with POR to conceive with their own oocytes, although further evidence is required to assess the influence of PRP on the live birth rate.

Keywords: Infertility, Ovary, Platelet-Rich Plasma, Pregnancy

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Introduction

The tendency of women to postpone their childbearing was increased because of the professional investment and looking for better living conditions. Today, we observe an increase in the first-time mothers' mean age, approximately 30-40 (1). This issue is accompanied by ovarian aging; physiological alterations that may lead to oocyte quantity and quality decline critical problem (2). In both ovaries, the total number of oocytes at birth is 1-2 million, and before reaching puberty, more than half of the oocytes will undergo atresia (3). The remarkable increase in the follicle degeneration was observed in the above 37 years old women, and on average, about 1000 oocytes are present at the menopause stage (4).

It is estimated that the prevalence of poor responses to gonadotropin stimulation among women undergoing *in vitro* fertilization (IVF) is about 9-25% of this population (5). Management of these patients is challenging, and to improve the pregnancy rate, different therapeutic approaches must be tried (6). Furthermore, it was not reported until now an effective and beneficial treatment to prevent, postpone, or reverse ovarian senescence. Various environmental factors can irreversibly decline oocyte quality and numbers, such as unhealthy dietary habits (7), cigarette smoking (8), and exposure to both the chemo and radiotherapy (9). Different treatment strategies have been used in reproductive medicine such as coenzyme Q10, dehydroepiandrosterone (DHEA) (10), antioxidant dietary supplements containing vitamins C and E (11), and melatonin to decrease oxidative stress and also improve ovarian reserve. However, the approval studies of the effectiveness of these therapies remain unclear, also meta-analysis studies have been inconclusive (12).

The role of platelets in triggering cell proliferation and tissue differentiation is therefore considered a promising strategy in regenerative medicine (13). Following activa-

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tion of platelets by external stimuli like hemorrhage and tissue damage, they can release bioactive molecules and various growth factors that subsequently induce inflammation, severe neovascularization, clotting, and local tissue repair (14). The most prominent healing properties of platelet have led to using platelet-rich plasma (PRP) in all aspects of regenerative medicine (15). However, its concentration is a powerful factor, it has a concentration of 7 times than current circulating serum, The effectiveness of PRP as a critical therapeutic strategy in different illnesses such as eye disease, myocardial infarction, nerve injuries, and cosmetic surgery is considerable (16). Here, we aimed to investigate the single intraovarian injection of PRP effects on the inducing fertility in poor responders to ovarian reserve.

Materials and Methods

Patient selection

Current before-after study was conducted between April and May 2021. Women with a poor ovarian response (POR) were referred to the infertility clinic of Al-Zahra hospital and Milad infertility clinic, Tabriz, Iran were invited to this study. In this study, the total number of participants was 49 women; 7 individuals were excluded because only one ovary was injected and access to another ovary was impossible. The other 7 participants were reluctant to further treatment after PRP injection because of personal reasons. The inclusion criteria were as follows; infertile women aged 30 to 42 years with at least one ovary and poor response criteria, including anti-Müllerian hormone (AMH) <1.1 ng/mL, number of antral follicles less <5-7, a history of cycle cancellation due to low follicular growth or oocytes obtained <3, and also persons willing to cooperate in this research. Exclusion criteria included; FSH >25, current or previous IgA deficiency, genital or non-genital cancers, treatment with anticoagulants, ovarian failure due to abnormal sex chromosomes, prior pelvic surgery resulting in pelvic adhesions, and most importantly, no willingness to cooperate in the current study. Patients with anemia, hemoglobin <10 g/dl, signs of thrombocytopenia, platelet count <10 5/µl, did not receive the PRP injection.

Informed written consents were obtained from the participants. Also, was thoroughly approved by the Ethics Committee of Tabriz University of Medical Sciences, Tabriz, Iran (IR.TBZMED.REC.1399.794).

The sample size was determined based on the number of oocytes; in line, a previous study conducted by Farimani et al. (5) in 2019 also used this oocytes number. Using G Power software, version 3.01 (Christian-Albrechts-Universität Kiel, Kiel, Germany) selected the family of test such as t tests. Statistical test was calculated as mean: difference between two dependent means (match mean) with α error: 0.05, power: 0.8, and effect sized=0.66. Totally, 22 patients were invited to this study, two cases more than calculation due to patients with incomplete data and/or early drop out.

To avoid any laboratory error and bias in the data, all of the experimental examinations were performed in the same laboratory.

Ovarian stimulation

All participants will undergo minimal stimulation protocol involving Letrozole. The patients were referred for undergone sonography on the first to the third day of their menstrual period. Ovarian stimulation was started with Letrozole (Femara, Novartis, Dorval, Quebec, Canada) PO administered daily (5 mg/kg) from the second or third day of menstruation to the day of ovulation triggering.Recombinant human FSH (rhFSH, Cat. No. sc7798, Cinal-F, Cinagen, Tehran, Iran) was started from the third day of Letrozole administration subcutaneously (225 IU). Human menopausal gonadotropin (hMG) ampule (Cat. No. ab200726, Abcam Co., USA) at a dose of 75 mg/kg was intramuscularly injected daily from the fourth day of Letrozole administration. Transvaginal ultrasound was carried out on the 7th or 8th day of stimulation. When one or more follicles reached a diameter of 14 mm, gonadotropin-releasing hormone (GnRH) antagonist (Merck Serono, Germany), and Cetrotide (ASTA Medica AG, Frankfurt am Main, Germany) 0.25 mg, was administrated to avoid premature ovulation and continued daily until the day of human chorionic gonadotropin (hCG) injection. After reaching the mean diameter of the follicle to 17-18 mm, the intramuscular hCG (10,000 IU, Cat. No. C5297, Sigma, USA) administrated to induce follicular maturation. Serum estradiol levels were measured on the day of hCG injection. Transvaginal ultrasound-guided oocyte retrieval was done under general anesthesia 35-36 hours after hCG injection.

Platelet-rich plasma preparation

PRP preparation has been done by segregation of PRP following the centrifugation. About 20 ml of blood sample was carefully collected under sterile conditions, and subsequently, PRP was prepared using a Royagen kit (Co. SN: 312569, Arya Mabna Tashkis, Iran) based on the manufacturer's instructions. Briefly, centrifugation was done at 830 g for 8 minutes after blood sample collection. Then, a 16 G needle was connected to a 5 ml syringe, inserted into the tube, and advanced to the buffy coat layer. Through rotating the needle tip, all of the PRP was collected. After collecting about 2-4 cc from one sample, PRP from the first tube applied the same process for the second tube (the total collecting PRP was 4-8 cc).

The prepared solution was shifted to the re-suspension tube and then shaken slightly for 30 seconds-1 minute.

Intraovarian injection

An antibiotic was administrated before oocyte pick-up and PRP injection. Furthermore, intravenous a single dose of Cefazolin (Jaber-eben Hayan, Tehran, Iran) administrated one hour prior to injection. During the first stage puncture, after oocyte pick up about 2 cc of autologous obtained PRP injected into the cortex of

both ovaries using a 35 cm 17 G single lumen needle. During PRP injection, we used color Doppler [DW-F3 (DW-C80), Jiangsu, China] to prevent damage to large blood vessels. After this process, the patients were transferred to the recovery room and carefully observed for about 30-40 minutes, and then discharged home on the same day. All embryos were frozen. Two months later, or after the third menses post PRP injection, and the lab test and transvaginal sonography were performed, then patients received a new ovarian stimulation cycle in the same way and with the same dose of previous pattern. Patients were discharged from the hospital and received Azithromycin (as Dihydrate, Abouraihan Pharma. Co., Iran) for five days. Patients, who were referred for a second ovarian stimulation, received a low dose estrogen (LD, OVOCEPT-LD, Abouraihan Pharma. Co., Iran) pill for a month if they have an ovarian cyst on the first, second, and third day of the ultrasound and are referred again in the next menses. This population was excluded and omitted from the study due to the prolonged interval between PRP injection and subsequent ovarian stimulation and the possibility of bias in this research.

The number of obtained metaphase II (MII) oocytes, mature oocytes, and the estradiol level on the day of human placental gonadotropin injection was evaluated. The level of ovarian reserve markers, including AMH, follicle-stimulating hormone (FSH), luteal hormone (LH), FSH/LH ratio. Also, these measurements were performed before and after treatment.

Statistical analysis

Data were carefully assessed for normality using the Shapiro-Wilk test, and results were expressed as a standard error of mean (SEM). A Paired-samples t test was performed in normal data to compare the effects of PRP injection in the both of the steps. A Wilcoxon signed-rank test was used to establish non normal data and univariate comparisons before and after of PRP treatment. Differences were considered significant where the $P<0.05$. The statistical SPSS software, version 22 (IBM SPSS, Armonk, NY, USA), was used for data analysis.

Results

Thirty-five women with mean age ± SEM (40.43 ± 0.26) and POR enrolled in this study (Table 1). The mean numbers of oocytes before and after PRP injection were 2.22 and 3.68, respectively. Our results showed that intra-ovarian injection of PRP led to a significant increase in the oocytes numbers ($P=0.0043$). Moreover, the number of embryos (3.17 ± 0.14) significantly was increased following PRP injection ($P=0.0001$). For women before the injection of PRP, the average number of oocytes (mean ± SD) was (0.64 ± 0.92) and for the same women after injection of PRP was (2.5 ± 2.1).

Table 1: Clinical features of our participants

Cases	Age (Y)	Oocyte number (Before PRP)	Oocyte number (After PRP)	Embryo number (Before PRP)	Embryo number (After PRP)
Case 1	42	2	2	1	2
Case 2	39	1	3	1	2
Case 3	40	2	3	1	3
Case 4	41	1	3	0	2
Case 5	41	2	4	2	4
Case 6	41	2	4	1	3
Case 7	39	2	3	1	3
Case 8	38	3	5	2	4
Case 9	43	1	3	0	2
Case 10	40	2	2	0	2
Case 11	41	2	3	1	3
Case12	39	3	5	3	4
Case 13	40	4	5	3	4
Case 14	38	3	4	1	3
Case 15	41	2	4	2	3
Case 16	41	2	2	1	2
Case 17	43	3	8	2	5
Case 18	42	3	3	2	3
Case 19	39	3	4	3	3
Case 20	42	1	3	1	2
Case 21	41	2	5	2	3
Case 22	44	2	4	1	3
Case 23	40	2	2	1	4
Case 24	37	2	2	1	3
Case 25	41	3	3	1	1
Case 26	38	4	4	2	1
Case 27	39	3	3	1	2
Case 28	41	2	2	1	1
Case 29	40	2	2	1	1
Case 30	39	2	3	2	2
Case 31	41	2	2	1	1
Case 32	40	2	2	1	1
Case 33	42	1	1	2	2
Case 34	42	2	2	3	3
Case 35	40	2	2	1	1
Mean ± SEM	40.43 ± 0.26	2.22 ± 0.13	3.68 ± 0.24***	1.41 ± 0.13	3.17 ± 0.14***

Data expressed as mean ± standard error of the mean (SEM). PRP; Platelet-rich plasma and *; P<0.001.**

As shown in Figure 1A, B, there was no significant difference in the serum FSH before and after PRP treatment (12.2 ± 0.31 and 12.51 ± 0.28, respectively) and also, LH (13.00 ± 0.25 and. 13.14 ± 0.26, respectively). Also, AMH level was not statistically significantly different after PRP treatment (0.38 ± 0.039) in comparison with before of treatment (0.39 ± 0.04, Fig.1C). We observed a statistically significant increase in the estradiol levels following PRP

treatment (404.1 ± 16.76), while it showed lower levels before treatment (237.7 ±13.14, P=0.0003, Fig.1D).

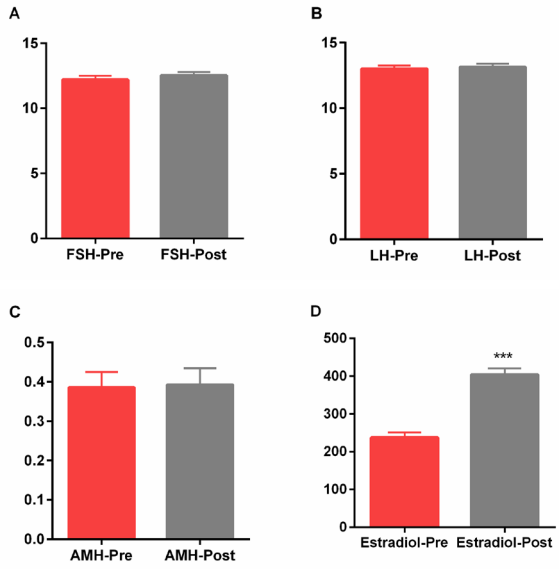

Fig.1: Alterations of serum hormone levels pre and post PRP injection. **A.** FSH, **B.** LH, **C.** AMH, and **D.** Estradiol levels before (pre) and after (post) PRP injection. Data were expressed as mean + standard error of the mean (SEM). ***; P<0.001, FSH; Follicle stimulating hormone, LH; Luteinizing hormone, AMH; Antimullerian hormone, and PRP; Platelet rich plasma.

Furthermore, in the current study, spontaneous pregnancy was observed in the 3 of 30 women within 4 months after PRP treatment. We concluded that PRP injection into the ovary by an expert person is safe. Any complications such as infection, bleeding, and hematoma did not observe during this research.

Discussion

Induction of torsion/detorsion causes histological PRP is a valuable therapeutic approach in the female factor infertility. In the current study, single intra-ovarian PRP administration significantly increased the oocyte numbers and embryos in the women with POR. Also, regarding the single injection of PRP, our results showed an increase in the estradiol levels of our participants.

To give birth to a child is one of problems women of reproductive age who suffered from the decline or loss of ovarian reserve. Ovarian failure is characterized by ovarian atrophy, reduction of follicles, and menopausal-level serum gonadotropins. PRP therapy has become a novel treatment in the multiple aspects of medicine, especially in the reproductive medicine and infertility (17). A systematic review study on the 663 subfertile women who received PRP injections reported that PRP is beneficial in the improving ovarian reserve parameters such as: serum AHM, serum FSH or antral follicle count (AFC) (18). Sills et al. (19) examined the effect of intra-ovarian injection of PRP on the women with a mean age of 42 ± 4 years. They found that administration of PRP improved ovarian function two months after trans-vaginal calcium gluconate-activated autologous injection PRP in all cases. It has reported that growth and survival rates of follicles in the media supplementation with PRP were significantly higher than those without PRP, indicating PRP may be a practical approach to induce follicular development (20).

Interestingly, the present study showed an number of oocytes and embryos following of a single injection of PRP, which can be a cost-effective and time-consuming approach. Farimani et al. (5) reported the same results. Here, we did not see any changes in the levels of serum FSH, LH, and AMH. In line with our study, Cakiroglu et al. (21) demonstrated that intra-ovarian injection of autologous PRP in the women with primary ovarian insufficiency had no significant effect on the FSH levels, and also, associated with minimal improvement in the AMH levels. Although, Aflatoonian et al. (22) did not observe a significant difference in the hormonal (LH and FSH) profile of women with POR or primary ovarian insufficiency after PRP injection. Previous studies showed an elevation in the Estradiol level, following intra-ovarian PRP treatment. A significant decrease in the FSH levels was observed six weeks after autologous PRP therapy (23). On another hand, Cakiroglu et al. (21) reported that PRP treatment increases the AMH level and AFC in the women with primary ovarian insufficiency. Therefore, regarding these studies, PRP's long-term effectiveness and serial measurements of FSH, LH, and AMH levels following PRP treatment should be more assessed in the context of this study. In our study, six of thirty-five women had spontaneous pregnancy three months after PRP therapy.

The healing regenerative properties of PRP are due to higher concentrations of various growth factors, including transforming growth factor-β, insulin-like growth factors 1 and 2 (IGF-1 and IGF-2), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor and hepatocyte growth factor (HGF) (24). Growth differentiation factor 9 (GDF-9), as an oocyte-derived protein, is one of the PRP factors that its critical role in the oocytes maturation associates with an increase in the primary and preantral follicle number (25). Furthermore, mutation of GDF-9 gene leads to premature ovarian failure (26). On the other hand, injection of PRP can activate postnatal oogenesis in the ovary that results in the new primordial follicle generation in the menopausal women's ovaries through the activation and stimulation of GnRH receptors (27). Melo et al. (16) reported that ovarian administration of PRP, effectively improved markers of the low ovarian reserve before assisted reproductive technology (ART) in the 83 women (median age 41 years) with low ovarian reserve. Also, they observed that PRP treatment was accompanied by a significant increase in the pregnancy rates, both of biochemical and clinical stages. Several studies suggested that treatment with PRP increases neoangiogenesis of the menopausal ovary and promotes ovarian stem cells development to mature follicles (23, 28).

The simultaneous injection of PRP and ovarian puncture surgery made this study principal novelty. These were resulted in a drastic therapy cost reduction, and also, ovarian

enlargement that facilitates the injection of PRP in these patients. This research was a before-after study, and we didn't find a similar previous study till now. Another strength of this study was a single injection of PRP, a low dose, while previous studies repeated the PRP injection more times. This study had its own limitations, such as small study population of mean age of 40 years therefore, it is recommended to conduct a large population over the age of 40.

Conclusion

Our findings showed the beneficial effects of single intra-ovarian injection of autologous PRP in the patients with POR. It could be considered as a cost-effective and time-consuming treatment strategy in the future clinical therapies. Injecting PRP into the ovaries raises the hope that women with a POR, were conceived through themselves eggs. However, further studies with a larger sample size are mandatory to assess the impact of PRP on pregnancy outcomes in infertile women.

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Authors' Contributions

N.N., L.S.; Participated in study design, clinical data collection, patients screening and selection. L.F., A.Gh.; Conducted patients' evaluation, molecular experiments and RT-qPCR analysis. K.H., P.H., B.N.; Were responsible for manuscript preparation, collaboration in the data collection, and data analysis. All authors read and approved the final manuscript.

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Original Article

Detection and Evaluation of Macrolide Resistance (Erythromycin) in *Mycoplasma hominis* Isolated from Endocervical Specimens of Patients Referring to Ibn Sina Infertility Treatment Centre, Tehran, Iran

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Abstract

Background: *Mycoplasma hominis (M. hominis)* is an important cause of bacterial infections of the genital tract. Macrolides are the first selective agents used to treat mycoplasma infections. However, widespread use of macrolides has led to a rapid and global emergence of macrolide-resistant strains. We evaluated macrolide resistance in *M. hominis* isolated from endocervical specimens of patients who referred to Ibn Sina Infertility Centre in Tehran, Iran.

Materials and Methods: In this cross-sectional descriptive-analytical study, 160 samples of Dacron endocervix swabs (80 infertile patient samples and 80 healthy controls) were collected and transferred to the laboratory. All samples were cultured in liquid pleuropneumonia-like organisms (PPLO) broth and PPLO agar solid media. After culturing and genome extraction, polymerase chain reaction (PCR) was performed using specific primers. Then, minimum inhibitory concentration (MIC) was obtained using the broth microdilution method. The MIC was recorded and reported for all samples positive for *M. hominis* against erythromycin.

Results: From the 160 endocervical specimens cultured in PPLO agar medium, 19 cases (23.75%) were positive. A total of 35 cases (42.5%) were positive using specific primers of *M. hominis* species. MIC results from all samples positive for *M. hominis* were measured against erythromycin. All of the *M. hominis* samples were resistant to erythromycin.

Conclusion: The results of the present study showed that a significant percentage of infertile women were infected with *M. hominis*. Also, MIC results from the broth microdilution method indicated that all strains positive for *M. hominis* were also resistant to erythromycin.

Keywords: Erythromycin, Infertility, *Mycoplasma hominis*, Resistance

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Introduction

Mycoplasmas belong to an unconstrained class called Mollicutes and the Mycoplasmataceae family; this family includes Mycoplasma spp. *Mycoplasma hominis* (*M. hominis*), Mycoplasma genitalium and *Ureaplasma urealyticum* (1, 2). These species may cause oligosyptomatic genital infections, including urinary tract infection, chorioamnionitis, pelvic inflammatory disease, and sperm cell disorders in both reproductive age men and women. Complications of these infections can lead to infertility. *M. hominis* is among the smallest human pathogens responsible for a wide range of infections. However, knowledge regarding the genetic mechanisms and pathogenicity of *M. hominis* is limited (3-5).

M. hominis is located in the human lower genital tract as commensal flora, even though it is linked with development of non-gonococcal urethritis, infertility, chorioamnionitis, adverse pregnancy outcomes, and neonatal diseases. *M. hominis* infections can be found in asymptomatic individuals as well as symptomatic patients (6). Due to the lack of a cell wall, mycoplasmas are not affected by many of the common antibiotics that target cell wall synthesis, such as beta-lactam antibiotics (e.g., penicillin and cephalosporins). Mycoplasmas are sensitive to factors that interfere with protein synthesis, such as tetracyclines, macrolides, aminoglycosides, and chloramphenicol, as well as fluoroquinolones that act as inhibitors of topoisomerases (7-10). The increasing number of macrolide- and fluoroquinolone-resistant *M. hominis* strains can lead to drug resistance and the emergence of incurable infections (11). Macrolides are first-line treatment of *Mycoplasma* pneumoniae. Widespread use of macrolides has

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led to rapid and global emergence of macrolide-resistant strains. The point mutation in domain V of the *23S rRNA* gene (nucleotide displacement at specific positions of domain V of *23S rRNA*) has been identified as the cause of macrolide resistance in mycoplasmas (11-13).

There is an increased prevalence of infertility in Iran. An elevated rate of genital infections caused by *M. hominis* in infertile women was reported by Seifoleslami et al. (14) Therefore, it is important to conduct observational studies on the prevalence and antimicrobial susceptibility of these bacterial species among infertile Iranian females. In this study, for the first time in Iran, we investigated the erythromycin macrolide resistance of *M. hominis* isolated from infertile women who referred to Ibn Sina Infertility Centre in Tehran, Iran. The objectives of this study were: i. Analysis of *M. hominis* prevalence by culture and molecular methods (e.g., presence of *23S rRNA* gene) from endocervical samples of the infertile women and ii. Evaluation of erythromycin macrolide resistance of *M. hominis* species isolated from these women.

Materials and Methods

Sample collection

In this cross-sectional descriptive-analytical study, 160 endocervical swab samples were collected from women who referred to Ibn Sina Infertility Centre, Tehran Province, Iran. The samples were assigned to two groups, patient (n=80) and control (n=80). The inclusion criteria were: married women with clinical signs of vaginosis, green vaginal discharge with a foul fishy odour, history of infertility (infertility after one year of intercourse), history of abortion, history of preterm delivery, and no antibiotic use during the past month. All the clinical examinations were performed by a gynaecologist. All participants completed a questionnaire for patient eligibility, and a written informed consent was obtained before they were screened for sampling eligibility. The control group consisted the healthy and fertile individuals.

Culture

Sampling was performed using endocervical Dacron swabs. Two swabs were obtained from each patient, one for culture and the other for molecular assessment. All swabs were transferred to the laboratory in 2 ml of liquid transfer medium under sterile conditions. Afterwards, 1 ml of the transfer medium was filtered through 0.45 µm pore size filters and transferred to main pleuropneumonia-like organisms (PPLO) broth (pH=7.8 ± 0.2). The samples were then incubated in a 5%-10% CO_2 atmosphere at 37 °C for one week. After three subcultures in liquid medium, 100 µl of each sample was cultured in PPLO solid agar, and the samples were incubated in 5-10% CO_2 at 37°C for 3-5 days. In this study, we used the standard *M. hominis* (ATCC: 23114) strain, which was prepared at Baqiyatallah University, Tehran, Iran.

DNA extraction

We used the polymerase chain reaction (PCR) technique, which is a highly sensitive, specific test to confirm the presence of all pathogens, regardless of culture test results. A DNA Extraction Kit (Sinaclon, Iran) was used to extract DNA from the samples. After DNA extraction, all samples were kept at -20°C prior to PCR.

Molecular method

In this study, specific primers were used to identify the *M. hominis* gene, 23S rRNA, as shown in Table 1 (15, 16). Primer BLAST was performed, and sensitivity and specificity were confirmed at the NCBI site.

PCR was conducted with a final volume of 25 µl for each sample, which included 12.5 µl Master Mix 2X (Master Mix 2X, Pishgam, Iran), 5 µl DNA template, and 0.5 µl of each primer pair in a total volume of 25 µl. PCR was performed according to the protocol for different genes (Table 2). Finally, PCR amplification products were subjected to 1% gel agarose electrophoresis. DNA extracted from a standard strain of *M. hominis* (ATCC: 23114) was the control.

Table 1: Sequences of the study primers

Gene	Primer sequence (5´-3´)	Product size	Tm (°C)
M. hominis species gene	F: CAATGGCTAATGCCGGATACGC R: GGTACCGTCAGTCTGCAAT	334	58
23S rRNA	F: TAACTATAACGGTCCTAAGG R: CCGCTTAGATGCTTTCAGCG	793	52

***M. hominis*; *Mycoplasma hominis* and Tm; Melting temperature.**

Table 2: Temperature protocol of PCR for *M. hominis* gene and the *23S rRNA* gene

Gene	Pre- denaturation	Denaturation	Annealing	Extension	Final extension
Gene species of *M. hominis*	95°C	95°C	56°C	72°C	72°C
	5 minutes	30 seconds	45 seconds	60 seconds	7 minutes
		←	35 cycles	←	
23S rRNA	94°C	95°C	55°C	72°C	72°C
	10 minutes	1 minute	1 minute	60 seconds	10 minutes
		←	40 cycles	←	

***M. hominis; Mycoplasma hominis* and PCR; Polymerase chain reaction.**

Minimum inhibitory concentration determination using the microdilution method

We determined the minimum inhibitory concentration (MIC) of erythromycin for the samples that were positive for the species gene of *M. hominis* and the *23S rRNA* gene. We used microdilution of the arginine PPLO broth that was enriched with horse serum and L-arginine. The assessment was conducted in 96-well microplates following preparation of the macrolide suspension (erythromycin). The microplates prepared for the *M. hominis* culture were incubated at 37°C for 48-72 hours. The final results were interpreted based on previous studies (16, 17). Antimicrobial susceptibility was determined through PPLO broth microdilution for clinical strains, as described previously. The specific cut-off points (mg/litre) that indicated susceptibility (S) or resistance (R) to erythromycin were S ≤1 and R ≥4 (11). The MIC of erythromycin was measured and recorded for all *M. hominis* positive samples. The MIC for erythromycin was measured based on colour changes of the arginine PPLO broth enriched with horse serum and L-arginine. Arginine PPLO broth is a phenol red medium and growth of *M. hominis* in arginine PPLO broth changes the colour of the medium from purple to darker red.

Ethical considerations

This study was approved by the Ethics Committee of Shahed University School of Medicine, Tehran, Iran (IR.SHAHED.REC.1398.014).

Statistical analysis

The data were analysed using IBM SPSS statistical software, version 20.0 (SPSS Inc., Chicago, IL) and presented as statistical tables. P<0.05 were considered significant according to the Chi-square test.

Results

In this study, 160 endocervical swab samples were collected from participants, of which 80 samples were collected from infertile women (experimental group) and 80 samples from healthy women (control group). The study participants ranged in age from 20 years to 49 years. As shown in Table 3, *M. hominis* was most prevalent in women 30-39 years of age in both groups.

Table 3: Frequency distribution of M. hominis in endocervical samples by age group

	Number of patients (n=160)		Patient group (n=80)		Control group (n=80)	
Age (Y)	Patient group	Control group	Culture	PCR	Culture	PCR
20-29	21	19	3	4	0	0
30-39	47	45	11	17	1	3
40-49	12	16	4	8	0	2
Total	80	80	18	29	1	5

***M. hominis*: *Mycoplasma hominis* and PCR; Polymerase chain reaction.**

Among the investigated endocervical specimens, 19 samples grew *M. hominis* colonies on PPLO agar, 18 (22.5%) were from the experimental group, and 1 (1.25%) from the control group (Table 4).

Table 4: Detection of *M. hominis* by culture on PPLO agar and PCR

Number	Age (Y)	Cultivation	PCR	Sample type
		Patient group		
1	20	+	+	Endocervical mucosa
2	25	+	+	Endocervical mucosa
3	27	-	+	Endocervical mucosa
4	29	+	+	Endocervical mucosa
5	30	+	+	Endocervical mucosa
6	31	+	+	Endocervical mucosa
7	31	-	+	Endocervical mucosa
8	32	+	+	Endocervical mucosa
9	32	-	+	Endocervical mucosa
10	33	+	+	Endocervical mucosa
11	33	+	+	Endocervical mucosa
12	34	-	+	Endocervical mucosa
13	35	-	+	Endocervical mucosa
14	35	+	+	Endocervical mucosa
15	36	-	+	Endocervical mucosa
16	36	-	+	Endocervical mucosa
17	37	+	+	Endocervical mucosa
18	37	+	+	Endocervical mucosa
19	38	+	+	Endocervical mucosa
20	39	+	+	Endocervical mucosa
21	39	+	+	Endocervical mucosa
22	40	-	+	Endocervical mucosa
23	42	+	+	Endocervical mucosa
24	44	+	+	Endocervical mucosa
25	47	-	+	Endocervical mucosa
26	47	+	+	Endocervical mucosa
27	48	-	+	Endocervical mucosa
28	48	-	+	Endocervical mucosa
29	49	+	+	Endocervical mucosa
		Control group		
1	29	-	+	Endocervical mucosa
2	36	+	+	Endocervical mucosa
3	37	-	+	Endocervical mucosa
4	39	-	+	Endocervical mucosa
5	48	-	+	Endocervical mucosa

PPLO; Pleuropneumonia-like organisms broth, PCR; Polymerase chain reaction, and *M. hominis; Mycoplasma hominis*.

Results of polymerase chain reaction detection of Mycoplasma hominis (*M. hominis*) species gene and 23S rRNA gene

Results of PCR on 160 Dacron endocervical swabs showed that 34 samples (42.5%) were positive for *M. hominis* by using first primers for the *M. hominis*

species gene and primers for the *23S rRNA* (Fig.1). Of 80 endocervical samples in the experimental group, 29 (36.25%) were positive for *M. hominis*, and from 80 endocervical samples in the control group, 5 (6.25%) were positive for *M. hominis* (Table 4).

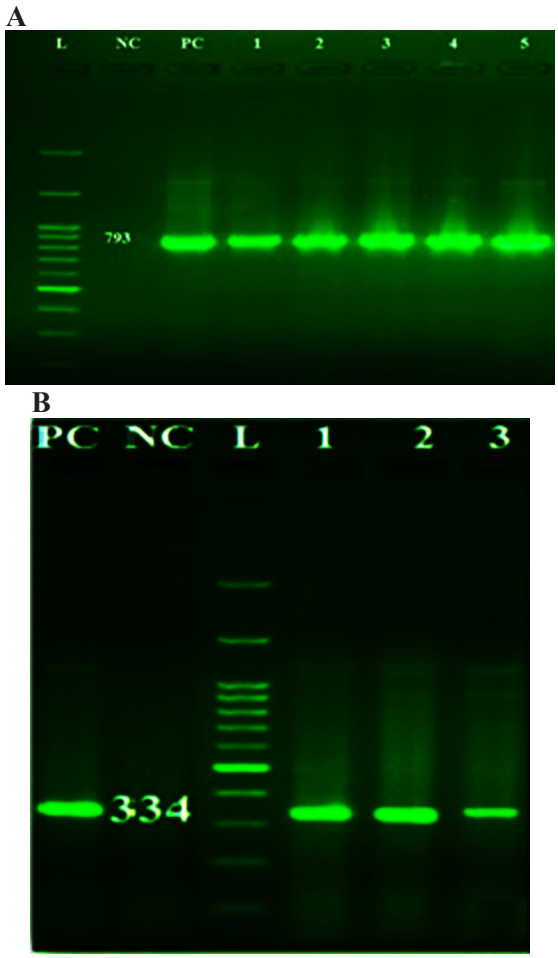

Fig.1: Results of 1% gel agarose electrophoresis. **A.** PCR results for the *23S rRNA* gene, L; DNA ladder (100 bp DNA Ladder, Pishgam, Iran), PC; Positive control, NC; Negative control, Lanes 1-5 are positive for *23S rRNA* gene. **B.** PCR results for the *M. hominis* species gene, L; DNA ladder (100 bp DNA Ladder, Pishgam, Iran), PC; Positive control, NC; Negative control, Lanes 1-3 are positive for species gene of *M. hominis*, and PCR; Polymerase chain reaction.

The frequency and distribution of *M. hominis* in both patient and control groups according to the culture and PCR methods indicated that positive culture of *M. hominis* in the infertile patients (P=0.004) and diagnosis by molecular PCR (P=0.003) were much higher compared to the control group. PCR results had a very high sensitivity compared to the culture method for detection of *M. hominis* (P=0.037).

Minimum inhibitory concentration of *Mycoplasma hominis (M. hominis)* positive specimens

We performed MIC on the positive samples to determine the susceptibility or resistance of erythromycin on these samples. According to the findings of our study, all of the *M. hominis* samples were resistant to erythromycin (Table 5).

Table 5: MIC ranges (µg/ml) for erythromycin against *M. hominis*

Patient group	MIC_{50}	MIC_{90}	Range	S (%)	I (%)	R (%)
1	256	256	≤1-≥512	0	100	0
2	128	128	≤1-≥512	0	100	0
3	128	256	≤1-≥512	0	100	0
4	128	128	≤1-≥512	0	100	0
5	128	256	≤1-≥512	0	100	0
6	256	256	≤1-≥512	0	100	0
7	128	128	≤1-≥512	0	100	0
8	256	256	≤1-≥512	0	100	0
9	128	256	≤1-≥512	0	100	0
10	256	256	≤1-≥512	0	100	0
11	64	128	≤1-≥512	0	100	0
12	256	256	≤1-≥512	0	100	0
13	128	128	≤1-≥512	0	100	0
14	128	256	≤1-≥512	0	100	0
15	256	256	≤1-≥512	0	100	0
16	128	128	≤1-≥512	0	100	0
17	256	256	≤1-≥512	0	100	0
18	128	128	≤1-≥512	0	100	0
19	256	256	≤1-≥512	0	100	0
20	128	128	≤1-≥512	0	100	0
21	256	256	≤1-≥512	0	100	0
22	128	256	≤1-≥512	0	100	0
23	256	256	≤1-≥512	0	100	0
24	256	256	≤1-≥512	0	100	0
25	128	128	≤1-≥512	0	100	0
26	64	64	≤1-≥512	0	100	0
27	128	256	≤1-≥512	0	100	0
28	128	128	≤1-≥512	0	100	0
29	256	256	≤1-≥512	0	100	0
		Control group				
1	128	256	≤1-≥512	0	100	0
2	256	256	≤1-≥512	0	100	0
3	128	128	≤1-≥512	0	100	0
4	64	128	≤1-≥512	0	100	0
5	128	128	≤1-≥512	0	100	0

MIC; Minimum inhibitory concentration, *M. hominis*; Mycoplasma homini, S; Susceptible, I; Intermediate, and R; Resistant.

The MIC of erythromycin was measured and recorded for all *M. hominis* positive samples. We determined the MIC for erythromycin was based on colour changes of the arginine PPLO broth enriched with horse serum and L-arginine from the growth of *M. hominis*.

Discussion

Infertility is one of the most important issues in medical science, which is commonly defined as a biological reproductive disability (10, 18). Some infections cause infertility amongst women, and some interfere with embryo

implantation in the uterus, causing miscarriage. Bacterial infections can also cause infertility, and *M. hominis* is one of the most prevalent bacterial pathogens associated with infertility over the past few years. *M. hominis* generally colonizes in the urogenital system and can be seen as a natural flora in the genital tract of many sexually active men and women. This pathogen has been isolated from vaginal secretions, amniotic fluid, placental tissue and umbilical cord blood during pregnancy (18, 19).

In this study, we recruited 160 women from which 80 infertile women comprised the experimental group and 80 fertile women were in the control group. From the 160 endocervical samples, 19 cases were positive for this pathogen when assessed by the culture method; 18 (22.5%) belonged to the patient group and 1 (1.25%) to the control group. The PCR test results from the 160 endocervical samples showed that 34 samples (42.5%) were positive for *M. hominis*. Out of the 34 positive samples, 29 (36.25%) were from the patient group and 5 (6.25%) from the control group. Both the culture and PCR method results showed a significant difference in frequency of the study microorganism in the patient group compared to the control group which indicated two main, important issues. First, *M. hominis* species were more prevalent in the patient group than the control group. Therefore, the presence of infectious bacterial species, such as *M. hominis*, could be associated with infertility. Second, the PCR method had much higher sensitivity than the culture method for detection of *M. hominis*.

Petrikkos and colleagues investigated and compared diagnostic methods of routine culture versus PCR. They found that 13 *M. hominis* negative samples by culture were positive by PCR, which indicated the higher sensitivity of PCR compared to the culture method (20).

Macrolides are the first selective agents for the treatment of mycoplasma infections. Extensive use of macrolides has led to the rapid and global emergence of macrolide resistance species. The presence of macrolide-resistant strains among infections caused by *M. hominis* has led to genetic mutations associated with macrolide resistance in these species and emergence of incurable infections.

In the present paper, the MIC of all the *M. hominis* positive samples with the *23S rRNA* gene against erythromycin was recorded and reported by using the broth microdilution method in 96-well microtiter plates. We found that all of the samples of *M. hominis* were resistant to erythromycin. MIC results by the microdilution technique was positive for *M. hominis* against erythromycin in all of the samples. The MIC was determined based on colour changes in the arginine PPLO broth medium, which was caused by the growth of *M. hominis*. The highest rate of *M. hominis* infection in the patient and control groups was observed in women who were 30-39 years of age.

Wang et al. conducted a study in China and found that all strains of *M. hominis* were 100% resistant to erythromycin due to the antimicrobial sensitivity of *M. hominis* to macrolides. *M. hominis* infection was most prevalent in 30-39 year-old age group; both findings were consistent with the results of the present study (21).

Pereyre and colleagues detected *23S rRNA* mutations associated with resistance against macrolides, including erythromycin and azithromycin. A point mutation at position 2057 (G2057A) was obtained in domain V of *23S rRNA* (16). These results were consistent with the results of our study.

Bayraktar et al. (6) conducted a study with 100 pregnant women; they reported that 50 patients were symptomatic and 50 were asymptomatic. Also, 29 subjects (29%) were positive for *M. hominis* and *Ureaplasma urealyticum*. Of these, 27 females were in the patient group (54%) and two were from the control group (4%). They successfully cultured *M. hominis* from five women (5%) and *Ureaplasma urealyticum* from 27 women (27%), which was consistent with the results of the present study. *M. hominis* and *Ureaplasma urealyticum* were 100% sensitive to doxycycline, tetracycline and pristinamycin; 90% to josamycin; 84% to clarithromycin; and 50% to erythromycin. The results of antibiotic susceptibility testing were significantly inconsistent with the results of the present study.

Zhou et al. (22) recruited a total of 5016 infertile males and 412 healthy males from 2011 to 2016. Culture, identification and antimicrobial susceptibility of *Ureaplasma uralticum* and *M. hominis* were evaluated in their study. A total of 30%-55% of the infertile men had *M. hominis* infections in their genital tracts. Two age groups were identified as high-risk for mycoplasmas: 26-30 years (37.8%) and 31-35 years (30.7%). The results of this study were in line with the results of our study.

Yang et al. (4) investigated 492 species of Ureaplasma and 13 strains of *M. hominis* in Hangzhou, China. They stated that the levels of resistance to levofloxacin, moxifloxacin and erythromycin were 84.69, 51.44, and 3.59%, respectively, in *Ureaplasma parvum* and 82.43, 62.16% and 5.40, respectively, in *Ureaplasma uralticum*. Among 13 *M. hominis* strains, 11 were resistant to levofloxacin and moxifloxacin, and five strains were resistant to clindamycin. The results of this study showed that new S21A mutations in the L4, G2654T and T2245C proteins were detected in the *23S rRNA* and *ermB* genes in the *erythromycin*-resistant Ureaplasma species. Fluoroquinolone resistance in the Ureaplasma and *M. hominis* species is relatively high in China; thus, the use of these antibiotics should be further controlled and limited. The results of this study were consistent with our study.

Ozturk and colleagues (5) isolated 72 strains of *M. hominis* from 220 clinical specimens. They observed that all of the selected strains were mostly resistant against pristinamycin (100%), followed by tetracycline (80%) and josamycin. (75%), and *M. hominis* was mostly resistant against levofloxacin (100%), clindamycin

(97%), roxithromycin (97%), and ciprofloxacin (94%). This study also reported resistance to erythromycin (81%) and clarithromycin (89%). The results of this study were almost similar to the results of our study, which indicated a high level of resistance against macrolide antibiotics.

Maldonado-Arriaga and colleagues (1) enrolled 37 infertile couples (74 samples) to diagnose genital mycoplasmas and evaluate antibiotic resistance. They observed that the prevalence of *Ureaplasma urealyticum*, *M. hominis* and coinfections were 71.4, 9.5 and 19%, respectively. In this study, most mycoplasmas showed significant resistance against fluoroquinolones, macrolides and tetracycline, with the highest resistance to macrolides in all strains. The results of this study were almost similar to the results of our study, which indicated a high level of resistance against the macrolide antibiotics.

Doroftei et al. reported that women aged 30-35 years were the most affected group, followed by 25-30 year-old women in their study. Cumulatively, the prevalence of *Ureaplasma urealyticum, M. hominis*, and coinfection with both, were 28.46% (n=117), 0.48% (n=2), and 2.91% (n=12), respectively. Drug susceptibility was evaluated in this study. Pristinamycin (100 vs. 100%) and Josamycin (100 vs. 98.00%) were the most efficient antibiotics in eradicating *Ureaplasma urealyticum* and *M. hominis*. High efficiency was observed with doxycycline (98.23%), minocycline (96.00%), tetracycline (96.48 vs. 68.00%), and erythromycin (70.17 vs. 92.00%). Based on antibiograms, clarithromycin (88.00%), roxithromycin (88.00%), levofloxacin (82.00%), and azithromycin (78.94%) could be used to treat these infections (23).

Conclusion

The results of the present study showed that a significant percentage of infertile women were infected with *M. hominis*. Also, all samples that were positive for *M. hominis* showed resistance against erythromycin according to MIC assessment by the broth microdilution method. This is the first study that investigated erythromycin macrolide resistance of *M. hominis* isolated from infertile women in Iran. Although this study examined a small population of infertile women, due to the increasing trend in infertility, further research is needed in Iran to achieve precise results in relation to increased antibiotic resistance in larger populations, especially macrolide antibiotics such as erythromycin. According to the MIC results obtained by the broth microdilution method, all of the strains that were positive for *M. hominis* and the *23S rRNA* gene were resistant to erythromycin. Since the target site for erythromycin is the S50 subunit of the bacterial ribosome, many cases of macrolide resistance in clinical isolates can be associated with specific nucleotide changes in domain V of the 23S rRNA gene in the ribosomal S50 subunit.

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Authors' Contributions

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

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Original Article

Changes of Serum Level of Homocysteine and Oxidative Stress Markers by Metformin and Inositol in Infertile Women with Polycystic Ovary Syndrome: A Double Blind Randomized Clinical Trial Study

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Abstract

Background: Hyperhomocysteinemia plays an important role in the anovulation in infertile women suffering from polycystic ovary syndrome (PCOS). However, long-term metformin therapy elevated homocysteine (Hcy) concentration in these individuals. Inositol increases serum insulin levels and improves ovulation. The aim of this study was to compare the effect of metformin and inofolic on the level of serum Hcy and oxidative markers in the infertile patients with PCOS.

Materials and Methods: Eighty PCOS infertile women undergoing *in vitro* fertilization in the Umm-al-Banin clinical center, Dezful, Iran from December 2018 to September 2019 were invited to participate in this double blind randomized clinical trial. They were divided into two groups; group A who received metformin (1000 mg twice/day) and folic acid (400 µg /day) and group B who used inofolic (inositol+ olic acid 200 µg twice/day) for 3 months.

Results: The mean Hcy levels increased significantly by metformin (P=0.02), but not by inofolic. There was a decrease in the total antioxidant capacity (TAC) after metformin administration (P=0.01). In both groups, a significant increase in folic acid levels was observed after treatment (P=0.04). Also, no significant change in vitamin B12 and malondialdehyde levels was observed in both groups (P=0.08).

Conclusion: These findings indicate an increase in the serum Hcy levels as well as a remarkable decrease in TAC following metformin treatment. Given the rise in blood Hcy in PCOS patients, inofolic and other medications containing inositol can be prescribed instead of metformin (registration number: IRCT20190508043516N1).

Keywords: Homocysteine, Inofolic, Metformin, Oxidative Stress, Polycystic Ovary Syndrome

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Introduction

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder that is responsible for a high proportion of infertility causes observed in 5-10% of women of reproductive age (1). PCOS cases are mostly characterized by the symptoms such as oligomenorrhea, hirsutism, anovulation (2), hyperandrogenism, hyperinsulinemia (following decreased tissue sensitivity to insulin) and high level of estrogen, which can impair the metabolic parameters and lead to the changes in lipid profile and oxidative stress biomarkers (3). Indeed, hyperinsulinemia can itself play a pathogenic role in this syndrome. In the long term it causes metabolic disorders such as glucose intolerance, type II diabetes and cardiovascular disease (CVD) (4). Therefore, previous studies have reported an elevated insulin rate in the serum of PCOS patients and subsequently a potent correlation was observed between insulin resistance and increased serum homocysteine (Hcy) levels in these women (5, 6). Hcy is an intermediary product of the methionine metabolism pathway and is catalyzed by the multiple enzymes along with the vitamin B12 and folic acid as a cofactor (7). Concentrations of Hcy in follicular fluid may be a useful marker in PCOS such as for fertilization rate, and oocyte and embryo quality (8).

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Metformin as an insulin sensitizer has been prescribed for PCOS patients. One study reported that metformin significantly increased Hcy level from 8.93 ± 0.49 to 11.26 ± 0.86 mmol/l in the serum of PCOS patients (9). It is possible that in diabetic patients, metformin administration can increase Hcy level by a reduction in the necessary cofactors such as plasma vitamin B12 and folic acid. While folic acid administration corrects Hcy elevation and vitamin B12 deficiency induced by metformin (5).

Folic acid (vitamin B9) controls blood Hcy levels (7). Its supplementation showed to prevent infertility and elevate ovulatory rates (10). Insulin sensitizers such as myoinositol have been advised to be prescribed for PCOS patients (11). This agent eventually stimulates the ovarian response to the endogenous gonadotropins by decreasing insulin secretion and androgens levels, therefore, enhance the ovulatory rates and spontaneous fertility (12). Studies support the idea of the insulin pathway impairment due to a defect in secondary inositol phosphoglycan (IPGs) (13). Also, in PCOS women deficiency or alteration in tissue inositol metabolism and IPGs may be due to insulin resistance (14). The supplementation with myoinositol and folic acid causes significant improvement in the serum level of insulin sensitivity and a reduction of Hcy after 3 months of treatment in PCOS participants (15). Also, a research concluded the use of myoinositol is capable of restoring ovarian function, metabolic and hormonal parameters, and subsequently fertility, in women with PCOS (16). Myoinositol therapy in women with PCOS causes better fertilization rates and a better embryo quality (17).

On the other hands, oxidative stress is an imbalance between reactive oxygen species production and the antioxidant defense system that ultimately causes oxidative damage to tissues. Total antioxidant capacity (TAC) is a combination of specific antioxidants such as vitamins E, C, and beta-carotene in the serum (18). Malondialdehyde (MDA) is also an indicator of lipid peroxidation and raise in the oxidative stress (19). Studies have designated an increase in oxidative stress in PCO patients (20). Some studies have reported that myoinositol is effective in reducing oxidative abnormalities in PCOS patients (21). Thus, metformin increases Hcy in women with PCOS (22). Therefore, we evaluated these two factors to investigate the effect of metformin and inofolic on the oxidative stress. However, to our knowledge there is no comprehensive investigation on the metformin consumption and its accompaniment with folic acid on the oxidative stress and Hcy in PCOS infertile women. Accordingly, this study aimed to investigate the effect of metformin and inofolic on the serum Hcy, folic acid and vitamin B12 levels in the PCOS infertile patients.

Materials and Methods

Subjects

This double blind randomized clinical trial was conducted on 80 infertile women with PCOS, who were elected with Block randomization method and attended for *in vitro* fertilization (IVF) or intra cytoplasmic sperm injection (ICSI) to the Umm-al-Banin clinical center in Dezful, Iran from December 2018 to September 2019. The PCOS diagnosis was based on two out of three Rotterdam criteria counting: clinical signs or biochemical excessive androgen level, oligomennerhea and/or anovulation, and morphology of ovaries in sonography termed as 12 or more small follicles (1).

The Ethics Committee of Dezful University of Medical Sciences approved the study protocol (IR.DUMS.REC.1397.037), registration ID in IRCT (IRCT20190508043516N1) and written informed consent was obtained from all the participants. Our inclusion criteria included women aged 22 to 38 years with a diagnosis of PCOS and serum levels of follicle-stimulating hormone (FSH) <10 IU / L on the third day of the cycle, minimum duration of infertility 3 years, who had no history of diabetes mellitus, hyperprolactinemia, congenital adrenal hyperplasia, thyroid disorders, cushing's syndrome, hypertension, folate and vitamin B12 deficiency, liver and kidney disorders.

All of the candidates <22 or >38 years, other causes of infertility, consumption of antioxidant drugs, were also excluded from the study. Also patients with current or previous history of using metformin within the last 3 months, other drug containing estrogen, progesterone and antiandrogenic effect such as combined oral contraceptive pill (OCP), antihypertensive drug were excluded

Intervention

This clinical trial study was conducted as a double-blind to remove probable biases in the evaluation of drug effectiveness. The research clinical group is responsible for concealment and allocation of the patients. Also, the samples will not know which drug product will be received. Only an Epidemiologist in the study was responsible for processing of the randomization and blinding to have access to blinded information on drug products. The other executive team will also remain as blind.

Patients were divided into two groups, 40 patients in each group, group A received 1000 mg metformin twice a day with 400 µg folic acid (Health Aid Co., UK) per day and group B consumed inofolic (LO.LI.pharma Co., Italy) 2 sachet per day (average amount per daily dose 1 sachet: Myo inositol 2000 mg, folic acid 200 µg) for a period of 12 weeks. Serum Hcy, MDA, TAC, vitamin B12, and folic acid levels were examined before and after consuming metformin and inofolic.

Outcome measured

Laboratory and biochemical analysis

Age, weight, body mass index (BMI, kg/m^2), menstrual pattern and laboratory study of homocysteine, folic acid, vitamin B12, malondialdehyde and TAC were measured in all of the participants for baseline parameters.

Women with cycles between 21-35 days considered regular cycle, 36 to 180 irregular cycles, cycles of less than 21 days of polymenorrhea, 36 to 180 days of oligomenorrhea, and cycles with intervals of 180 days or more were considered amenorrhea (2).

Blood samples were collected from each patient before and after treatment (12 weeks after beginning). Serums were separated immediately after sampling to avoid Hcy levels being increased and were stored at -70°C until assayed. Then Hcy levels were measured by UV Enzymatic Assay using the Hcy EIA Kit (DRG International Corporation, USA) with accuracy of 1 µmol /L.

Also, folic acid and vitamin B12 levels were measured by Cobas immunoassay (Roche Co., Germany). Oxidative stress factors (malondialdehyde and total plasma antioxidant) were respectively measured by Nalondi™ Lipid Peroxidation (MDA) assay. MDA assay has relied on a reaction with thiobarbituric acid (the TBARS assay) to generate a product that can be measured colorimetrically at 532 nm. Also, the total antioxidant defense was measured by Naxifer™ TAC assay kit with the ferric reducing ability of plasma (FRAP) assay.

Statistical analysis

The results were expressed as the mean ± SD. Difference between mean serum TAC, MDA, vitamin B12, folic acid, and Hcy levels before and after metformin or inofolic consumption was assessed by paired t test. All statistical tests were performed using the Statistical Package for the Social Sciences software (SPSS 16.0, SPSS Inc., Chicago, IL, USA). In all cases, $P \leq 0.05$ was considered statistically significant.

Results

The patients were randomly divided into the following two subgroups based on their drug consumption. The Consort statement flow diagram is presented in Figure 1.

Specific biochemistry tests

The baseline parameters were not significantly different between the two groups before treatment. The levels of serum Hcy, TAC, MDA, vitamin B12, and folic acid were obtained and compared before and after metformin and inofolic treatment (Tables 1, 2). The assay showed the level of all parameters before starting the treatment within the normal range in both groups. According to the findings, after metformin treatment for 3 months mean Hcy (normal range: 3.7-13.9 µmol/L) concentration increased in the serum (P=0.02). Also after inofolic treatment for 3 months mean Hcy concentration decreased in the serum but not significantly. The correlation between metformin and inofolic consumption with serum malondialdehyde concentration did not show a noticeable difference, but despite insignificant changes, it revealed a relative tendency to decrease serum malondialdehyde levels with inofolic consumption after the aforesaid time (P=0.12). Correlation between metformin consumption and total plasma antioxidant capacity showed a remarkable decrease in the TAC levels (485 Mm) in group A (P=0.01) but no substantial difference was observed in the serum TAC density after supplementation with inofolic in the group B. In addition, the levels of vitamin B12 did not show a significant difference in the subgroups after treatments. The mean serum level of folic acid was considerably increased in both groups (P=0.08) and indicated the same effect on the mean folic acid content.

Table 1: Basal clinical parameters of infertile PCOS patients in metformin and inofolic groups

Variables	Metformin	Inofolic	P value
Patients (n)	40	40	-
Age (Y)	35.37 ± 5.4[a]	34.21 ± 4.9[a]	0.07
BMI (kg/m^2)	27.82 ± 3.4[a]	28.43 ± 2.3[a]	0.1
Hcy (µmol/L)	12.21 ± 4.17[a]	11.43 ± 3.23[a]	0.06
Folic acid (ng/mL)	10.43 ± 5.86[a]	9.18 ± 4.41[a]	0.2
Vitamin B12 (Pg/mL)	219 ± 8.45[a]	220 ± 7.03[a]	0.09
TAC (µm)	531.34 ± 6.75[a]	584.82 ± 5.07[a]	0.1
MDA (nmol/mL)	4.86 ± 1.74[a]	4.11 ± 1.68[a]	0.1

[a]; In each column indicate no significant (P<0.05) difference. BMI; Body mass index, Hcy; Homocysteine, TAC; Total antioxidant capacity, and MDA; Malondialdehyde.

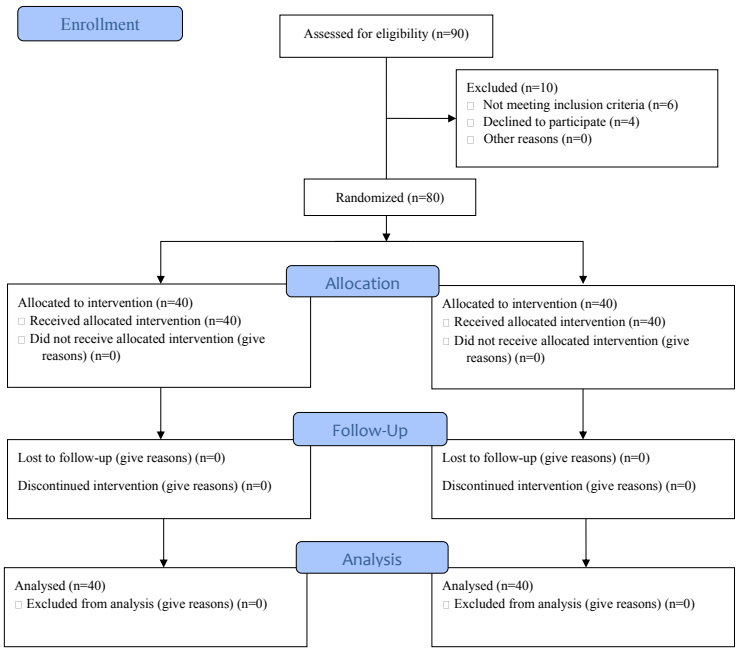

Fig.1: Consort flow diagram.

Table 2: Clinical parameters of infertile PCOS patients in metformin and inofolic groups before and after treatment

Variables	Group	Before treatment	After treatment	Difference	P value
Hcy (µmol/L)	A	12.21 ± 4.17a	13.39 ± 4.54[b]	1.18 ± 0.56	0.02
	B	11.43 ± 3.23[a]	10.94 ± 2.14[a]	-0.43 ± 0.14	
Folic acid (ng/ mL)	A	10.43 ± 5.86[a]	15.95 ± 6.31[b]	5.52 ± 1.13	0.04
	B	9.18 ± 4.41[a]	15.37 ± 5.7[b]	6.19 ± 1.25	
Vitamin B12 (Pg/mL)	A	219 ± 8.45[a]	237.4 ± 7.46[a]	18.40 ± 4.21	0.08
	B	220 ± 7.03[a]	227.29 ± 8.37[a]	7.29 ± 0.76	
TAC (µm)	A	531.34 ± 6.75[a]	485.87 ± 6.42[b]	-45.47 ± 6.16	0.01
	B	584.82 ± 5.07[a]	586.25 ± 7.8[a]	1.43 ± 0.37	
MDA (nmol/mL)	A	4.86 ± 1.74[a]	5.12 ± 1.5[a]	0.26 ± 0.08	0.12
	B	4.11 ± 1.68[a]	3.69 ± 1.31[a]	-0.42 ± 0.12	

Data are presented as mean ± SD. Assessed by paired t test. A; Metformin group, B; Inofolic group, Hcy; Homocysteine, TAC; Total antioxidant capacity, MDA; Malondialdehyde, and PCOS; Poly cystic ovary syndrome.

Discussion

Various studies have indicated that PCOS patients mostly experience high levels of androgen, insulin resistance, hyperinsulinemia, high level of estrogen and obesity that can affect metabolic parameters and oxidative stress (1). Previous studies have indicated that ROS and oxidative stress biomarkers show an increased amount of oxidative stress in PCOS patients (23). Previously, scientists declared a folic acid and vitamin B12 deficiency after a long-term metformin therapy in type 2 diabetes and PCOS patients. High levels of Hcy in PCOS patients might be explicable by these traits (6). Several studies have shown that serum folate depletion is one of the reasons for decreased fertility in women due to lack of ovulation (24). The mechanism by which folic acid affects fetal protection is not known, but may be due to the regulation of Hcy metabolism (9). Methylenetetrahydrofolate reductase, (MTHFR), is an enzyme that breaks down the amino acid homocysteine. The *MTHFR* gene that codes this enzyme has the potential to mutate, which can either interfere with the enzyme's ability to function normally or completely inactivate it. These mutations can lead to high levels of Hcy in the blood, which may contribute in numerous health conditions (7). Hcy is a cytotoxic amino acid that is catalyzed by multiple enzymes along with the cofactors such as folic acid. This study was designed with 80 infertile PCOS women to appraisal the effect of metformin and inofolic consumption on the alteration of serum Hcy, folic acid, vitamin B12, TAC, and MDA concentration.

Regidor et al. (25) study, reported there was 15% increase in pregnancy rates and significant decrease in testosterone and dramatic increase in progesterone levels after folic acid intake in PCOS women. Also, the patients who were taking myoinositol showed a higher rate of fertility and ovulation in this study. In a similar study, a 4-month treatment with metformin in patients with type 2 diabetes showed a dramatic decrease in serum folate concentration. The results of both studies are in contrast to our observations. There is no evidence of increase in serum folic acid concentrations during metformin therapy in previous studies, which is in contrast to our findings and could be due to the comparison of folic acid with metformin consumption.

On the other hand, our results in the present study indicated a significant increase in serum Hcylevels with metformin. In a study by Esmaeilzadeh et al. (5) investigating Hcy levels after 4 to 6 months of metformin intake, the results showed that metformin significantly increased serum Hcy levels in women with PCOS, suggesting that elevated serum Hcy levels may be one of the inherent side effects of metformin in this group. Many studies have reported metformin as a common drug in type 2 diabetes, with increased circulating Hcy concentration probably by decreasing plasma vitamin B12 and folic acid (26-28). In addition, metformin analysis showed that higher doses of metformin (more than 2000 mg/day) were associated with increased serum Hcy levels compared to lower doses (8). These results are also consistent with the present study and show that the effects of increased Hcy on metformin use is dose-dependent. Therefore, inofolic is a safe complement in PCOS women compared to metformin and folic acid. Folic acid uptake also appears to decrease with increasing Hcy concentration.

Studies have shown that folic acid and vitamin B12 levels are highly correlated, both of which affect reproductive quality and the health of women and fetuses. Folic acid and vitamin B12 are also absorbed in the body, but folic acid intake and serum concentrations are not mentioned in any of the related studies, and the results are consistent with our studies that metformin and inofolic consumption significantly increased folic acid levels. It did not show the amount of vitamin B12 in the serum (29). The results of our present study are probably because of long-term use of metformin on serum vitamin B12 concentration and suggest that the use of this drug on a temporary and short-term basis has no effect on vitamin B12 levels, thus it's administration can reduce related complications.

The present study showed a relative tendency to decrease serum malondialdehyde levels in inofolic compared to metformin consumption. Therefore, inofolice could reduce oxidative stress and its consequences such as

abnormalities in PCOS patients.

In a study conducted by Bułdak et al. (30) the effect of metformin on malondialdehyde in blood monocytes was examined on healthy non-smoker individuals and found that metformin reduced malondialdehyde in individuals. It is likely that the greater the sample size, the greater the decrease in malondialdehyde after ingestion. Since malondialdehyde increases in oxidative stress and decreases in inofolic group, therefore inofolic it can reduce the effects of oxidative stress in PCOS women and eventually can improve ovulation in these patients.

In one study, oxidative stress was measured in women with PCOS after taking myoinositol and metformin. The results showed no effect of metformin after 12 weeks of administration on malondialdehyde levels, which is consistent with our observations (31).

One study on diabetic rats found that 4 to 6 weeks of metformin prevented the increase in TAC in either treatment, monotherapy or combination therapy with insulin. This may be due to the action of metformin in controlling ROS production in diabetic rats, which is consistent with our observations (32). In another study in by Li et al. (6) that examined the levels of oxidative stress and Hcy in women with PCOS, the results showed a decrease in the TAC after metformin use in lean PCOS women. These observations are consistent with our results.

Conclusion

Considering the effect of metformin on serum Hcy levels and remarkable decrease in plasma TAC in infertile PCOS women, repeated monitoring of Hcy levels is recommended in patients with metformin intake and also suggest the use of inofolic and other medications containing inositol can be prescribed instead of metformin.

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Authors' Contributions

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

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Original Article

Macrophages and Natural Killer Cells Characteristics in Variously Colored Endometriotic Lesions: A Cross-Sectional Analytic Study

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Abstract

Background: Dysregulation of the immune response contribute to a significant role in endometriosis. This research examined macrophages and natural killer (NK) cells numbers in endometriotic lesions and their association with the different lesion colors: red, black, and white. To investigate the amount of the CD68 and CD56 in eutopic endometrium and different type of the endometriotic lesions.

Materials and Methods: A cross-sectional analytic study was conducted. Women suspected endometriosis requiring laparoscopic surgery between July 2016 and January 2017 were recruited. Their lesions were classified as red, black, or white and these lesions were excised by standard laparoscopic surgery. Twenty-four endometriotic lesions from each color group were obtained from 45 women who met the inclusion criteria. One type of lesion was collected from 25 women. Two different lesion types and three-color lesion types were collected from the same women in 13 and 7 subjects, respectively. Immunohistochemistry staining with anti-human mouse cluster of differentiation (CD) 68 monoclonal antibody for macrophages and mouse anti-human CD56 monoclonal antibody for NK cells were performed.

Results: The number of CD68 macrophages in red lesions was higher than in black and white lesions [median (25^{th}-75^{th} percentile); 10 (5-19.4), 0 (0-6.9), 0 (0-2.5) cells per mm^2, respectively, adjusted P=0.001 for red vs. black lesions and red vs. white lesions, and adjusted P=1.000 for black and white lesions]. The number of CD56 NK cells was not significantly different among red, black, and white lesions [median (25^{th}-75^{th} percentile; 5 (2-16.5), 3.8 (0-14.4), 1.3 (0-6.9) respectively, adjusted P=1.000 for red vs. black lesions and black vs. white lesions, and adjusted P=0.617 for red vs. white lesions].

Conclusion: The dynamic changes in the immune cells in ectopic endometrium were specific to the macrophages but not to the NK cells, as demonstrated by the highest number of CD68 macrophages in the red lesion, the earliest established ectopic endometrium. NK cells in endometriosis may have a role in the uterus.

Keywords: Endometriosis, Endometrium, Killer Cells, Macrophages, Natural

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Introduction

Endometriosis is an important gynecologic disease in women of childbearing age; however the pathophysiology of endometriosis is still poorly understood. Retrograde of menstruation into the peritoneal cavity established by Sampson is the most widely believed theory for the origin of the disease. At present, studies of certain genetic, environmental, and immunological factors enhance clinical practice of eradication of ectopic endometrial cells and may or may not allow the implantation of endometriotic lesions onto peritoneal tissue or pelvic organs. Current endometriosis treatments are not satisfactory due to the high rate of recurrence after surgery and the requirement of long-term pharmacological therapy. Treatments tailored to pathophysiology, for example an abnormal immunologic response, might yield a better outcome.

Endometriosis is associated with abnormal immunologic responses to both innate and adaptive mediated-immune cells, including T and B cells. Endometrial cells that retrograde during menstruation from the intrauterine cavity to the peritoneal cavity lyse and release chemokine and hypoxic substances. Macrophages then ingest these endometrial cells. However, these recruited macrophages also release certain cytokines leading to the recruitment of

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more macrophages to the peritoneal cavity, inducing anti-apoptosis of endometrial cells, and stimulating neo-angiogenesis. Thus, exaggerated activities of macrophages contribute to the survival of ectopic endometrial cells and the development of endometriotic lesions (1). The number and proportion of macrophages in the peritoneal fluid are significantly increased in women with endometriosis (2). Angiogenesis caused by vascular endothelial growth factor (VEGF) and induced by hypoxia is partly regulated by macrophages and might contribute to the persistence of ectopic endometrial tissue. Macrophages expression of cluster of differentiation (CD) 68 was significantly increased in eutopic endometrium of endometriosis women during the proliferative phase of the endometrium (3). Therefore, results from previous studies support the role of macrophages in the pathogenesis of the disease.

Natural killer (NK) cells, a component of innate immunity, release cytotoxic cytokines to kill malignant and infected cells. NK cells react with heat shock protein-70 and human leukocyte antigen (HLA)- G found on endometrial cells and release cytokines in order to kill them during retrograde menstruation (4). Many studies have provided evidence of NK cell dysfunction in women with endometriosis through the decreased cytotoxic activity and increased inhibitory activity of peripheral blood and peritoneal fluid NK cells (5). Dysfunction of NK cells might lead to endometrial cell survival and implantation and likely promotes the development of the endometriotic lesion. However, to the best of our knowledge, the amount and activity of NK cells in different endometriotic lesions of the peritoneum have not been reported yet.

Each color of the lesion (red, black, or white) of superficial peritoneal endometriotic has been studied for their association with different levels of inflammation, angiogenesis, and immunologic response. The red lesion is the most active, earliest lesion, resembling eutopic endometrium. VEGF level, microvessel density score, and surrogate markers of angiogenesis, are significantly higher in the red endometriotic lesions than black or non-opaque lesions in all phases of menstruation (6). Studies have also demonstrated that the inflammatory response, proliferating cell nuclear antigen index, endoglin or CD105 [a marker of transforming growth factor (TGF)-β type 1), interleukin-1 (IL-1) receptor type 1, hepatocyte growth factor (HGF), and HGF receptor] expressions are higher in red lesions than black and white endometriotic lesions (7, 8). Such findings suggest that red lesions are the most active lesion among all colors and confirm the retrograde menstruation theory and the occurrence of the red endometriotic lesion before black lesion.

Most studies have focused on markers of angiogenesis and inflammation, but very few have investigated dysregulated immune cells within endometriotic lesions. It was hypothesized that different types of endometriotic lesions would have differences in the expression of macrophages and NK cells. The present cross-sectional analytic study was conducted to compare the expression of CD68 macrophages and CD56 NK cells in red, black, and white endometriotic lesions.

Materials and Methods

This cross-sectional study was done at the Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynaecology, Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok, Thailand. It was approved by the Ethics Committee on Human Rights Research related to Research involving Human Subjects of Ramathibodi Hospital, Mahidol University (MURA 2016/364).

Study subjects

Women between 18 and 45 years old who had symptoms and signs of pelvic pain, dysmenorrhea, and infertility or ovarian cyst compatible with endometriosis were recruited in this study. All subjects provided informed consent before recruitment. These women underwent laparoscopic surgery from July 2016 to January 2017. The present study recruited women who had regular menstrual periods (cycle length within 28 ± 7 days in the 3 months before enrollment), were in the proliferative phase of their menstrual periods on the day of operation and had no history of any hormone therapy at least 3 months before the surgery. Each participant was questioned about parity, history of surgery, any previous pelvic inflammatory disease, pre-operative symptoms, duration of symptoms, and underlying disease as the baseline assessment. All operative data, including pre- and post-operative diagnosis, operation, and the stage of endometriosis, were also recorded. Subjects who had no peritoneal endometriosis, according to laparoscopic finding or histopathology, were excluded (Fig.1).

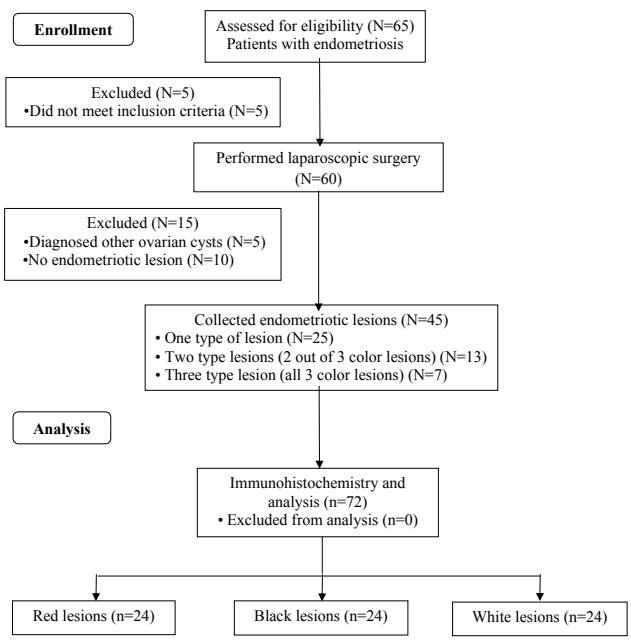

Fig.1: The study flow chart. N; Number of participants and n; Number of endometriotic lesions.

Sample size calculation

For the sample size estimation, we performed a pilot study for the expression of macrophages in different colors of endometriotic lesions using the n4Studies software with two independent means (two-tailed test) (9). Since the main aim of the present study was to compare the number of macrophages among three groups-red, black and white lesions-we chose means and standard deviations (SDs) of black and white lesions to calculate. The difference of means between a pair of black and white was less than both pairs of red and black and red and white.

$$n_1 = \frac{(z_{1-\frac{\alpha}{2}} + z_{1-\beta})^2 \left[\sigma_1^2 + \frac{\sigma_2^2}{r}\right]}{\Delta^2}$$
$$r = \frac{n_2}{n_1},\ \Delta = \mu_1 - \mu_2$$

The mean ± SD (σ) value in the black lesion group (μ) was 1.00 ± 0.30. Mean ± SD (σ) in the white lesion group (μ_2) was 0.80 ± 0.10. The ratio (r), alpha (α), Z (0.975), beta (β), and Z (0.800) values were 1.00, 0.05, 1.96, 0.200 and 0.84, respectively. The sample size for each group was 20 subjects. Allowing for a 20% dropout rate, 24 samples were recruited to each color group.

Endometriotic lesions collection

All operations were performed according to the standard laparoscopic surgical technique. Endometriotic lesions were carefully excised by laparoscopic scissors along with attached peritoneum no more than 2 mm from the lesions. Electro-cauterization was avoided to preserve tissue quality. Macroscopic appearances of endometriotic lesions were classified into red, black, or white in accordance with the revised American Society for Reproductive Medicine (rASRM) classification (10) confirmed by a second observer during operation. A red endometriotic lesion has red, red-pink, or clear morphology. The black endometriotic lesion has either a black or blue morphology. In addition, a white endometriotic lesion is a lesion containing a white, yellow-brown morphology, or peritoneal defects. The menstruation phase was confirmed by pathological dating of the eutopic endometrial tissue.

Tissue processing and immunohistochemistry

All lesions were fixed overnight in 10% formalin, followed by 70% alcohol, and embedded in paraffin. The tissues were then cut and prepared in a 3-mm thick slide for subsequent histopathological and immunohistochemical assesments. Every tissue was stained with hematoxylin and eosin (H&E) for histological study and examined by a pathologist to confirm the diagnosis. Tissues that were not diagnosed with endometriosis were discarded. The criterion for the diagnosis of endometriosis was tissues composed of both the endometrial gland and stroma.

An immunohistochemical study was performed with mouse anti-human CD68 monoclonal antibody (514H12,1:100; Novocastra, UK) for macrophages and mouse anti-human CD56 monoclonal antibody (CD56, predilution; Novocastra, UK) for NK cells. CD68 is a transmembrane glycoprotein receptor found in the endosome surface of monocytes and macrophages (lysosomal-associated membrane protein) and has been used as the principal marker of macrophages in most studies (11). CD56 is a cell membrane protein of an unknown function found on human lymphoblastoid cells, including NK cells (12). CD56 has been widely used as a marker of NK cells. The deparaffinization of paraffin-embedded tissue sections was performed in xylene solution. Tissue slide sections were incubated with Bond Dewax Solution (Leica Biosystems, Bannockburn, IL) for 60 minutes at 60°C. The slides were then incubated for 20 minutes at 100°C in Bond Epitope Retrieval Solution. The Bond Polymer Refine Detection kit (Leica Biosystems, Bannockburn, IL) was used for immunohistochemistry analysis (13). Briefly, the tissue slides were incubated with primary antibody for 45 minutes at room temperature. Hydrogen peroxide solution at 3% concentration was added for 5 minutes and then washed with Bond Wash Solution. The tissue slides were incubated for 9 minutes in the post-primary polymer. Polymer poly-HRP IgG was added for 7 minutes and then rinsed with bond wash solution before applying the diaminobenzidine chromogen for 4 minutes. Counterstaining of the slides with hematoxylin solution was done for 5 minutes. A tonsil tissue was used as a positive control slide.

Measurement of CD68 macrophages and CD56 NK cells number of endometriotic lesions

Positive CD68 macrophages and CD56 NK cells will appear as brown chromogenic granules. Two investigators,who were blinded for the type of tissues, counted the number of positive cells under microscopy with ×200 magnification independently. The cells were randomly counted in ten different fields, 200×200 microns each, and reported as (cells per mm^2). Results from two investigators were checked for sample correlation and recounted if differences occurred.

Statistical analysis

All data in the present study were statistically analyzed using an SPSS program version 23.0 (Statistical Package of the Social Security, IBM, Armonk/USA). The baseline demographic data of all women was analyzed as a whole and as a group by the color of the lesion. The number of CD68 macrophages and CD56 NK cells was compared between groups. Data were presented as median with 25^{th}-75^{th} percentile, or number with percentage as appropriate. The normality of the data was assessed by the Shapiro-Wilk test. Categorical variables were assessed with the Chi-square test. Continuous non-normally distributed variables between groups were compared with the Kruskal Wallis test. A $P<0.05$ was used to determine statistical significance. Multiple comparisons were used to assess the number of CD68 macrophages and CD56 NK cells between each group. Adjusted $P<0.05$ was chosen as the cut off for statistical significance.

Results

Sixty women suspected of endometriosis and admitted to Ramathibodi Hospital for laparoscopic surgery were recruited into the study if they met the inclusion criteria. All of them had no underlying medical condition, except two women with a thyroid nodule, one woman with hyperthyroidism, and the other one with hepatitis B carrier. Fifteen women were excluded because the diagnosis during surgery was not an ovarian endometriotic cyst and no endometriotic lesions were present. Twenty-four endometriotic lesions were obtained for every three groups. Twenty-five women had only one type of lesion. Thirteen and seven women had two and three matched different lesions, respectively. All tissues were histologically diagnosed as endometriosis (Fig.1). The demographic data of endometriosis women are shown in Table 1. The median (25^{th}-75^{th} percentile) age of the subjects was 38 (33.3-40.0) years. Their presenting symptoms included chronic pelvic pain (4.4%), dysmenorrhea (57.8%), an ovarian cyst (37.8%), and infertility (35.6%). Forty percent had a history of previous surgery. None had past medical illnesses related to pelvic inflammatory disease. The distribution of the rASRM stage was severe (60%), moderate (13.3%), mild (8.9%), and minimal (17.8%). There was a significant difference in the number of CD68 macrophages in the eutopic endometrial gland, eutopic endometrial stroma, red lesions, black lesions and white lesions (P<0.001, Fig.2). The red endometriotic lesions contained the highest number of CD 68 macrophages when three ectopic endometrial tissues were compared; red vs. black vs. white [median (25^{th}-75^{th} percentile); 10 (5-19.4), 0 (0-6.9), and 0 (0-2.5), respectively]. The eutopic endometrial stroma had higher number of CD 68 macrophages than the eutopic endometrial gland [median (25^{th}-75^{th} percentile); 20 (7.5-35) and 0 (0-2.5), adjusted P<0.001] (Table 2).

Table 1: Baseline demographic data of women with endometriosis

Characteristics	All women (n=45)
Age (Y)	38 (33.3-40.0)
Parity	
0	43 (95.6)
1	2 (4.4)
Previous history of surgery	18 (40.0)
Symptoms	
Chronic pelvic pain	2 (4.4)
Dysmenorrhea	26 (57.8)
Ovarian cyst	17 (37.8)
Infertility	16 (35.6)
Duration of symptoms in months, median (25^{th}-75^{th} percentile)	8 (4-21)
Stage of endometriosis	
I	8 (17.8)
II	4 (8.9)
III	6 (13.3)
IV	27 (60.0)

Data are presented mean ((25^{th}-75^{th} percentile) or n (%). Statistical analysis was performed by SPSS version 23.0.

Table 2: The number of CD68 macrophages and CD56 natural killer cells in eutopic endometrium and different types of endometriotic lesions

Immune cells	Endometrial gland	Endometrial stroma	Red lesion	Black lesion	White lesion	P value
Macrophages (cells/mm^2)	0 (0-2.5)	20* (7.5-35)	10* (5-19.4)	0**,*** (0- 6.9)	0**,*** (0-2.5)	<0.001
NK cells (cells/mm^2)	5 (0-10)[a]	70* (17.5-220)	5** (2-16.5)	3.8** (0-14.4)	1.3** (0-6.9)	<0.001

[a]; Positive cells were expressed as median (25^{th}-75^{th} percentile), *; Significant difference from endometrial gland, adjusted P<0.001, **; Significant difference from the endometrial stroma, adjusted P=0.001, *; Significant difference from the red lesions, adjusted P=0.001, and NK; Natural killer. Statistical analysis was performed by SPSS version 23.0.**

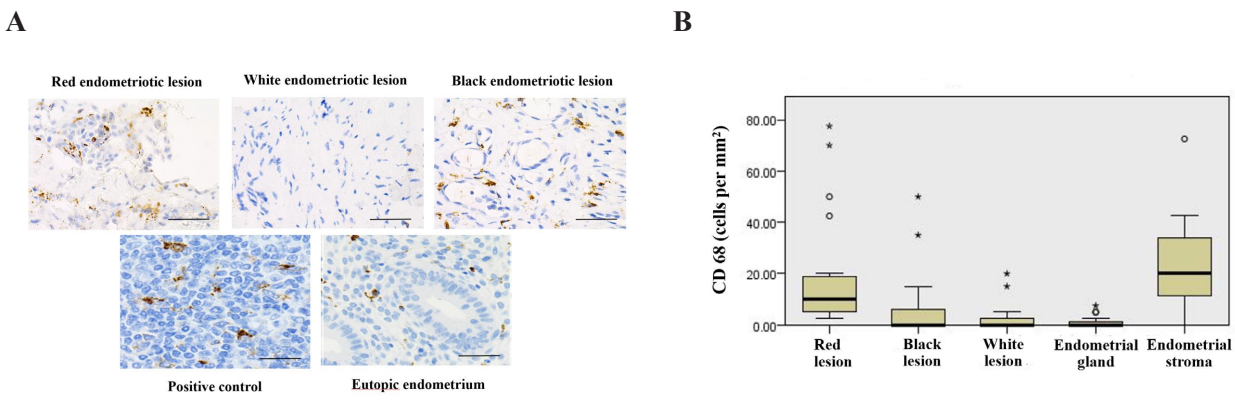

Fig.2: The immunohistochemistry by anti-CD68 to identify macrophages in eutopic endometrium and different types of endometriotic lesions. **A.** The immunohistochemistry images of each type of endometriotic lesions (scale bar: 50 µm), **B.** Boxplot graph of macrophages number of each type of endometriotic lesions.

There was a significant difference in the number of CD56 NK cells in the eutopic endometrial gland, eutopic endometrial stroma, red, black and white lesions (P<0.001, Fig.3). The number of CD56 NK cells was not significantly different among red, black, and white endometriotic lesions [median (25^{th}-75^{th} percentile); 5 (2-16.5), 3.8 (0-14.4), and 1.3 (0-6.9), respectively]. The eutopic endometrial stroma exhibited a higher number of CD56 NK than the eutopic endometrial gland [median (25^{th}-75^{th} percentile); 70 (17.5-220) and 5 (0-10), adjusted P<0.001].

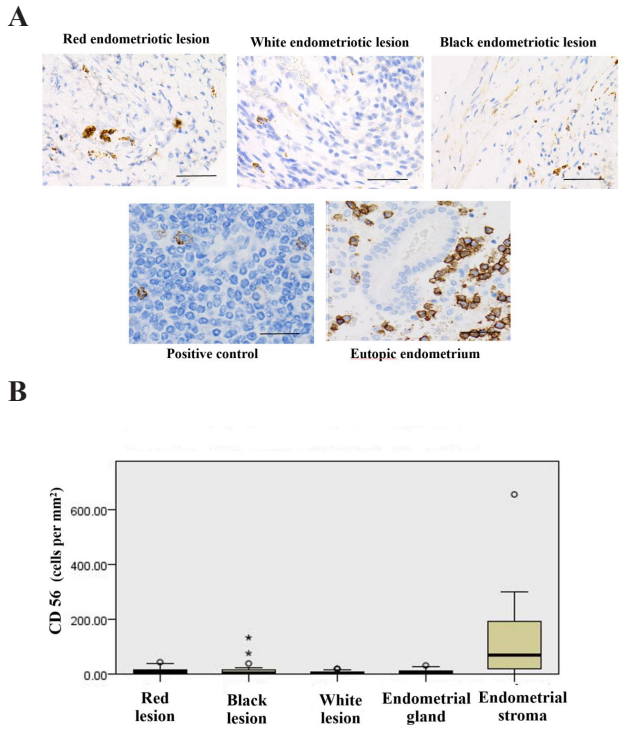

Fig.3: The immunohistochemistry by anti-CD56 to identify natural killer cells in eutopic endometrium and different types of endometriotic lesions. **A.** The immunohistochemistry images of each type of endometriotic lesions (scale bar: 50 µm), **B.** Boxplot graph of macrophages number of each type of endometriotic lesions.

Discussion

The study investigated macrophages and NK cells in peritoneal endometriosis by comparing the number of CD68 and CD56 positive cells according to the different colors of endometriotic lesions; black, red, and white. The number of CD68 macrophages in red endometriotic lesions was significantly higher than in the black and white endometriotic lesions. The expression of CD68 macrophages in red lesions was similar to the stroma of the eutopic endometrium. CD56 NK cell abundance was not significantly different among all color types of endometriotic lesions. Nevertheless, CD56 NK cells were more abundant in the endometrial stroma than in all endometriotic lesions.

The dynamic features and metabolic activities of peritoneal endometriosis have been studied in both animal models and humans. Multiple studies have reported that red lesions show increased vascularization (14), increased expression of VEGF (15), increased epithelial mitotic and proliferation activity (16), higher incidence of complex glands, and increased matrix metalloproteinase (MMP)-1 (17) and MMP-2 (18) when compared to white and black lesions. The study of dynamic change of lesions investigated in the monkey model of endometriosis demonstrated that the red lesion had a higher proliferation index, endothelial cells numbers, and vascularity compared with the black and white lesions, but similar to the endometrium (19). These data support the hypothesis that red endometriotic lesions are the initial stage of peritoneal lesions and consist of more active metabolic function than the other types of lesions. Black and white lesions are assumed to be advanced endometriosis and healed endometriosis or quiescent lesions, respectively (16). Many studies have reported that red lesions consist of a more pro-inflammatory process and immune cells than the other lesion types. Increased a pro-inflammatory transcription factor nuclear factor-kappa B (NF-kB) (20), IL-1 receptor type 1 (7), a receptor for a macrophage-derived pro-inflammatory IL-1, and a pro-inflammatory macrophage migration inhibitory factor (MIF) were found in red lesions (21). MIF was believed to function to retain macrophages in the lesions. Therefore, the inflammatory process, specifically macrophage and macrophage-related cytokines, plays an important mechanism involving the pathogenesis of early endometriosis development.

Macrophages in endometriosis play multiple dynamic roles (or phenotypes), for example, growth of lesion, neurogenesis and angiogenesis in endometriosis. Women with endometriosis have an increased number of CD68 macrophages in eutopic endometrium (3), an increased number of macrophages and a higher level of pro-inflammatory in peritoneal fluid (22) compared to healthy controls. The present study found that the number of CD68 positive cells, a marker for macrophages, is significantly higher in red lesions than in black or white lesions and similarly to eutopic endometrium. The results from the present study are consistent with those of Khan et al. (23). Endometrial macrophages derived from women with endometriosis contributed lower expression of CD163, a marker of wound healing, than those from women with no disease (24). Macrophages may increase the deposition of the retrograded endometrial tissue in the peritoneal cavity since the expression of MMP-9, reflecting tissue remodeling, was demonstrated to co-localized with CD 68 macrophages in the endometrium of women with endometriosis (25). A decrease in the phagocytotic activity of macrophages enhanced the growth of endometriotic lesions, as demonstrated *in vitro* by Shao et al. (26). Macrophages contribute to neurogenesis in endometriosis. Macrophages were found densely in the high nerve fiber density area of the endometriotic lesions, and the *in vitro* study demonstrated that the outgrowth of a nerve fiber by chemokines secreted by macrophages was estrogen-dependent (27). The mouse endometriosis model demonstrated that deletion of endothelial growth factor receptor 1 (*VEGFR1*) gene decreased

endometriotic lesion and vascularity. VEGFR1 positive cells in endometriotic lesions derived from macrophages in bone marrow (28). Previous data demonstrated the progressive change of the macrophage phenotype over time in endometriosis. Initially, macrophages expressed pro-inflammatory cytokine inducible nitric oxide synthase but they then switched to tissue modeling markers, that is, CD204 and arginase, after one to two weeks of induction of the endometriotic lesions (29). In addition, the present study showed the same direction of the dynamic change in terms of the macrophage numbers which were found more in the active red lesions than less active, black and white lesions.

Cytotoxicity of NK cells is a response of NK cells using activating and inhibitory receptors on its cell surfaces to target cells. The balance between both activating and inhibitory receptors affects the action of NK cells on the target cells. NK cell attacks the target cell when it binds to the NK cell's activating receptor. However, the NK cell does not act on the target cell when it binds to the NK cell's inhibitory receptor. An important function of NK cells in the peritoneal cavity is to get rid of the refluxed eutopic endometrium bearing a non-classic HLA-G during menstruation. NK cells use the killer immunoglobulin-like receptor (KIR) 2DL4 (CD158d) to bind to HLA-G on endometrial cells and destroy endometrial cells (30). Many studies have reported that the numbers of cytotoxic NK cells were reduced in the peritoneal fluid and circulation of patients with endometriosis association with an overall decrease in NK cell activity (12, 31, 32), while another study reported that the number of NK cells in blood circulation was increased (33). The expression of inhibitory receptors on the cell surface of NK cells, such as killer immunoglobulin-like receptor 2DL1 and immunoreceptor tyrosine-based activation motif-killer immunoglobulin-like receptor (ITAM-KIR), were up-regulated in women with endometriosis compared to healthy women (34-36). The dysfunctional NK cell cytotoxicity might allow reflux endometrium to survive in the peritoneal cavity.

No dynamic changes in the number of NK cells in the ectopic endometrium of women with endometriosis were demonstrated from the present study. This indicates that NK cells might not play a differential role in the dynamic progression of endometriosis lesions. However, the expression of NK cells was more prominent in the eutopic endometrium than in peritoneal ectopic lesions. The results of the present study were comparable to Drury's study. They collected the eutopic endometrium from women with and without endometriosis, 30 subjects for each group, and ectopic endometrium from 22 women with endometriosis, having matched eutopic endometrium for seven women. They reported strikingly low NK cells numbers in ectopic lesions when compared to uterine NK (uNK) cells. Dynamic change of uNK cells across the menstrual phase cycle was also demonstrated (37).

The role of uterine NK (uNK) cells is not well understood (12). The number of uNK cells is low during the proliferative phase of the endometrium, but the number of uNK cells is increased in the secretory phase (38). Moreover, uNK cells proliferation was increased during early pregnancy and associates with good pregnancy outcomes (39). However, a recent retrospective study did not demonstrate the association between endometriosis and an increased number of uNK cells (40). The present study compared NK cell abundance between different peritoneal lesions and eutopic endometrium, but no tissues from women without endometriosis were compared side by side. Future work should compare the uNK cell abundance of women with and without endometriosis and study the functional role of NK cells in the uterus.

Knowledge from the present study could be useful for designing the potential therapeutic intervention, for example, a drug for blocking macrophages or blocking recruitment of macrophages, and the appropriate timing for prescribing these medications. The limitation of the present study was the sample of all colors could not be simultaneously collected from the same subject because one endometriosis woman could have more than one color of lesions. Moreover, only the number but not the activity of both macrophages and NK cells were studied.

Conclusion

The dynamic changes in the immune cells in ectopic endometrium are specific to the macrophages but not to the NK cells, as shown by the highest number of CD68 macrophages in the red lesion, the earliest established lesion of peritoneal endometriosis. NK cells in endometriosis may contribute their role to the uterus, and they have been reported to be a significantly low number in ectopic endometrium in women with endometriosis.

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Authors' Contributions

A.S., N.A., Y.T.; Participated in study design, data collection, drafting and statistical analysis. M.S., S.S., W.W., K.D., T.C., A.J.; Conducted laboratory results. A.S., Y.T.; Participated in the finalization of the manuscript. All authors read and approved the final manuscript.

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Original Article

Relationship between Serum Vitamin D in Male, Sperm Function and Clinical Outcomes in Infertile Men Candidate for ICSI: A Cohort Study

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Abstract

Background: Today, vitamin D deficiency (VDD) is one of the major health issues around the world and VDD is associated with several diseases. This study was conducted to find the relationship between vitamin D status in male's serum with sperm function and clinical outcomes in infertile men candidate for intracytoplasmic sperm injection (ICSI).

Materials and Methods: In this cohort study, different parameters of male fertility such as sperm parameters, oxidative stress, and sperm chromatin status were evaluated in sperm samples of 30 infertile couples candidate for ICSI. Clinical outcomes like fertilization, embryo quality, and implantation were also assessed. Data were analyzed using SPSS Statistics 25.0 software. Besides, assessment of the correlation between aforementioned parameters with the level of serum vitamin D, in this study, ICSI candidates were divided into three groups [individuals with sufficient vitamin D levels (>30 ng/ml), insufficient vitamin D levels (between 20-29 ng/ml), and VDD (<20 ng/ml)]. The aforementioned parametesr were also compared between these study groups.

Results: Analysis of all the data revealed a significant correlation between the level of vitamin D with sperm concentration (P=0.000, r=0.5), sperm count (P=0.03, r=0.31) and sperm reactive oxygen species (ROS) level (P=0.000, r=-0.77). Moreover, comparing clinical outcomes within study groups showed a significant difference in implantation rate between sufficient and other groups (insufficient and deficient) (P=0.02).

Conclusion: Considering the association between sperm concentration and level of ROS with vitamin D and, higher implantation rate in individuals with vitamin D sufficient group compared to other two groups, our data call for vitamin D supplementation as part of male infertility treatment. But considering our sample size, further research is needed to verify these findings.

Keywords: DNA Fragmentation, Infertility, Oxidative Stress, Sperm Motility, Vitamin D

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Introduction

Vitamin D deficiency (VDD) is known as a major health issue and affects the normal functions of many organs (1). It is believed that the effect of VDD is more profound in organs in which vitamin D metabolizing enzymes and vitamin D receptors (VDRs) are present (2, 3). Given the presence of VDRs as well as its metabolizing enzymes in male and female reproductive systems, VDD is also likely to affect human fertility (4). The presence of VDRs and several cytochrome P450 enzymes (CYPs) which are known as vitamin D metabolizing enzymes such as CYP2R1, CYP27R1, CYP24A1 in Sertoli cells, germinal cells, Leydig cells, spermatozoa, and epithelial cells of the male reproductive tubules highlights the importance of vitamin D in male fertility (5). It is clear that expression of CYP24A1 is positively correlated with total sperm count, concentration, motility, and morphology (5) while the presence of vitamin D,unlike in follicular fluid, is limited in the semen. So, researchers believe vitamin D released during ovulation via follicular fluid, may act as chemoattractant, and facilitate the process of *in vivo* fertilization (6). So, activation of VDRs in sperm could increase intracellular calcium in the human sperm and mediate sperm motility, sperm capacitation, acrosomal reaction, and sperm attachment to the oocyte (7). In addition, vitamin D increases lipase activity by lowering triglycerides, providing the energy needed for sperm (8). Furthermore, a recent study by our group showed that dietary VDD not only may affect spermatogenesis but also could impact chromatin status and DNA integrity subse-

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quently reduction of fertility potential in men (9). These findings also validate the results of some studies showing the association between VDD and male infertility in humans (7, 10).

While the limited number of studies assessing the association between vitamin D levels and sperm parameters, chromatin status, DNA integrity, oxidative stress, and fertility in humans, a previous study on rats showed the importance of the level of vitamin D in their diet for sperm DNA integrity and fertility potential (11). Lack of vitamin D could be associated with infertility in mammals. Moreover, another study by Azizi et al. (12) revealed that sperm DNA fragmentation and ROS do not have a significant relationship with vitamin D. Therefore, this study aims to assess sperm quality, chromatin integrity, and ROS in individual candidates for intracytoplasmic sperm injection (ICSI) with different levels of vitamin D deficiency.

Materials and Methods

This cohort study was conducted on 30 couple candidates of ICSI that referred to Shiraz Infertility Treatment Center between April 2019 and October 2019. This study was approved by the Ethics Committee of Azad University, Science and Research Branch of Tehran, Iran (IR.IAU.SRB.REC.1397.102). Prior to participation, individuals were informed about the study, and they were asked to sign an informed consent form.

Serum vitamin D

For assessment of serum vitamin D levels, a vitamin D total kit (Roche Diognosis, USA) was used. This assay is intended for the quantitative determination of a total 25-hydroxyvitamin D in human serum and plasma. The functional sensitivity of this test is determined to be 4.01 ng/ml. The limits and measurement ranges are between 3.00-70.00 ng/ml. The value below the limit of detection is reported as <3 ng/ml and values above the measurement range are reported as >70 ng/ml. Accordingly, individuals were divided into three groups based on previous literature cut off values (13): individuals with sufficient vitamin D levels (>30 ng/ml), insufficient vitamin D levels (between 20-29 ng/ml), and VDD (<20 ng/ml) (14, 15).

Inclusion criteria

Couples with at least one parameter below the cutoff values defined by World Health Organization (WHO, 2010), were considered as a male factor and were included in the study. In these cases, females presented normal menstrual cycles with normal hysterosonography. In addition, to reducing female confounding factors, females with age higher than 35 years old, and women with infertility causes such as low levels of anti-mullerian hormone (AMH), polycystic ovary syndrome (PCOS), and endometriosis were excluded in the study. Besides, men under vitamin D supplement treatments within the past 3 months, women with vitamin D deficiency, men with seminal infections, systematic disease or endocrine disorders, and men with azoospermia and severe oligozoospermia were excluded in this study.

Semen analysis

Semen samples were collected into sterile containers after 3-7 days of sexual abstinence, and were assessed according to WHO (2010) criteria or as described below. Sperm concentration was assessed by a sperm counting chamber with 10 µm depth (Sperm meter, sperm processor, Garkheda, Aurangabad, India). Ten micro-liters of liquefied semen were loaded on the chamber and the number of sperm were counted and expressed as million per ml. Sperm motility was assessed by light microscopy as sperm were considered progressive, non-progressive, and immotile. For assessment of sperm morphology, Papanicolaou staining was used according to WHO (2010) protocol. In this method, at least 200 sperm were counted in each sample at ×100 magnification. A portion of the remaining semen sample was processed by density gradient centrifugation (DGC) method for ICSI technique, and the remainder was used for assessment of functional tests such as intracellular ROS, sperm DNA fragmentation, and protamine deficiency.

Cytosolic reactive oxygen species

Cytosolic ROS was assessed using Dichlorofluorescin (DCF) staining by a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) according to a previous study (16). Briefly, two million sperm per ml phosphate-buffered saline (PBS, Sigma, Louis, MO, USA) was separated from the semen sample and incubated with 0.5 µl DCFH-DA for 40 minutes at room temperature in a dark condition. Then, sperm DCF was evaluated by flow cytometry. For verification of the procedure, a positive control tube was considered. Initially, ROS was induced by adding H_2O_2 to sperm samples before incubation with DCFH-DA stain, and then 0.5 µM DCFH-DA was added to the sperm sample. The result was expressed as the percentage of DCF positive spermatozoa.

Protamine status

Sperm protamine deficiency was assessed using Chromomycin A3 (CMA3) staining according to Iranpour et al. (17). Briefly, 100 microliters of semen samples were washed by PBS buffer and then fixed in Carnoy's fixative solution at 4°C for 5 minutes. After preparing smears, slides were treated with 100 microliters of 0.25 mg/ml CMA3 (Sigma, St. Louis, MO, USA) in McIlvaine buffer. The slides were then rinsed in PBS buffer. Finally, microscopic analysis was performed using an epifluorescence microscope (Olympus, Japan) equipped with appropriate filters (460-470 nm) at ×100 magnification. For each sample, at least 200 sperm were assessed and sperm with bright yellow color were considered as positive or protamine deficient sperm, while sperm with dim yellow color were considered as negative or sperm with normal protamine content.

Sperm DNA fragmentation (TUNEL assay)

Sperm DNA fragmentation was assessed by Terminal Deoxynucleotidyl Transferase dUTP nick end labeling (TUNEL) commercial kit (Promega, Germany) according to manufacturer's instructions (18). Briefly, the semen sample was washed with PBS and fixed with paraformaldehyde. Subsequently, cells were permeabilized with Triton x100 solution in PBS for 5 minutes, and then washed with PBS and the procedure was continued according to GMP stated in the kit. The status of sperm DNA integrity was analyzed by a FACS-Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) and at least 10,000 sperm were counted. The result for each case was expressed as a percentage of DNA fragmentation.

Ovulation induction and intracytoplasmic sperm injection

For ovarian stimulation, gonadotropin-releasing hormone (GnRH) antagonist was used for superovulation, using Cinal-F (Sinagen, Iran) and Menogon (Ferring, Germany) along with Cetrotide (Serono, Germany). The cycle was monitored using vaginal ultrasound. Ovulation was triggered by the administration of 10000IU human chorionic gonadotropin (HCG, Poyesh Daro, Iran). G-V series Vitrolife culture media (Vitrolife, Sweden) was used for performing ICSI and culturing embryos. Following vaginal aspiration of the follicle, ICSI was carried out according to standard protocols. Briefly, follicles were aspirated with the aid of transvaginal guided ultrasound. The aspirated cumulus oocyte complex was treated with hyaluronidase to remove cumulus cells. Maturity of oocyte was defined and MII oocyte was inseminated with a motile and morphologically normal sperm under 200 magnification. Inseminated oocytes and embryos were cultured at 37ºC in 6% CO_2 and 6% O_2 under humidified conditions.

Fertilization rate and embryos quality

After 16-18 hours post-ICSI, oocytes were assessed for the presence or absence of two pronuclei (2PN). The fertilization rate was calculated by dividing the ratio of fertilized oocytes by the total number of injected oocytes multiplied by 100. Embryos were graded on days 2 and 3 after insemination based on the 3 scoring system. Grade A: equal-sized blastomeres with blastomeric fragmentation less than 5%, and having at least 4 blastomeres on day 2 and 8 blastomeres on day 3. Grade B: 5-15% blastomeric fragmentation, having at least 4 blastomeres on day 2 and 8 blastomeres on day 3. Grade C: unequal blastomeric size with the fragmentation of more than 15%, and having less than 4 blastomeres on day 2 and 8 blastomeres on day 3. For the assessment of chemical pregnancy, the level of βHCG was measured. Clinical pregnancy was defined by ultrasonography findings showing at least one embryo with a fetal heartbeat, 5 weeks after embryo transfer. Implantation was defined by the number of observed gestational sacs divided by the number of transferred embryos.

Statistical analysis

Obtained data from sperm parameters, sperm functional tests, and clinical outcomes of participating couples were analyzed by IBM SPSS Statistics 25.0 software (SPSS.Inc., Chicago, USA) and the graphics were designed by GraphPad Prism (GraphPad Software,San Diego, California, version 8.00). The data represented were reported as mean ± SD. The one-way ANOVA (Tukey's post hoc test) was used to compare study parameters within groups. In addition, for assessment of the relationship between vitamin D with other parameters, two-tailed Pearson correlation test was used. $P<0.05$ was considered significant. Furthermore, a chi-square test was used for analyzing the mean of chemical and clinical pregnancy. The Kolmogorov-Smirnov test and Shapiro-Wilk test were utilized to evaluate the normality of data.

Results

In this study, semen samples of 30 infertile couple candidates for ICSI were analyzed. The mean of sperm concentration, motility, abnormal morphology, and semen volume were 44.0 ± 23.77 (106/ ml), 40.83 ± 16.19 (%), 95.5 ± 3.24 (%), and 3.7 ± 1.81 (ml), respectively. The mean of male and female ages was 35.25 ± 4.78 and 28.25 ± 4.47, respectively.

Vitamin D level in subgroups

The mean value of measured serum vitamin D in couples within sufficient, insufficient, and deficient groups and other demographic characteristics are presented in Table 1 respectively; the difference of male serum vitamin D between the three groups was statically significant ($P\leq0.001$). The number of couples in the sufficient, insufficient group, and deficient groups were 11, 8, and 11, receptively (Table 1).

Comparison of age, sperm parameters, and functional tests within study groups

In this study, the ages of the males and females in the sufficient, insufficient, and deficient vitamin D groups were compared, and no significant difference was observed. In addition, semen volume, sperm concentration, sperm total count, motility, and abnormal morphology were compared within these groups, only sperm concentration was significantly lower in the deficient vitamin D group compared to sufficient vitamin D group ($P=0.000$, Table 2).

Mean of sperm DNA fragmentation, ROS, and protamine deficiency were also compared within groups (Fig.1). The means of sperm DNA fragmentation were 7.72 ± 3.3, 7.42 ± 2.0, and 9.45 ± 4.5, in the sufficient, insufficient, and deficient vitamin D groups, respectively. The mean of this parameter was insignificantly higher in the deficient group compared to sufficient and deficient vitamin D groups ($P>0.05$). Regarding sperm ROS, the mean of this parameter was significantly higher in the deficient group compared to sufficient and deficient vitamin D groups ($P=0.000$). Moreover, we did not observe any significant difference in sperm protamine deficiency within sufficient, insufficient, and deficient vitamin D groups (Fig.1).

Table 1: Comparison of couples vitamin D and ages between study groups

Age and vitamin D supplement	Sufficient vitamin D (>30 ng/ml) (n=11)	Insufficient vitamin D (20-29 ng/ml) (n=8)	Deficient vitamin D (<20 ng/ml) (n=11)	P value between groups
Male age (Y)	35 ± 5.38	35 ± 3.11	35 ± 5.51	0.94
Female age (Y)	27.45 ± 5.31	28.37 ± 4.56	28.9 ± 3.75	0.75
Male vitamin D (ng/ml)	39.3 ± 6[a]	26.36 ± 3.3	15.02 ± 3.65[a]	<0.001
Female vitamin D (ng/ml)	39.86 ± 7.85	37.56 ± 6.5	42.29 ± 9	0.47

All data were presented as mean ± SD. Common letters indicate a significant difference between the two groups in each column (ANOVA followed by Tukey's multiple comparisons). ROS; Reactive oxygen species.

Table 2: Comparison of sperm parameters and functional tests between study groups

Parameters	Sufficient vitamin D (>30 ng/ml) (n=11)	Insufficient vitamin D (20-29 ng/ml) (n=8)	Deficient vitamin D (<20 ng/ml) (n=11)	P value between groups
Volume (ml)	3.63 ± 1.75	401 ± 2.03	3.54 ± 1.86	0.8
Sperm concentration (10^6/ml)	63.36 ± 12.66[a]	41.12 ± 28.63	26.81 ± 12.70[a]	<0.001
Sperm total count (10^6/ejaculate)	234.72 ± 138	164 ± 161.78	102.72 ± 88.52	0.08
Abnormal sperm morphology (%)	96.1 ± 2.45	96.37 ± 1.84	94.36 ± 4.43	0.32
Sperm motility (%)	46.36 ± 15.32	40.62 ± 15.22	35.45 ± 17.24	0.3
Sperm DNA fragmentation (%)	7.72 ± 3.31	7.42 ± 1.98	9.45 ± 4.52	0.41
ROS positive sperm (%)	22.12 ± 10.07[a]	27.66 ± 10.04	58.59 ± 11.28[a]	<0.001
Sperm Protamine deficiency (%)	16.81 ± 6.09	21 ± 5.47	22.45 ± 15.8	0.45

All data were presented as mean ± SD. Common letters indicate a significant difference between the two groups in each column (ANOVA followed by Tukey's multiple comparisons). ROS; Reactive oxygen species.

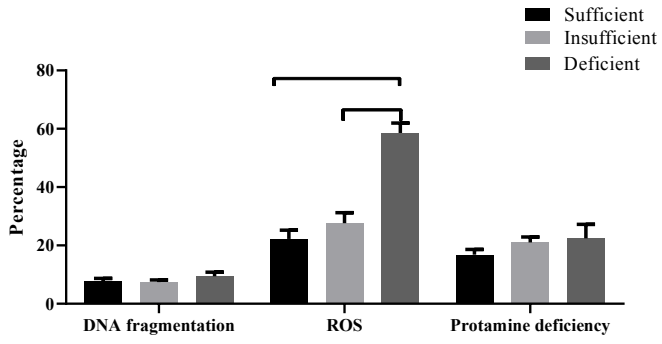

Fig.1: Comparison of sperm DNA fragmentation, reactive oxygen species (ROS), and protamine deficiency between sufficient, insufficient, and deficient vitamin D, groups. Mean value compared using ANOVA followed by Tukey's multiple comparisons. The number of participants in the sufficient, insufficient, and deficient vitamin D, group were 11, 8, and 11, respectively (P<0.001).

Correlation of vitamin D with sperm parameters

Among sperm parameters and sperm functional tests, only sperm concentration, sperm total count, and ROS level showed significant correlations with vitamin D level (Table 3).

Comparison of intracytoplasmic sperm injection outcomes between study groups

The mean number of oocytes, matured oocytes, percentage of fertilization, embryo quality with grade A on day 3 and mean number of embryos transferred were compared within sufficient, insufficient, and deficient vitamin D groups (Table 4). No significant difference was not observed in these parameters within groups. Besides, the outcomes of clinical and chemical pregnancy and implantation rates in all cases were followed (Fig.2), and the results showed the higher mean percentage of chemical and clinical pregnancy in the sufficient group (63.6% for chemical pregnancy and 54.5% for a clinical pregnancy), compared to insufficient (50% for chemical pregnancy and 25% for clinical pregnancy), and deficient (63.6% for chemical pregnancy and 27% for clinical pregnancy) vitamin D groups, respectively. The mean for implantation rate was significantly higher in the sufficient group (53.03 ± 47.6) compared to insufficient (12.5 ± 35.35), and deficient (12.12 ± 21.2) vitamin D groups, respectively (P=0.02).

Table 3: The relationship between vitamin D level and sperm parameters and sperm functional tests

Sperm parameters	Correlation coefficient (r)	P value
Sperm concentration (10^6/ml)	0.5	<0.001
Sperm total count (10^6/ejaculate)	0.31	0.03
Abnormal sperm morphology (%)	0.24	0.2
Sperm motility (%)	0.24	0.2
Sperm protamine deficiency (%)	-0.26	0.15
Sperm DNA fragmentation (%)	-0.17	0.36
ROS positive sperm (%)	-0.77	<0.001

P≤0.05 was considered significant, using two-tailed Pearson correlation, n=30.

Table 4: Comparison of intracytoplasmic sperm injection (ICSI) outcomes between study groups

ICSI outcomes	Sufficient vitamin D (>30 ng/ml) (n=11)	Insufficient vitamin D (20-29 ng/ml) (n=8)	Deficient vitamin D (<20 ng/ml) (n=11)	P value between groups
Retrieved oocytes (No.)	12.63 ± 6.56	16.37 ± 12.12	13.36 ± 7.6	0.63
Mature oocytes (No.)	7.36 ± 3.82	9.75 ± 7.32	8.9 ± 6.15	0.65
Fertilization rate (%)	75.98 ± 26.97	69.07 ± 30.65	78.49 ± 22.42	0.75
Embryo quality with grade A (%)	49.94 ± 37.87	29.28 ± 33.35	53.72 ± 27.56	0.43
Transferred embryo (No.)	2.1 ± 0.55	1.87 ± 0.85	2.18 ± 0.75	0.64

All data were presented as mean ± SD (ANOVA followed by Tukey's multiple comparisons).

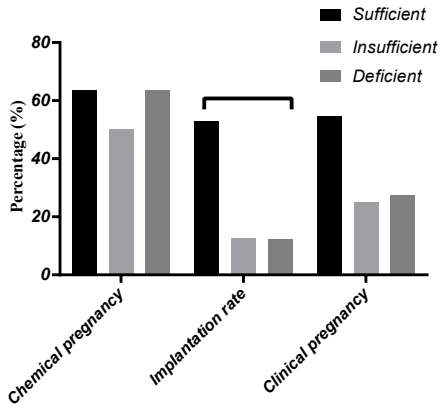

Fig.2: Comparison of chemical and clinical pregnancy and implantation rates within sufficient, insufficient, and deficient vitamin D, groups. for chemical and clinical pregnancy Mean value compared using chi-square test. No significant difference was shown within groups (P=0.8 for chemical pregnancy, P=0.3 for clinical pregnancy), for implantation rate Mean value compared using ANOVA followed by Tukey's multiple comparisons which shows a significant correlation between sufficient and deficient groups (number of participants in sufficient group=11, in insufficient group=8, in deficient group=11) (P=0.02).

Discussion

Vitamin D has been found to have various impacts on fertility, levels of sex hormones, and various organs including the uterus, prostate, and testis (19). Vitamin D levels higher than 30 ng/ml have been reported to be associated with improved fertility rates (20). In this study, we investigated the effect of vitamin D on sperm parameters, sperm chromatin status, oxidative stress, DNA damage, in infertile men candidates for ICSI. The findings of this study showed that individuals with higher levels of vitamin D had a significant difference in sperm count and sperm concentration compared to subjects with low levels of vitamin D.

Regarding the relation between vitamin D levels and sperm parameters, considerable disagreement exists among studies. Similar to the results of this study, Hammoud et al. (21) showed a significant correlation between vitamin D level and sperm concentration. A significant difference for sperm concentration between vitamin insufficient/deficient with vitamin D sufficient group was also reported in that study. In contrast, Abbasihormozi et al. (9) showed a significant correlation between vitamin D level and sperm concentration. Considering sperm morphology, Blomberg et al. (5) did not observe a relationship between sperm morphology and vitamin D. Contrary to this, de Angelis et al. (22) reported a significant correlation between these two parameters. Several differences can account for the controversies observed between different research groups, these include sample size, type of patient selected, vitamin D supplementation, different social, economic backgrounds. Indeed, it has been shown that there is a relation between vitamin D level, vitamin B6, and acid folic. In this study, we selected individual candidates for ICSI, while other studies may have included general infertile and fertile groups (23).

It is of note that in the animal model these factors are accounted for, VDD has been reported to reduce fertility rate and reduced sperm count, and vitamin D repletion can rectify this shortcoming (11). In this regard, it has been shown that VDR knock-out mice show a decrease in sperm count and motility and histological abnormalities of the testis (19). These results indicate that VDD may play an important role in spermatogenesis and sperm maturation.

In this study, we also assessed the relationship between vitamin D level with protamine deficiency, DNA fragmentation, and ROS production. Only a significant negative relation between sperm ROS with vitamin D level was observed in this study. In this regard, VDR is closely related to the nuclear matrix, and it is believed that VDD plays a significant role in stabilizing chromosomal structure and thereby protecting DNA from insults and breaks (24). Interestingly, it has been proposed that the sperm nuclear matrix is crucial in the regulation of DNA fragmentation and degradation and therefore, one may speculate that vitamin D and its receptor may act as guardians of genomic in sperm. Therefore, a significant negative relation between vitamin D and sperm protamine deficiency as well as DNA fragmentation was expected to observe in the present study (24, 25). This could be due to the small sample size which is the main limitation of this study. However, what was interesting in this study is the strong significant relationship observed between ROS production and vitamin D level. Aquila et al. (8) reported that vitamin D has a direct effect on many sperm functions including sperm motility, capacitation, acrosomal activity, and even the metabolic performance of sperm. Indeed, there exists significant evidence that shows a key role for ROS in these events (26). Considering the fact that ROS is

increased in the vitamin D deficient individuals, it may be suggested that the capacity of sperm in these individuals to decrease ROS production is reduced. Indeed, vitamin D plays an enhanced role in promotors regions of many enzymes involved in spermatogenesis and in enzymes with anti-oxidative activity (7). ROS decreases fluidity of plasma membrane by lipid peroxidation of unsaturated fatty acids in sperm which can decline sperm's function (27). It has been thought that vitamin D can protect protein and cell membranes from oxidative stress by preventing pro-oxidative insults (28). Lack of antioxidant protection and free radical productions in sperm can cause oxidative stress (29). Therefore, the increase in ROS production could be related to the reduced antioxidant capacity of these sperm, related to the diminished vitamin D level in these individuals.

Increased ROS production is commonly associated with an increase in DNA fragmentation (30). In this study, a trend toward increased DNA fragmentation in individuals with reduced vitamin D levels was observed, however, the increase was not significant. Aitken et al. studies suggest a lag of several hours between ROS increase and DNA oxidation in sperm (29, 31). It is also suggested that vitamin D could reduce chromosomal aberrations, prevent telomere shortening, inhibit telomerase activity and decline biological damages which are induced by oxidative stress (32). So probably, it is vitamin D itself that protects DNA from being damaged by ROS.

After evaluating clinical outcomes, we observed a significant difference in implantation rate within study groups, despite a similar number of embryos transferred between the groups. The implantation rate was significantly higher in the vitamin D sufficient group than the other two groups. Indeed, effect of male on embryo development appeared after day 3 when the embryo also becomes dependent on gene expression from male genome (33). Interestingly, Ozkan et al. (34) showed an association between vitamin D and implantation rate. But another study showed negative effect of vitamin D in IVF outcomes (35). Due to these controversies, the importance of vitamin D in clinical outcomes remain to be clarified. According to the results of this study, it could be concluded that vitamin D has the potential to increase fertility potential by improving the sperm parameters. It may be suggested that infertile men could be checked for vitamin D level and if necessary, vitamin D could be supplemented in their diet for two to three months before ICSI, to improve sperm parameters as well as both fertilization and implantation rates.

Conclusion

In this study, we assessed the relationship between serum vitamin D levels with sperm parameters, sperm function, and clinical outcomes of infertile male candidates for ICSI. The result of the present study shows that there is a significant negative and positive relation between sperm ROS and sperm concentration with serum vitamin D level, respectively. Also, the implantation rates were significantly lower in the vitamin D insufficient and deficient groups compared to the sufficient group. In light of our results, it can be concluded that vitamin D has the potential to improve fertility in infertile men by improving sperm concentration and reducing ROS level, which consequently may account for an improved implantation rate. But, based on our sample size further trials are needed to bonify these observations.

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Authors' Contributions

M.H.N.-E., M.H.-M.; Conceived the idea, planned and designed the manuscript. M.H.N.-E., M.H.-M., F.S.; Contributed extensively in interpretation of the data and the conclusion. M.H.N.-E., F.S.; Were responsible for overall supervision. M.H.-M.; Wrote the first draft, which was then revised by F.S., M.H.N-E. All authors read and approved the final manuscript.

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Original Article

Comparison between *SPATA18* and *P53* Gene Expressions in The Sperm Cells Obtained from Normospermic and Asthenospermic Samples: A Case-Control Study

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Abstract

Background: Improving sperm motility results in increasing the success of a treatment cycle. Recently, sperm RNA has been used for diagnostic purposes such as whole seminal fluid, sperm analysis, and sperm quality test in patients undergoing *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI). SPATA18-P53 pathway is considered an essential pathway related to sperm mitochondria, which controls mitochondrial quality by eliminating its oxidative proteins. Oxidative stress may decrease sperm motility and affect sperm quality negatively due to an increase in P53 expression. SPATA18 protein is found in satellite fibers related to outer dense fibers in the middle piece of sperm. The downregulation of SPATA18 in the asthenospermia group can represent this gene's critical function in sperm motility and fertility. The present study aimed to assess the relationship between *SPATA18* and *P53* gene expression in sperm cells obtained from normospermia and asthenospermia.

Materials and Methods: In this case-control study, the quantitative real-time polymerase chain reaction (RT-PCR) technique was used to measure the *SPATA18* and *P53* gene expression level in sperm samples collected from 21 patients and 63 healthy individuals. Further, the sperm DNA fragmentation assay (SDFA) kit was applied to determine the relative apoptosis level in cells and evaluate the biochemical information related to the patients' sperm samples. Furthermore, all the participants completed the consent form, and the ethics committee confirmed the study.

Results: Based on the results, the *P53* and *SPATA18* gene expression levels in most of the samples, in which motility was less than 40%, increased and decreased ($P \leq 0.001$), respectively.

Conclusion: The *SPATA18* and *P53* gene expression levels increased and decreased in the asthenospermic patients, respectively, compared to the control group. Thus, the *P53* and *SPATA18* expression levels can be used as an appropriate marker for diagnosing sperm motility in males.

Keywords: Apoptosis, Asthenosperm, Normosperm, *P53*, *SPATA18*

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Introduction

Recently, sperm RNA has been applied for diagnostic purposes such as whole seminal fluid, sperm analysis, and sperm quality test in patients undergoing *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI). Sperm RNA is considered a potential marker for diagnosing sperm abnormalities and fertility capability in infertility clinics (1). Based on the latest information provided by the World Health Organization, normal sperm parameters are considered 15 million sperms with 4% normal morphology and 40% motile sperm or at least 32% with progressive motility per ml of seminal fluid (2). The infertility distribution caused by male factors varies between 20-70%, and the percentage of infertile males ranges between 2.5-12% (3-9).

Asthenospermia is a common reason for male infertility, which is diagnosed by reduced sperm motility in new ejaculation (8). The maximum frequency of genetic factors recognized in male infertility (25%) is observed in asthenospermia (10). Three main factors, including decreased sperm count, motility power, and abnormal sperm morphology, are raised in male infertility. Selecting normal and mature sperms is considered useful in assisted reproduction techniques (11-13).

Mieap is considered another name for *SPATA18*. The genomic position for *SPATA18* gene is on the chromosome 4 (GRCh38/hg38), with the 46,002 bp linear DNA length and the 14 exons. The genomic position for *P53* gene is on the chromosome 17 (GRCh38.p13), with the length of 19149 bp linear DNA and 12 exons. It is assumed that *SPATA18* is a *P53* inducible protein, the transcription of which is directly regulated by the *P53* tumor suppresser. Mieap controls mitochondrial

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quality by repairing or removing unhealthy mitochondria through the Mieap-induced accumulation of lysosome-like organelles within mitochondria (MALM) or Mieap-induced vacuole (MLV), respectively (14, 15). Inactivating *P53* or *Mieap* disturbs MALM and MLV severely, leading to the accumulation of unhealthy mitochondria. Mitochondria are necessary for intracellular signaling and cellular energy supply after stress (16, 17).

Crosstalk is available between the nucleus and mitochondria during stress events (16). Reactive oxygen species (ROS) are produced as side-products during the oxidative phosphorylation process, the overproduction of which can play a role in mitochondrial damage and stress (16, 18). Mitophagy is considered an effective mechanism for controlling mitochondrial quality since it can optionally remove unwanted or damaged mitochondria (16, 19). Mitophagy plays a role in basal mitochondrial turnover and eliminates damaged mitochondria under stress (16, 20).

Additionally, these organelles are regarded a major source of intracellular ROS, including highly reactive free oxygen radicals like hydroxyl radical (OH•) and superoxide anion (O2•−), as well as stable non-radical oxidants such as hydrogen peroxide (H_2O_2) (21). ROS is commonly created as the by-products of oxidative phosphorylation (21, 22). The generation of excessive ROS in mitochondria (mtROS) causes oxidative damage to lipids, proteins, and DNA and may lead to apoptosis (21, 23). Further, ROS accumulation can cause various diseases like degenerative disorders and cancer. Based on recent reports, elevated levels of mtROS can increase cancer cell invasion and metastasis through activating different major signaling pathways and transcription factors (21). Increasing oxidative stress, such as oxygen free radicals and ROS, can negatively affect sperm quality (24). Mitochondria include cardiolipin (CL), which is considered an organelle-specific phospholipid that carries 40 fatty acids with a strong preference for unsaturated chains (25-27). Further, TPCL is associated with acrosome, a sperm-specific organelle, during spermiogenesis, along with a subset of authentic mitochondrial proteins such as Suox, Ant4, and *SPATA18* (27).

Proteins such as caspase -1, 3, 7, 8, and 9 and aquaporin-7 are involved in regulating mitochondrial function in the apoptotic pathway and decreasing the sperm volume. Apoptosis may be associated with decreasing sperm motility (28). *SPATA18* protein is found in satellite fibers related to outer dense fibers in the middle piece of sperm. Decreasing *SPATA18* expression in the asthenospermia group can represent this gene's critical function in sperm motility and fertility (29, 30).

Based on Dan et al. (16), *SPATA18* expression is an essential player in the mitophagy process after DNA damage.

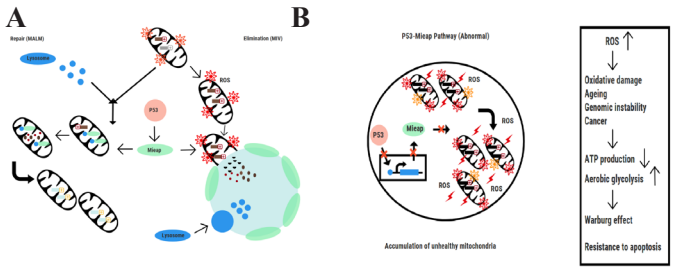

Fig.1: The Cross talk between *P53* and Mieap in mitochondria. **A.** The hypothetical model of *P53*-Mieap pathway for mitochondrial quality by which Mieap controls mitochondrial quality by repairing or removing unhealthy mitochondria through generating MALM or MIV, respectively (14, 15), and inactivating *P53* or Mieap disturbs MALM, and MLV severely leads to the accumulation of unhealthy mitochondria and **B.** Hypothetical model for inactivating *P53*-Mieap pathway by which ROS surfaces increase under severe oxidative stress such as oxygen free radicals, leading to DNA damage and apoptosis induction, and finally the disruption in the function of *P53*-Mieap (14). MALM; Mieap-induced accumulation of lysosome-like organelles within mitochondria, MIV; Mieap-induced vacuole, and ROS; Reactive oxygen species.

Considering the changes in *SPATA18* gene expression in normospermic and asthenospermic cells and the effect of the gene expression on *P53*-induced apoptosis, the present study aimed to assess the possibility of gene expression and its effect in generating apoptosis in sperm cells.

Materials and Methods

Sampling, classification, and characterization of samples

In this case-control study, the count and motility of the sperm samples of the 84 participants, who were referred to Bu Ali Laboratory in Zanjan for 6 months from June-November of 2020, were written on the day of sample preparation and the data were sorted from minimum motility to maximum one. From the total 84 samples were assessed in the present study, of which 21 and 63 were related to the patients and healthy individuals, respectively. Also, to appropriately compare gene expression levels between the asthenospermia and normospermia samples, the normospermic samples were divided into three subgroups, each of them including 21 samples based on their motility range, including [41-55] subgroup A, [55-69] subgroup B, and [69-83] subgroup C. Since the population included 21 asthenospermic samples and 63 normospermic ones, the control group with 63 members was divided into three subgroups to compare the 21-member asthenospermia group with each of the control subgroups statistically.

Primer design

Table 1 presents the sequence of primers using Oligo 7 software. In this table, the content of each reaction and the time and temperature of each cycle is shown.

In Table 2, the *P53* and *SPATA18* gene expression levels are compared between the asthenospermia group with the asthenospermic samples and the three normospermia subgroups. Table 3 shows the sperm DNA fragmentation assay (SDFA) results concerning gene expression in two groups of fair to low and good fertility potential.

The sperm samples were analyzed using HFTCASA Computer Aided Semen Analysis System software, 8.00 (31). A t test was implemented to compare the results using SPSS 22 (IBM Company, USA). Which was evaluated using the Kolmogorov-Smirnov test. To compare two groups from t test and to compare 3 groups from one-way analysis of variance. The abbreviation ANOVA is used. The significance level was considered 0.05, and the SDFA results were calculated using REST 2009 software. In this software, REST RG mode is used for data analysis.

Total RNA was extracted using EZ-10 Spin Column Total RNA Mini-Preps Kit (BioBasic Inc., USA) based on the Sperm RNA Company's guidelines.

cDNA synthesis

The cDNA complementary strand was created using the RNA extracted by the Takara kit based on the company's guidelines. Additionally, cDNA was synthesized immediately after extracting RNA based on the Takara Company's kit protocol (cat.no RR037Q).

Real-time polymerase chain reaction

The Rotor-Gene (Q) real-time PCR machine (QIAGEN) was used in the present study.

Steps of real-time polymerase chain reaction

Real-time PCR was conducted for the target and control genes in two separate, paired tubes. The mixture volume is provided in Table 1.

Halosperm® G2 kit (HT-HSG2, halotech)

Of the 84 samples, 24 were randomly assessed using the SDFA kit. Additionally, the Halosperm® G2 kit (HT-HSG2, Halotech) was used based on the company's guidelines for sperm DNA fragmentation assay (SDFA).

Reference rage of SDFA

• Samples with SDF<15%: These samples were assayed to have a deficient fragmentation level and ranked good.
• Samples with 15%<SDF<30%: These samples were assayed to have a low fragmentation level and ranked medium.
• Samples with 30%<SDF: These samples were assayed to have a high fragmentation level and ranked abnormal.

Results

A significant difference was observed regarding the *P53* and *SPATA18* gene expression levels between the asthenospermia group and the subgroups A, B, and C of normospermic samples. As shown in Table 2A, by decreasing motility rates in the asthenospermic samples led to lower *SPATA18* and higher *P53* gene expression levels compared to the normospermic samples.

Table 1: The sequence of primers and the condition of optimized Real-Time PCR reaction

Primer	Primer length (5´-3´)	Length of created piece (bp)
GAPDH	F: GGTCATCATCTCTGCCCCCT R: AGGCAGGGATGATGTTCTGG	276
SPATA18	F: GTTCAGCGATTCCTATTCCCAGGC R: TCGACCCCACATAAGATGGTGTCA	192
P53	F: ATAGTGTGGTGGTGCCCTATGAGC R: TTCCAGTGTGATGATGGTGAGGAT	134

Component	Vol./reaction (µl)	Final concentration (µM)
2X Master Mix RealQ Plus	10	1x
Forward primer	0.5 (0.25-2.5)	0.1 (0.05-0.5)
Reverse primer	0.5 (0.25-2.5)	0.1 (0.05-0.5)
PCR-grade H_2O	7	-
Template cDNA	2	0.1
Total	20	-

Cycles	Duration of cycle	Temperature (°C)
1 for activation TEMPase	15 minutes	95
40	15 seconds	95
	30 seconds	52
	30 seconds	72

Table 2: The obtained P value of *P53* and *SPATA18* gene expression levels in the studied groups to determining statistical significance. The comparison of the *P53* and *SPATA18* gene expression levels between the asthenospermia and three normospermic subgroups samples, and the simultaneous assessment of significance of expression *P53*, *SPATA18* genes in all the three subgroups (a, b, c) and total samples (d)

	Gene	P value	Gene	P value
		P53		*SPATA18*
a. The mean difference of the asthenospermia and normospermia subgroup A	12.86 $_{\Delta CT}$	0.023	-14.06 $_{\Delta CT}$	0.001
b. The mean difference of the asthenospermia and normospermia subgroup B	36.72 $_{\Delta CT}$	0.025	-14.10 $_{\Delta CT}$	0.001
c. The mean difference of the asthenospermia and normospermia subgroup C	33.90 $_{\Delta CT}$	0.004	-14.05 $_{\Delta CT}$	0.001
d. The mean difference of the asthenospermia and normospermia total subgroups	12.86 $_{\Delta CT}$	0.023	-14.07 $_{\Delta CT}$	0.00000*

*** P value decreases to zero to five decimal places**

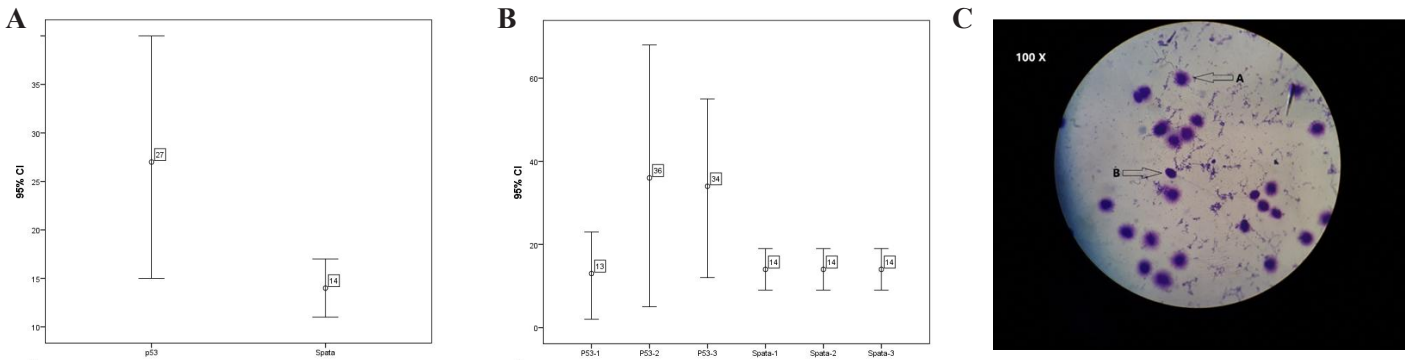

Fig.2: The results of the comparison of *P53* and *SPATA18* gene expressions obtained by both RT-PCR and SDFA methods. **A.** The mean difference of the asthenospermia and normospermia groups regarding *P53* and *SPATA18* genes in all the three subgroups. **B.** The mean difference of the asthenospermia and normospermia groups in *P53* and *SPATA18* genes in each of the three subgroups. **C.** Sperms (A) with halo (healthy and without DNA fragmentation) and (B) without halo (with DNA fragmentation) with ×100 magnification. CI; Confidence Interval of mean, SDFA; Sperm DNA fragmentation assay, and RT-PCR; Real-time polymerase chain reaction.

Table 3: The SDFA results concerning gene expression in two groups of fair to low and good fertility potential by using REST 2009 software

Gene	Reaction efficiency	Expression	P value
GAPDH	0.7124	1.000	
P53	0.6548	1.617	0.748
SPATA18	0.6014	7.012	0.078

The *P53* and *SPATA18* gene expression levels were compared between the asthenospermia group and the three subgroups of normospermic samples. Based on the real-time PCR technique results, the *P53* gene expression level increased (P≤0.01 up, especially Table 2 subgroup C) whereas the *SPATA18* expression level decreased (P≤0.01 down, Table 2 all subgroups). Furthermore, based on the analysis of expression level of total normospermic with asthenospermic samples, the P≤0.05 and P<0.001 were obtained for *P53* and *SPATA18*, respectively, and the difference was significant due to the high sample size (Table 2). However, no significant difference was observed concerning gene expression between the good and fair to low fertility potential groups (Table 2). In the healthy groups, the possibility of asthenosperima in males increased by decreasing their motility range to 40. Comparing the data related to the three groups demonstrated that the *P53* and *SPATA18* expression levels in patients with motility between 12.2 and 30 increased and decreased, respectively.

Thus, the *SPATA18* gene expression level hypothesis was accepted in normospermic and asthenospermic cells by considering the effect of its expression in generating *P53*-induced apoptosis and assessing the *SPATA18* and *P53* gene expression levels, which was different in sperm cells.

Based on the results, a significant difference was observed between the asthenospermia and normospermia groups. Figures 2A and 2B display the mean difference of the asthenospermia and normospermia groups regarding *P53* and *SPATA18* genes in all and each of the three subgroups, respectively.

Discussion

In the recent decade, the recognition of male reproductive function and the effect of malefactors on infertility has progressed significantly. Based on previous studies, germ cells, anatomic and hormonal disorders, and genetic abnormalities can be considered reasons for infertility in males (32). Sperm abnormalities can emerge in different forms, such as azoospermia (seminal fluid with no sperm), oligospermia (a low concentration of sperm), asthenospermia (low sperm motility), teratozoospermia (decreased sperms with normal morphology), or a combination of them (33).

The motility level is inversely and directly related to *P53* and *SPATA18* expression, respectively. Accordingly, low motility results in increased and decreased *P53* and *SPATA18* gene expression, respectively. Low motility hinders sperm motility, and *P53* prevents *SPATA18* activation and directs cells toward cell death by increasing their expression.

Thus, inactivating *SPATA18* and increasing *P53* expression during infertility can probably intensify the condition.

Male *P53* -/- knockout mice in Zalzali et al.'s (34) study showed a decreased sperm count and abnormal sperm motility and morphology. Their results implicated the central role of cell cycle gene *P53* in some events like sperm development and differentiation. However, unknown pathways and the absence of probable known downstream effectors like Cdkn1a highlight the complicated roles of these genes in sperm biology. Nakamura and Arakawa (35) examined the *SPATA18* role in mice fertility. They found that although the *SPATA18* -/- knockout mice were fertile in vivo, the sperm of these mice was severely impaired *in vitro* because of sperm motility failure and the oxidized proteins were dramatically accumulated in the midpiece of *SPATA18* -/- sperms.

Several studies have been conducted on *P53* and *SPATA18* gene expression, although their results are consistent and inconsistent with those of the present study. Other study declared that *SPATA18* expression was reduced by the direct effect of the *P53* regulatory response. However, almost all the other studies reported that this relationship was inverse (14, 30, 36-40).

After surveying the literature to find out the underlying hypothetical mechanism, the model presented by Kitamura et al. (14) was used to interpret the results. Based on the model, increasing *P53* expression and decreasing *Mieap* resulted in inducing *Mieap* by *P53*. Mieap functions by two methods. Increasing disturbance in mitochondria, they continued with lysosomes by destroying damaged mitochondria or repairing and returning mitochondria to their natural activity. Further, since knocking down the *Mieap* gene disturbs repairing, an increase in *P53* and a decrease in *SPATA18* in these sperms disturb repair of mitochondria. Consequently, destroying mitochondria leads to the reduction of sperm energy and low motility.

According to Moradi et al. (36), the *P53* level increased significantly in asthenospermic samples. The present study results demonstrated that the *P53* and *SPATA18* gene expression levels increased and decreased in the asthenospermic samples, respectively. However, in their study the ROS level in sperm samples and the activity of thioredoxin reductase (TrxR) and identified sperm DNA fragmentation were assessed by using TUNEL assay, while the SDFA kit was used in the present study to assess the DNA fragmentation level.

Ghandehari-Alavijeh et al. (37) provided a model in which hypoxia markers such as *P53* were significant in infertile males due to asthenospermia compared to fertile ones. Moreover, its high expression, such as *P53*, led to apoptosis, which is in line with the present study results showing that *P53* in the asthenospermic samples was high and directed sperm cells toward apoptosis. However, Ghandehari-Alavijeh et al. (37) assessed reactive oxygen species (ROC) in sperm cells, while the SDFA kit was used in the present study to evaluate the DNA fragmentation level.

Furthermore, considering the data obtained by comparing the sperms without and with DNA fragmentation, the *P53* and *SPATA18* expression levels decreased and increased, respectively, although the difference was insignificant.

Belloc et al. (39) reported that the decomposed fragmented sperm DNA level was more in males with asthenospermia (sperm motility defect) than in males with oligozoospermia or teratozoospermia, which is related to motility. Thus, their motility was low, and consequently, their *P53* and *SPATA18* gene expression levels increased and decreased, respectively.

Conclusion

Based on the results, low motility hinders sperm motility and *P53* prevents *SPATA18* from activation and directs cell toward DNA breaking by increasing its expression, which is considered the apoptosis background. Accordingly, *SPATA18* inactivation and increased *P53* expression during infertility can probably intensify the condition. In other words, the motility level is directly and inversely related to *SPATA18* and *P53* expression, respectively. Thus, lower motility leads to increased and decreased *P53* and SPATA18 gene expression, respectively. The viability and motility of sperm samples can be found using the gene expression panel of sperms in the future.

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Authors' Contributions

A.P.; Methodology, formal analysis, investigation, resources, and writing (original draft and editing). S.M.A.; Methodology, conceptualization, and supervision. G.A.T.; Formal analysis and resources. All authors read and approved the final manuscript.

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Commentary

Fully Masculinized 46,XX Individuals with Congenital Adrenal Hyperplasia: Perspective Regarding Sex of Rearing and Surgery

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Abstract

Current guidelines for gender assignment for all 46,XX congenital adrenal hyperplasia (CAH) continue to be female. This decision is most challenging for individuals with a 46,XX karyotype born with (CAH) having severely masculinized genitalia (Prader 4 or 5). They may be at significant risk for quality of life (QoL) and psychological health. More outcome information currently exists for such individuals assigned male than female. Most available data for those raised females do not indicate the extent of masculinization at birth, so there are minimal outcome data to compare with those raised males. Gender dissatisfaction among those raised females may be related to the degree of prenatal androgen excess in the brain evidenced by external genital masculinization. Also, additional brain maturation after birth, especially during puberty, is impacted by postnatal androgen excess resulting from inadequate androgen suppression. The purpose of this perspective is to suggest that both female and male assignment be considered. Most who have been raised male at birth have positive adult outcomes. This consideration should occur after discussions with full disclosure to the parents. The lack of more outcome data highlights the need for further information. This perspective also suggests that surgery should be deferred whether assigned female or male at least until gender identity is apparent to preserve the potential for male sexual function and prevent irrevocable loss of sensitive erotic tissue. While the gender fluidity is recognized, it is important to consider potential subsequent need for gender reassignment and extent of masculinization, particularly at the time of gender determination.

Keywords: Congenital Adrenal Hyperplasia, Differences of Sex Development, Disorders of Sex Development, Gender, Intersex

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According to the Endocrine Society Clinical Practice Guidelines, all genetically female individuals with congenital adrenal hyperplasia (CAH) should be assigned and raised female, even those with fully masculinized external genital maturation (1). This perspective is written to stimulate further assessment of this issue based upon outcome data and to plea for publication of more detailed outcome data. Data suggest that levels of fetal androgen play an important role in the brain sexual differentiation and has an enduring influence upon behavior (2). In situations such as CAH involving atypical androgen concentrations among genetic females during fetal life, increased male-typical juvenile behavior is well known. Alterations in gender identity and sexual orientation also occur in this situation. In addition, there is indirect evidence that elevated androgen levels occur after birth, both among genetic females with CAH and among some with polycystic ovarian syndrome (PCOS) (3). This provides the basis for a perspective regarding the current care of newborns. We realize that our perspective may change if a treatment of CAH becomes available that results in suppression of androgen excess continually throughout childhood and adolescence. The Penn State Hershey Medical Center Institutional Review Board, Hershey, Pennsylvania, United States of America indicated that because new data regarding patients are not reported that this perspective is exempt from full committee review and approved this submission.

Currently, most demographic data about this group of patients do not include the extent of masculinization such as Prader Staging (4). However, these outcomes appear to be related to the severity of the CAH. In contrast, among those raised male because of extensive masculinization, there is considerable positive outcome information regarding employment, intimate and general social

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relationships and sexual satisfaction, although these men are infertile. The most recent summary of these outcomes was reported in 2020 (5).

This small portion of those with 46,XX and CAH who have male genitalia comprise an important focus for the current controversial viewpoints regarding the care of intersex, referred to as disorders (or differences) of sex development (DSD). This involves neonatal assignment of sex of rearing and genital surgery during infancy and childhood. Herein, we aim to present a perspective regarding the difficult choices that must be made for CAH patients with a 46,XX karyotype and male external genitalia.

The focus of this discussion is based largely upon the available adult outcome of individuals whose clinical diagnosis of CAH was missed at birth and delayed until after sex was assigned. This refers to the clinical diagnosis based on physical findings and laboratory values, while genetic mutations were not verified among all. Because of the variability of severity in CAH, a rigid diagnostic algorithm should not apply. It is our viewpoint, particularly for this masculinized group with 21-hydroxylase CAH. We agree that unique aspects of each of these individuals should be considered as discussed in publications (6, 7).

It has been presumed since the first use of glucocorticoids, about 70 years ago, that good adrenal suppression in the CAH affected patients would result in a well-adjusted adult life including fertility potential. Yet, this still has not been verified for those born with nearly or completely masculinized genitalia. Further, to date, no fully adequate therapy has been developed to suppress adrenal androgen excess throughout the 24-hour day without glucocorticoid excess.

The International Consensus Conference on Management of Intersex Disorders (Oct 28-30 2005) concluded that there were insufficient data to present the consideration of male assignment so the long-standing recommendation of a female sex of rearing was simply repeated. The most recent Endocrine Society Clinical Practice Guideline regarding CAH states that this issue is controversial while recognizing the need for full disclosure (1). In spite of the lack of fertility data among those born with essentially male genitalia raised female, the guideline further states that among the minority who were born with considerable masculinization who try to conceive may experience “near normal” pregnancies.

In the last 15 years, there has been considerable additional data illustrating positive adult outcome among those raised males such as satisfactory sexual relationships (5). This study of 128 affected that included 46 of whom were adults age 18 or over. Also, there were 11 who died in infancy, 35 who were reassigned female and 36 still minors. These 46 adult individuals were all assigned male based on Prader 4 or 5 staging (4) before the diagnosis of CAH was made. Among these adults raised male, many were in long-term relationships with females, only 2 were reported to have gender identity problems. The other 44, although infertile, reported male gender identity and a good adult outcome based on intimate, including sexuality, relationships, general socialization and employment. Most reported being regularly sexually active with sufficient sexual arousal and potency.

Almasri et al. (8) designed a systematic analysis on the more than 1200 raised female reported female identity over the 35 years prior to Nov. 2017. These 46,XX patients with CAH deficiency were not categorized according to Prader staging (4). Among this group, 11.3% had a male gender identity (11.3%) and 23.8% homosexual. Although without verification, it can be assumed that those born with the greatest masculinization are more likely to develop problems with gender identity. Therefore, it is not far from mind that a disproportionate portion with gender identity was found in the study analysis. Although, identification a consistent gender with related rearing, sex is the golden goal in DSD management.

It is well-documented based on a review of 30 publications that patients with CAH raised female are more likely to have a sexual orientation than normal females (9). Since a homosexual outcome should not be considered a negative outcome, sexual orientation is currently not seen to be a factor for consideration.

The current standard of care must involve full disclosure to patients and for minors their parents. Therefore, parents of genetically female CAH individuals born with markedly masculinized genitalia (Prader 4 or 5) should be provided with current outcome information for those raised both female and male. The goal of the assignment of a sex of rearing is that it be consistent with gender identity that is subsequently expressed. Because of the indirect evidence that androgen excess impacts the central nervous system (CNS) development prenatally, there may be a greater tendency among those with evidence of greater exposure because of male genital external development toward a male gender identity.

This situation involves the question of whether genital surgery can occur before gender identity is manifest based on parents’ decisions. It is clear from the outcome among those raised and identifying male that the male external genitalia provide for sexual function as a male. Penetrative intercourse has been reported to be satisfying and fulfilling. When raised as a male, there is no need for external genital surgery during infancy. It is appropriate to consider insertion of prosthetic testes and oophorectomy after male gender is clearly manifest, at or after the age of puberty. Conversely, the difficult surgical challenge to construct female genitalia when the sex of rearing is female suggests that surgery should be deferred in this situation until the female sex of rearing is clear.

No comparison of outcome between those raised male and female for only those with Prader 4 or 5 (4) outcome data is currently available. A recent publication (10) cites a 2005 review (11) that addressed the issue of sex of

rearing among 46,XX individuals with CAH. However, in this 2005 publication, 23 publications reported that 250 and 33 46,XX individuals were raised female and male respectively. The 250 raised female included those with Prader stages 1 to 5, without information regarding Prader 4 and 5. Among those raised male, 19 of the 33 raised male had Prader stage 4 or 5 masculinization. This portion among those raised male is disproportionate since this occurs in less than 5 percent of cases. Hence, a comparison of the 13 among the 250 raised female (5.2%) and 4 of 33 (12.1%) raised males reported to have gender dysphoria is an inappropriate comparison of different populations. Based on this previous conclusion, the 2019 summary used these data as a basis for again making the conclusion that raising all 46,XX patients as females was appropriate for all, including those markedly virilized. However, in retrospect, it appears that the groups were not comparable since those raised males almost certainly had a greater portion with Prader 4 or 5 and those cases were published because of their unique circumstance rather than as a series to evaluation gender dysphoria. Therefore, this conclusion based on non-representative data is likely inaccurate.

The basis of our opinion is the perception that the excessive androgen has a significant impact upon gender development, making it more likely that those with Prader 4 or 5 raised female will have gender identity problems. Ideally, to make an informed decision about whether male assignment is an appropriate consideration for those currently born with CAH and essentially masculinized genitalia requires comparably detailed outcome information for those who were raised female and male. Currently, such information is unavailable. Nevertheless, full disclosure mandates that parents of infants born with essentially male genitalia be informed of the current status of outcome information while considering male in addition to female sex of rearing.

Regarding surgery, if being raised male is a viable option and/or if a portion of those raised females will develop a desire to be male, no external genital surgery should be done until gender identity has been reasonably established. If a male gender identity is clearly established, hypospadias repair, if needed, is all that would be needed to function throughout life as a male. A delayed surgery approach frees the family of the painstaking decision about the need for feminizing surgery by precluding removing a functional neurologically intact penis. A basic issue related to genital surgery among infants with intersex (DSD) is that genital tissue not be removed that can not be replaced if gender development occurs contrary to sex of rearing. This certainly applies if the outcome in such a child is male.

Gender reassignment surgery has become a valuable resource in improving QoL for transgender patients (12). For pediatric patients, especially infants with intersex genitalia, it provides a basis for parents to make difficult decisions since gender identity is still developing. Since feminizing surgeries for patients with CAH historically have resulted in a high level of sexual dysphoria, it suggests the need for individual considerations for gender assignment. Because of critical tissue conservation, gender reassignment and alignment surgery in late adolescence or adulthood may be a better choice than during infancy.

The objective of the more specific outcome data is to prevent patients from having to undergo multiple gender reassignments or have a poor QoL outcome as a result of the feminizing surgery. It needs to be recognized that masculinization of the genitalia and the CNS can influence gender development and such needs to be recognized in outcome studies. Similar to transgender individuals, gender reassignment among these individuals should not be viewed as negative but as a positive step to improve quality of life. Assignment of these infants should not be based on karyotype, but must consider the extent of masculinization of the CNS and that effect on gender identity.

Fertility potential must be considered for these severely masculinized patients, regardless of sex assignment. While fertility among those born with Prader4/5 genitalia who were raised female continues to be potentially possible, it appears to be infrequent and may be related to the difficulty of suppressing androgen excess as well as the impact of androgen excess on body image and social relationships. Parenthood potential can be compared among those 46XX persons with CAH and Prader 4 or 5, transgender males, and females with long-term untreated PCOS (3). While germ cell retrieval assisted reproductive techniques (ART) may be unlikely given the exposure of the gonads to hormone that diminish retrieval of the germ cell, there may be surrogacy options. Similar to transgender individuals, these possibilities must be presented to parents as part of full disclosure.

Important for the care of these patients and families is psychological counseling initially and intermittently geared to level of understanding. An initial psychological assessment of parents as soon as possible after the birth of their child to identify their strengths and vulnerability (13). Throughout life, the need for psychological support must be evaluated periodically. Such would be included in the "good practices" approach (14). Ongoing psychological care should involve periodic discussions of such topics as cognitive and emotional responses and altered interpretations of religion or other cultural values. Outcome information must attempt to assess the impact of counseling or lack thereof. A basic tenant is that, if the parents and patient understand the pathophysiology of CAH in everyday terms, there will be motivation to treat as well as possible.

The limitation of this perspective is the lack of complete comparable outcome data for those individuals raised female versus male. This has been implied above together with the need for such published reports. Further, while it appears that those raised female born with severe masculization have very low fertility rates or infertility,

it is unknown whether current medical care and surgical procedures will result in a better fertility outcome in the future. Moreover, the outcome in terms of QoL is not only multifaceted, while assessment testing procedures are lacking.

Individuals with CAH born with severely masculinized genitalia (Prader 4 or 5) appear to be at great risk for poor outcome impacting various aspects of QoL. Among individuals with CAH and a 46,XX karyotype, the occurrence of gender dysphoria may well be related to the degree of prenatal androgen excess evidenced by external genitalia masculinization and also postnatal androgen excess, resulting from lack of continual androgen suppression by glucocorticoid therapy. Currently available adult outcome data suggest that those 46, XX CAH patients raised male with essentially male genitalia may have a better adult outcome than those born with similar genitalia who were raised female including a clear gender identity and adult outcomes. Thus, until there are more complete adult outcome data among those raised female, male assignment should be considered as it may improve the chances for a higher QoL and to reduce the likelihood of adult gender dysphoria. Until gender identity becomes apparent, such individuals probably should have surgery deferred to preserve the potential for male sexual function and sensitive erotic tissue. Fertility potential if raised male is similar to that of transgender males.

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Authors' Contributions

C.P.H., P.A.L.; Have cared for patients with intersex disorders and contributed to this presentation including interpretation of publications, since 2006. U.B.; Provided a surgical and international perspective. C.L.J.; Provided a theoretic approach. All authors read and approved the final manuscript.

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International Journal of Fertility and Sterility (Int J Fertil Steril)
Guide for Authors

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International Journal of Fertility & Sterility is a peer review and quarterly English publication of Royan Institute of Iran. The aim of this journal is to disseminate information through publishing the most recent scientific research studies on Fertility and Sterility and other related topics. Int J Fertil Steril has been certified by the Ministry of Culture and Islamic Guidance since 2007. It has also been accredited as a scientific and research journal by HBI (Health and Biomedical Information) Journal Accreditation Commission since 2008. **This open access journal holds the membership of the Committee on Publication Ethics (COPE) and the International Committee of Medical Journal Editors (ICMJE).**

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