The Effect of Vitamin D Deficiency on the Frozen-Thawed Embryo Transfer Outcome

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ABSTRACT
Background: Vitamin D (vit D) regulates the female reproductive system and inflammation reduction. Vit D deficiency has been reported to be related to various diseases, such as infertility in females.
Objective: This work aimed to study the effect of vitamin D deficiency and vaginal inflammation on the pregnancy rate following frozen embryo transfer.
Subjects and Methods: This prospective study included 188 infertile females who underwent ICSI protocol between February 2021 and January 2022. The individuals were categorized into two groups: Group I (Vit D deficiency, vit D < 30 ng/mL)**,** and group II (Normal vit D ≥ 30 ng/mL). Vitamin D levels in serum and vaginal inflammation were detected, and pregnancy outcomes were compared between the groups. The impact of vitamin D deficiency and vaginal inflammation on the pregnancy rates was investigated.
Results: Among participants, 74.3% had vitamin D deficiency, and 25.5% had normal vit D levels. 37.23% of patients had a positive β-hCG, while 62.77% had negative β-hCG. 14.9% of cases had no vaginal infection, while 85.1% had a vaginal infection. There was a statistically significant correlation between lower pregnancy rates in Egyptian females and vit D deficiency (p<0.001). However, there was no correlation association between vaginal inflammation and pregnancy rates (p > 0.05). Logistic regression analysis showed that reduced pregnancy outcome could be due to vit D (P<0.001). **Conclusion:** Lower pregnancy rates following ICSI among Egyptian women may be related to vitamin D deficiency, while vaginal inflammation has no impact on pregnancy rates.
Keywords: Frozen embryo transfer (FET), Intracytoplasmic sperm injection (ICSI), Infertility, Pregnancy rates, Vitamin D.

INTRODUCTION
Infertility is considered a serious health issue that affects over 70 million women globally. According to the World Health Organisation, between 10 and 25 % of couples (48 to 180 million) are thought to suffer from infertility [1]. Assisted reproductive technology (ART) is widely used to treat infertility. The most effective assisted reproductive technique currently available is frozen-thawed embryo transfer (FET), which has overcome many of the fresh embryo transfer technique drawbacks such as superfluous embryos following oocyte retrieval and Intracytoplasmic sperm injection (ICSI) to improve pregnancy rates [2].
Vit D is an essential hormone that is not only responsible for phosphate and calcium homeostasis [3], but also vitamin D exhibits many other biological effects, such as regulating hormone synthesis, immune system response, and reduction of inflammation [4].
Vit D has a crucial role in the reproductive process. It binds to the vitamin D receptor (VDR) that is found in most female reproductive systems and tissues such as the placenta, fallopian tubes, uterus, endometrium, and ovaries. Additionally, it stimulates the production of progesterone, estrogen, placental prolactin, and FSH, and controls human chorionic gonadotropin secretion [5]. This implies that vitamin D is essential for mediating its biological effects in female reproductive tissues [6].
According to the American Association of Clinical Endocrinologists guidelines (AACE), vit D deficiency refers to a serum level of vit D < 30 ng/mL, which is common in 30–81% of adults worldwide while 30 ng/mL or above refer to Vit D sufficient [7]. Vit D deficiency can result from impaired vit D 1α-hydroxylation, impaired vit D 25-hydroxylation, inadequate exposure to sunlight, inadequate vit D intake from food, malabsorption, and vit D resistance resulting from mutations in the VDR gene [8].
Low vit D has been revealed to be related to numerous non-skeletal diseases including cardiovascular disease, autoimmune, cancer, obesity and diabetes, and infertility in both males and females [9]. Vit D is essential for maintaining a healthy pregnancy and for several reproductive processes that occur throughout pregnancy, such as fertilization and implantation. Miscarriage, recurrent implantation failure, and some conditions accompanied by pregnancy like preeclampsia are also associated with low vitamin D [10].
The vaginal microecosystem, immune response, and biological metabolism are significantly influenced by the vaginal flora. Flora protects the vagina against harmful microorganisms, including those responsible for urinary tract infections, vaginosis, candidiasis, and sexually transmitted diseases (STDs) [11]. Vaginal inflammation (VI) is inflammation of both the vagina and vulva, characterized by the disruption of the natural

Received:12/10/2023
Accepted:12/12/2023

vaginal bacteria, namely Lactobacillus species that produce hydrogen peroxide, as well as resulting in a rise in the prevalence of anaerobic bacteria. Associations between poor reproductive outcomes in IVF patients and the presence of VI have been proposed. There is evidence that bacterial infection of the uterus would result in decreased endometrial receptiveness, unsuccessful implantation, and hence lower pregnancy rates [12]. The relation between vitamin D deficiency, pregnancy outcomes, and fertility is still poorly understood. So, the objective of the recent work was to examine the impact of both vit D deficiency and vaginal inflammation on the ICSI outcomes.

MATERIALS AND METHODS

Subjects: This study included 188 Egyptian females aged between 25-35 years old who had BMI of 23-27 kg/m^2 with endometriosis and infertility for three to five years from those attending private fertility were enrolled in this study and underwent the ICSI protocols between February 2021 to January 2022. All participants were prepared for our standard FET protocol.

Exclusion criteria: Female who had endometrial thickness blew 7 mm, systemic diseases, endometriosis, repeated abortion, recurrent implantation failure, uterine diseases, inherited or acquired uterine abnormalities, and a history of endocrine disorders. All participants in this study were subjected to a comprehensive assessment that included full medical history and performing a physical examination. Additionally, transvaginal ultrasound scanning (TVUS) was conducted to examine the uterus, including the measurement of endometrial thickness, evaluation of the uterine cavity, vagina, and cervix, as well as monitoring the growth of follicles.

Clinical manipulations

Ovarian hyperstimulation: Firstly, the ovary was stimulated by 0.1 mg/day of Decapeptyl (Germany) on the 21st day of the cycle. After downregulation by E2 < 50 pg/mL, the ovary was hyperstimulated via the administration of daily 160-200 IU of hMG (Fengyuan, China) to stimulate follicular growth from cycle day 2 and lasts until the follicles reach 18 mm in diameter. The size and number of the growing follicles were accurately recorded. Finally, 9,000 IU of hCG (Choriomon, ''IBSA, Switzerland") was given for oocyte maturation by deep intramuscular injection.

Oocyte retrieval: The 18-mm follicles were retrieved from the patients 36 hrs after β-hCG injection, using an ultrasound-guided needle Wallace® 17g 330 mm. The oocyte-cumulus complex was aspirated, washed, and placed into fertilizing global total media (Life Global, Bulgaria) and kept till denudation.

Oocyte denudation: The oocytes were denudated to remove the coronal cells using 80 IU/mL hyaluronidase enzyme (Life Global, Bulgaria) for 45 seconds. An inverted microscope (Olympus1x71) was used to assess the oocytes' maturity. Mature oocytes metaphase II (MII) was cultured in an incubator (Galaxy, R170-200P, UK) at global media.

Sperm Preparation: Sperm was prepared for ICSI protocol by removing inactive cells and seminal fluid by sperm gradient. 1mL of liquefied semen was added above 1 mL of density gradient 90% SpermGrad™ gradient solution contained a colloidal suspension of silica particles, the pellet was suspended in 3 mL of sperm washing media (G-IVFTM) after the mixture was centrifuged and the pellet was suspended again in sperm washing buffer. Sperm that had normal morphology were chosen and placed into a polyvinyl pyrrolidone (PVP) drop for immobilization [13].

Intracytoplasmic sperm injection: Under an inverted microscope, each MII oocyte was injected with a single sperm using an injection needle, the selected sperm was chosen depending on its normal motility and morphology, and it was injected in the center of the oocyte. All oocytes were warmed in a culture dish and incubated overnight in a CO_2 incubator. Oocyte fertilization was assessed after 18 hours of injection. Fertilized oocytes were recorded and confirmed by two visible pronuclei in the ooplasm**.**

Cryopreservation and thawing (warming) procedure: The embryos were frozen and thawed according to previously established protocols [14].

For freezing: the embryos were transported to 300 µL of equilibration solution (ES) [HEPES within Basic Culture Media, 7.5% ethylene glycol (EG) and 7.5% DMSO (Kitazato, Japan] for 9-15 min at RT. Afterwards, the embryos were vitrified for 30 seconds into 300 µL of vitrification solution 1 (VS 1) composed of a combination of three solutions 10% sucrose, 15% DMSO, and 15% EG. They were then transported to 300 µL of VS 2 composed of 10% sucrose, 15% DMSO, and 15% EG for 30 seconds. The samples were subsequently gathered within 5 to 10 seconds and promptly transferred into a Cryotop device (Kitazato, Japan) to be quickly preserved in liquid nitrogen [15].

For thawing: The embryos were moved from the cryogenic device to the thawing solution (TS, Kitazato, Japan) with the addition of 1 ml sucrose in basic culture media for one minute. Then, they were transferred to the diluting solution (DS, Kitazato, Japan) with the addition of 0.5 ml sucrose in basic culture media for 3-min. Afterward, the embryos were placed in a washing solution (WS) for 5-min. Finally, they were cultured overnight at 37 °C and 7% CO_2 [16].

Embryo transfer and outcomes: High-quality embryos with successful blastocyst formation were transferred to the patient. After 6-weeks of gestation, an ultrasound examination confirmed the presence of an intrauterine sac, which was used as an indication of clinical pregnancy.

Laboratory assessment: 3 mL of blood were obtained from every participant for level of vitamin D analysis. The samples were kept at room temperature (RT) for 15 minutes until coagulation and then centrifuged. Serum Vit D was measured by Enzyme Immunoassay kit to quantitatively determine Human 25(OH) Vit D following the manufacturer protocol (MyBioSource, # MBS580159, USA). Serum vit D level < 30 ng/mL is considered vit D deficiency according to the AACE [7]
2.5 mL blood was collected from each subject on day no 14 of frozen embryo transfer to measure β-HCG. Blood was allowed to clot, and the serum was separated by centrifugation at RT. Free β-hCG in serum was measured using the Enzyme Immunoassay kit (MyBioSource, #MBS495047, USA) to determine Human β-HCG quantitatively following the manufacturer protocol.

Microbial isolation: Each participant underwent two sterile vaginal swabs that were taken from the posterior vaginal fornix. The first sample was used to detect yeasts (pseudohyphae), and after being smeared onto Sabouraud's Dextrose Agar, the sample was incubated at 25°C. The second one was allocated for culturing aerobic and anaerobic bacteria. The sample was cultured on MacConkey agar and chocolate agar and then was incubated for 24 hours at 37°C. If there was no growth after 24 hours, further incubation for 48 hours was carried out.

Pap smear histopathological examination: Vaginal swabs were taken for Pap smear from patients of all groups. Vaginal scraping from the posterior fornix with a cervical spatula was done and the sample was placed directly on a glass slide, then the material spread on a labeled slide and fixed immediately then stained with hematoxylin & eosin [17].

Statistical analysis
Data were analyzed using the SPSS computer program (version 26; SPSS Inc., Chicago, IL, USA). Continuous variables were reported as mean ± standard deviation (SD). Categorical variables were reported as numbers and percentages. The Chi-square test (χ^2) was applied to examine the association between categorical variables. The logistic regression analysis was applied to analyze the impact of vit D levels and vaginal inflammation on pregnancy rates in females undergoing FET.

Ethical approval: This research was approved by the Scientific and Ethics Committee of Al-Azhar University, Faculty of Medicine, Egypt (IR. No.: His. _3953fed. Research-Vitamin D. Frozen. Infertility. Embryo Transfer. Vaginal inflammations. _00000103). All participants gave their consents before FET. The study was carried out following the Helsinki Declaration protocol.

RESULTS
This study involved a total of 188 women with average age of 29.64 ± 3.19 years. Eighty-five (45.2%) subjects were between 25 and 29 years old, and 103 (54.8%) aged between 30 and 35. Women were categorized into two groups: Group I included 140 women (74.3%) had vit D deficiency (< 30 ng/mL) with a mean of 15.02 ± 5.96 ng/mL, with a mean age of 30.45 ± 3.06 years, with a mean BMI of 25.03 ± 5.96 kg/m^2, and mean infertility of 3.93 ± 0.80 years. Group II included 48 women (25.5%) exhibited normal vit D levels (≥30 ng/mL) with mean 33.48 ± 3.62 ng/mL, a mean age of 29.63±3.41 years, a mean BMI 23.89±5.96 kg/m^2, and mean infertility years of 4.05±0.77. Our results exhibited a non-significant difference in the tested group's participants according to age of participants, BMI, and infertility years (**Error! Reference source not found.**).

Table (1): Comparison of demographic and clinical data between the studied patients

		Vitamin D level Vitamin D deficiency N=140	Normal Vitamin D N=48	P-value
Vitamin D (ng/mL)	Mean ± SD	15.02±5.96	33.48±3.62	< 0.001*
Age (y)	RANG Mean ± SD	25-35 30.45±3.06	25-35 29.63±3.41	0.1702
BMI (kg/m)	RANG Mean ± SD	23-27 25.03±5.96	23-27 23.89±5.96	0.2979
Infertility years	RANG Mean ± SD	3-5 3.93±0.80	3-5 4.05±0.77	0.4594

70 (37.23%) patients had a positive β-hCG, and 118 (62.77%) had negative β-hCG. Regarding vaginal infection, 14.9% of cases had no vaginal infection. 85.1% had vaginal infection, 38.8% showed Candida, 26.6% showed E coli, 10.1% had Streptococcus, 7.4% had Klebsiella, and 2.1% had MARSA.

Among females included in the study, no significant association was found between age and vitamin D levels (p>0.05). Also, there were no significant differences (p>0.05) observed between females with low vit D and others with normal levels regarding vaginal infection. Otherwise, the study demonstrated a significant correlation between vit D levels with pregnancy rates among Egyptian females.

Women whose vit D was below 30 ng/mL had significantly lower pregnancy rates compared to those with normal vitamin D (19.3% (27/140) versus 89.6% (43/48), *p* < 0.001) (Table 2).

Table (2): Associations between vitamin D status and the studied biomarkers

	Vitamin D levels		
	Vitamin D deficiency (<30 ng/mL) N=140	Normal vitamin D (≥30 ng/mL) N=48	P value
Age (years)			
25-29	69(49.3%)	16(33.3%)	0.055
30-35	71(50.7%)	32(66.7%)	
Vaginal infection			0.689
negative	20(14.3%)	8(16.7%)	
positive	120(85.7%)	40(83.3%)	
β-HCG			
negative	113(80.7%)	5(10.4%)	< 0.001*
positive	27(19.3%)	43(89.6%)	

Chi-square test for comparing Vitamin D deficiency and Normal vitamin D.

There was no statistically significant association between pregnancy outcomes and age in our work (p > 0.05). Also, a non-significant relation between pregnancy outcomes and vaginal infection among subjects (p> 0.05) was found (Table 3).

Table (3): Associations between pregnancy rates and the studied biomarkers

	Pregnancy rates		
	Negative β-HCG N=118	Positive β-HCG N=70	P value
Age (years)			
25-29	57(48.3%)	28(45.2%)	0. 269
30-35	61(51.7%)	42(54.8%)	
Vaginal infection			
negative	18(15.3%)	10(14.3%)	0.857
positive	100(84.7%)	60(85.7%)	

Chi-square test for comparing between Negative β-HCG and Positive β-hCG

Logistic regression analysis showed that vitamin D was the only factor that affected clinical pregnancy rates. Reduced pregnancy rates were linked to a deficiency in vit D with an odds ratio of 0.28 (0.10-0.77) versus normal vit D, P<0.001. Pregnancy odds in women will decline by 72% due to its deficient vitamin D compared to those with normal vit D. On the other hand, vaginal infection and age were not implicated in pregnancy rates (**Error! Reference source not found.**).

Table (4) Logistic regression analysis

Variable	OR	SE	95% CI	P-value
Vitamin D deficiency <30 ng/mL	0. 28	0.524	0. 10-0.77	<0.001*
Vaginal infection	1.375	0.578	0.443-4.273	0.581
Age	1.136	0.435	0. 485-2.663	0.768

OR: odds ratios; SE: standard errors of regression coefficient; 95% CI: 95% confidence intervals

Histopathological examination of vaginal Pap smear revealed: In the control group women who had enough vitamin D, Hematoxylin and Eosin-stained Pap smear showed multiple most superficial cells with flat cells, acidophilic cytoplasm and pinhole nucleus, and small numbers of parabasal and intermediate cells (Figure 1). In the control group women who had enough vitamin D, Hematoxylin and Eosin-stained Pap smear showed multiple most superficial cells with flat cells, acidophilic cytoplasm and pinhole nucleus, and small numbers of parabasal and intermediate cells (Figure 1).

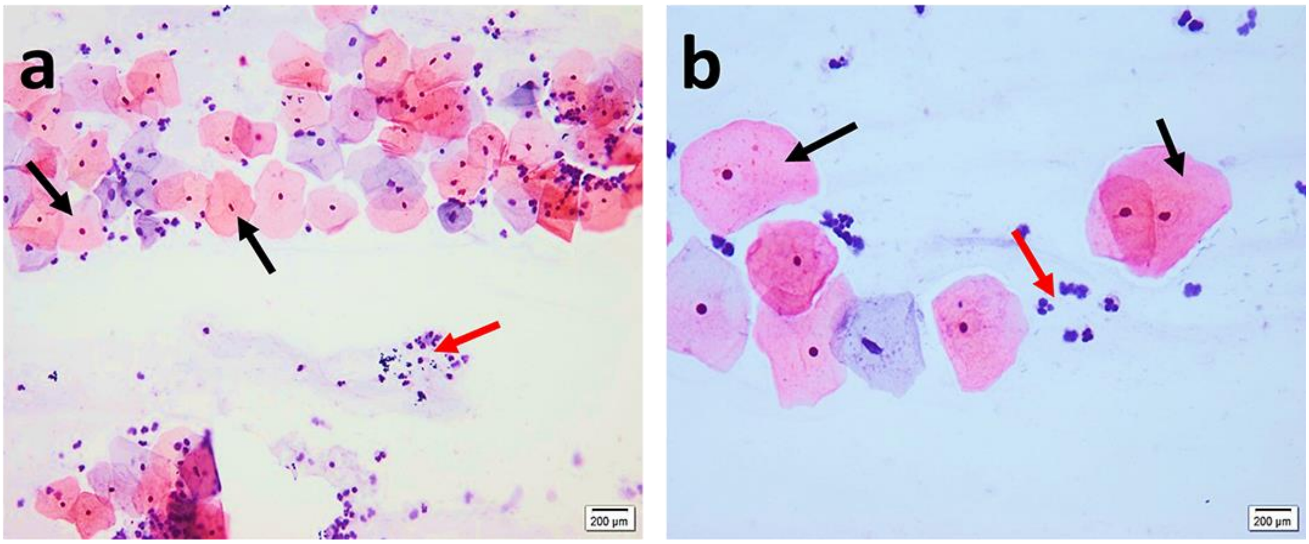

Figure (1): Photomicrograph of vaginal Pap smear in control group women who have a sufficient amount of vitamin D showing multiple most superficial cells with flat cell, acidophilic cytoplasm, and pin hole nucleus (black arrows) and small numbers of the parabasal and intermediate cell (Red arrows). (H & E a. x200 – b. x400).

In patients in vitamin D deficiency group, Hematoxylin, and Eosin-stained Pap smears showed multiple isolated condensed parabasal cells with multiple degrees of chromatin condensation indicating atrophy and inflammation (Figure 2).

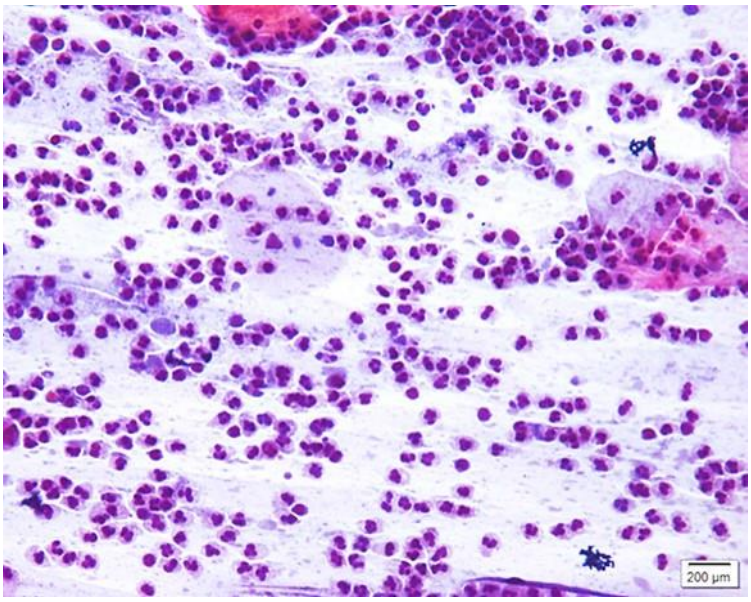

Figure (2): Photomicrograph of vaginal Pap smear in patients with vitamin D deficiency group showing multiple isolated condensed parabasal cells with multiple degrees of chromatin condensation indicating atrophy and inflammation. (H & E x400).

DISCUSSION

Vit D deficiency is a major health challenge among women in reproductive age [18]. Vit D has been proposed to be involved in female fertility. According to recent studies lower pregnancy rates may be related to a deficiency in vit D [16,19], while additional research didn't demonstrate any effect on reproductive outcomes [20], the results from clinical studies are conflicting.

Previous research has demonstrated the relation between vaginal inflammation (VI) and a corresponding number of gynecologic conditions, such as bacterial vaginosis (BV) and aerobic vaginitis (AV) [21]. Deficient vit D has been suggested to contribute to the prevalence of VI and, later, adverse pregnancy [22].

In our study, 38.8% of vaginal infection species were due to Candida, 26.6% were due to E. coli, 10.1% had Streptococcus, 7.4% had Klebsiella, and 2.1% showed MARSA. This coincides with **Anh *et al.* [23]** and **Willems *et al.* [24]**, who found that 75%, and 50% of all women at least suffer from Candidiasis and recurrent episodes respectively once in their lifetime. VVC is caused mainly by Candida which colonizes the vaginal lumen without causing any noticeable symptoms. Contradictory to our result, **Ravel *et al.* [25]** studied the vaginal microbiota prevalence in 396 women at reproductive-age and found Lactobacillus as the most prevalent genus of bacteria found in the vagina.

In our work, there was no statistically significant association between vitamin D levels and age. In agreement with our results, **Ganeb *et al.* [26]** investigated the impact of decreased vitamin D levels on the mental status of 55 healthy Egyptian participants aged between 20 and 40 years. They showed no significant correlations between vitamin D levels and age. Also, **Lagunova *et al.* [27]** have reported that no significant relationship was found between vitamin D-binding protein (DBP) and age, possibly due to differences in vitamin D intake. Older people may be interested in eating food with high vitamin D concentrations; most take vitamin D and calcium supplements to prevent it.

On the other hand, **Elsayed *et al.* [28]** examined the vitamin D deficiency impact on skeletal issues of 90 healthy volunteers and showed that serum 25(OH) D was positively associated with age. Also, **Khazaei *et al.* [29]** found a significant relationship between vitamin D and the age of subjects.

Our results showed that no correlation was observed between vit D and vaginal infection. These results are in agreement with **Palpitany *et al.* [30]** findings, that the study didn't address any relationship among vitamin D and BV in 100 pregnant women in first-trimester pregnancy. Similarly, our results agree with **Turner *et al.* [31]** report, who found no association between low vitamin D and the prevalence of bacterial vaginosis in non-pregnant or pregnant women. Contrary to our results, **Bodnar *et al.* [32]** prospective cohort study that included 469 women to investigate the relation between vitamin D level and BV prevalence in women. The findings revealed a strong correlation between vitamin D deficiency and BV especially among black women. Women with BV had lower vitamin D concentrations.

According to our results, there was a statistically remarkable association between vitamin D level and pregnancy status. Women with sufficient levels of vitamin D had a better chance of getting pregnant in comparison with those who had low levels. These results coincide with **Liu *et al.* [33]** in which scientists examined the influence of vitamin D levels on the effectiveness of IVF. The findings of the study indicated a direct relationship between vitamin D levels and the rate of fertilization. This indicates that the presence of vitamin D can influence the process of fertilization and subsequently affect pregnancy outcomes. The researchers also observed that vitamin D played a role in the follicle's development, facilitating the maturation of oocytes before its retrieval. This led to enhancing of the capability of sperm penetration and interaction of the zona pellucida leading to improved fertilization rates. In contrast, **Franasiak *et al.* [34]** studied the correlation between pregnancy rates and vitamin levels in 529 women who underwent IVF. However, there was no evidence indicating any influence of vitamin D levels on the rates of pregnancy following the transfer of euploid blastocysts.

Vit D is believed to pose a key role in pregnancy, especially in placental function, and is implicated in implantation. Furthermore, Vit D is essential for anti-mullerian hormone regulation, development of oocytes, and steroidogenesis of ovary [35,36]. Deficiency of vitamin D is linked to pregnancy complications like preeclampsia, characterized by impaired placental development [37]. Low levels of vitamin D have been observed to potentially exert an adverse influence on endometrial receptivity and rates of implantation, oocyte maturation, or embryo quality in infertile females undergoing IVF/ICSI and subsequently a deleterious potential impact on reproductive outcomes [38,39]. So, it was suggested that the clinical outcomes of ART are related to vitamin D that could improve the outcomes of ART [33].

Our results showed no significant association between age and pregnancy rates. Our results agree with the previous report, which examined the factors affecting clinical pregnancy rates in 368 infertile women, aged between 18 and 36 years who were undergoing IVF/ICSI. **Polyzos *et al.* [40]** failed to conduct a notable correlation between the age of women undergoing IVF and their pregnancy rates. Contrary to the findings shown in our study, **Tan *et al.* [41]** evaluated the IVF outcomes of 2,900 women of different age groups ranging from < 30 to ≥ 45 years. The study demonstrated a correlation between age and lower fertility in women, as indicated by a significant decrease in the cycle numbers leading to embryo transfer as age increased ($p < 0.001$). Additionally, there was a notable decline in oocyte numbers across different age groups ($p < 0.001$), and women below the age of 30 had the highest pregnancy outcomes. With increasing age, a decline in fertility starts.

Our study failed to find an association between vaginal infection and pregnancy outcomes. Women with infertility problems did not show a higher prevalence of inflammation associated with BV. In line with our findings, **Liversedge *et al.*** [42] conducted a study involving 301 females undergoing in-vitro IVF to examine the BV impact on implantation and fertilization, their research revealed that BV did not negatively affect the fertilization rate. Additionally, the difference in implantation rates between participants with normal flora and those diagnosed with BV was not significant. Contrary to our results, **Mangot-Bertrand *et al.*** [43] examined the influence of VI (specifically BV) on the pregnancy rate of 307 French women treated by IVF and showed that BV might have a favorable effect on pregnancy outcome, BV was associated with poorer fertilization results, but these results were not significant.

Similar to **Basirat *et al.*** [44] findings, our study revealed that in patients with vitamin D deficiency, the histopathological analysis indicated a decreased thickness of the vaginal epithelium. This resulted in a deterioration of the maturation process due to inflammation.

CONCLUSION

Lower pregnancy rates following ICSI among Egyptian women may be related to vitamin D deficiency. Vitamin D would improve the odds of having a high pregnancy rate. Therefore, vitamin D should be assessed routinely in reproductive-age women.

Conflict of Interest: None to be declared.
Funding Source: None.

ABBREVIATIONS

- **β-HCG:** Human chorionic gonadotropin
- **ART:** Assisted reproductive technology
- **AV:** Aerobic vaginitis
- **BV:** Bacterial vaginosis
- **FET:** Frozen-thawed embryo transfer
- **ICSI:** Intracytoplasmic sperm injection
- **STDs:** Sexually transmitted diseases
- **TVUS:** Transvaginal ultrasound scanning Vit D receptor
- **VDR:** ***VI*:** Vaginal inflammation
- **VIT D:** Vitamin D.

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