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Cross-sectional Study

Correlation between 25-hydroxy vitamin D levels in women and in vitro fertilization outcomes: A cross-sectional study

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ABSTRACT

Background: Vitamin D has recently raised a great deal of controversy, not because of its traditional role of absorbing calcium and maintaining bone health, but because of its unconventional role as an endocrine factor and the extent of its impact when linked to its specific receptors (VDR) found in different tissues. Research has raced trying to find its different roles in those tissues and its association with different clinical or medical conditions, and among these cases, its role in reproductive functions and fertility in women, these studies conflicted between supporting and denying the role of vitamin D in reproductive function and rejecting this hypothesis according to the results of their study.

Materials and methods: The in vitro fertilization process allowed us to study the possible hypotheses, as this technique provides an opportunity to study the relationship between vitamin D levels with the in vitro fertilization outcomes, thus providing us with an idea of the relationship of vitamin D with fertility in women. In order to study this relationship, we designed our research as a cross-sectional study to confirm or deny this claim. Vitamin D was measured in the blood and in the follicular fluid for all cases using the electrochemiluminescence immunoassay (ECLIA) for the assay of total vitamin D, then IVF outcomes were compared with the levels of vitamin D in the blood.

Results: the levels of vitamin D are not related to the criteria of eggs such as the number of eggs and the maturity rate (MR) of eggs, but they are correlated in a statistically significant manner with the fertility rate (FR), and at the same time the levels of vitamin D in the blood were completely independent of the clinical pregnancy rate (CPR).

Conclusion: blood vitamin D levels will affect the FR when its levels in the blood drop below a specified value, vitamin D did not correlate with the CPR. In the long run, there is scope for more research projects on vitamin D. Future research could include case-control studies of patients on vitamin D supplementation, and the study of its correlation with IVF outcomes.

1. Introduction

Recently, there is a renewed interest in vitamin D in terms of its synthesis, metabolism, mechanism of action, and association with the functions of various organs. The reason behind this growing interest is due to the global vitamin D deficiency situation, and novel research and hypotheses regarding its hormonal and non-hormonal mechanism of action in humans. Another evidence could be the number of annual citations in the PubMed database concerning vitamin D, which amounted to about 4800 from May 2008 to May 2009. This increase is considered double that of the previous decade [1,2].

The main source of vitamin D in humans is from the skin, where

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Abbreviations: UVB, Ultraviolet B rays; VDREs, Vitamin D Response Elements; CPR, Clinical Pregnancy Rate; IVF, In vitro Fertilization; TESA, Testicular Sperm Aspiration; GnRH, Gonadotropin Releasing Hormone; PCOs, Polycystic Ovarian syndrome; ECLIA, Electrochemiluminescence immunoassay; IOM, Institute of Medicine; WHO, World Health Organization; PTH, Parathyroid Hormone; 25(OH)D, 25 hydroxy vitamin D; BMI, Body Mass Index; MR, Maturity Rate; FR, Fertility Rate; RT-PCR, Reverse transcription Polymerase chain reaction; AMH, Anti-Müllerian Hormone; VBP, vitamin-bindingprotein.

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vitamin D_3 is produced via UVB rays through a photochemical non-enzymatic reaction that converts7-dehydrocholesterol to pre-vitamin D_3 [1,3], which subsequently undergoes a non-enzymatic thermal isomerization conversion process to vitamin D_3. Subsequently, in order to be activated, vitamin D_3 passes into the blood circulation where it is exposed to two hydroxylation processes located in the liver and kidneys respectively. The hormonally effective end product is 1,25-hydroxyvitamin D_3, or the so-called Calcitriol, which affects the target organs in either a genetic or non-genetic way [4]. As for vitamin D_2 (Ergocalciferol), it is synthesized by plants via UVB rays from ergosterol in plants and fungi.

Vitamin D is first metabolized in the liver to 25-hydroxyvitamin D (Calcidiol) via hydroxylation enzymes (the most notable of which is CYP2R1), then it is converted in the kidneys to 1,25-hydroxyvitamin D, the active hormonal form, by the hydroxylation enzymes CYP27B1 and CYP24A1. This active form is considered as a ligand for the Vitamin D Receptor (VDR), which acts as a transcription factor that binds to sites on DNA called Vitamin D Response Elements (VDREs). Thousands of these binding sites regulate hundreds of genes in a cell-specific manner [4].

Vitamin D has many important functions, the most prevalent of which are: (i) Regulation of calcium and phosphate uptake for bone mineralization, and thus its importance for bone growth and remodeling through osteoblasts and osteoclasts. (ii) Reducing the inflammatory response through modifying some processes in the cell such as cellular growth. (iii) Its role in tumors and the development of tumor cells such as breast, colorectal, lung, pancreatic, and prostate tumors [5–9]. (iv) Its role in cardiovascular diseases [10]. (v) Its role in multiple sclerosis [11]. (vi) Studies have also noted its vital role in type 2 diabetes mellitus [12]. (vii) Its role in weight loss and management [13].

25-hydroxyvitamin D levels are considered the main indicator of vitamin D stability, as it has a biological half-life of 15 days, in contrast to 1,25-hydroxyvitamin D which is not a good indicator of vitamin D levels due to its short biological half-life estimated at several hours.

Data on the status of vitamin D and its effect on fertility in women are conflicting and inconsistent. As some studies have demonstrated that increasing levels of vitamin D could improve clinical pregnancy rates (CPR) and the results of in vitro fertilization (IVF), other studies failed to find this association. In contrast to studies that prove the role of vitamin D in various body functions in humans, its role concerning reproduction function needs further investigation and study.

The data supporting the function of vitamin D in female fertility in general and embryo implantation, in particular, is not solid. The available pieces of evidence about the IVF procedure connection with vitamin D deficiency, and its effect on female fertility, are conflicting [14].

In vitro fertilization (IVF) can be defined as a multi-stage procedure that assists conception, improves fertility, and helps to avoid genetic problems during procreation. It is one of the most effective assisted reproductive technologies, through which eggs are removed from the ovaries and fertilized with sperms in the laboratory, then the embryo or the fertilized eggs are transferred to the uterus. One IVF cycle extends for about three weeks or more according to the followed protocol. The success of the IVF process and ultimately childbearing depends on many factors, the most significant of which are age and the cause of infertility. We resort to the IVF procedure in several cases, including those related to females (such as Ovulation Disorders, Endometriosis, Uterine Fibrosis, etc.), and others related to males (such as impaired sperm production or function), and there are mixed factors associated with both sexes, and unexplained infertility cases [15].

The chances of conception using the IVF technique depend on several factors, most importantly: the age of the female, the condition of the embryos, the reproductive history, and the case of infertility. In addition, there are lifestyle factors such as smoking, obesity, excessive alcohol consumption, and various dietary habits.

Therefore, our study aims to clarify the relationship of 25-hydroxy vitamin D levels with IVF outcomes in order to understand its role in the reproductive process in women.

2. Materials and methods

2.1. Sampling

The study samples were collected from Orient Hospital-operations department and fertility laboratory in Damascus for assisted reproduction, infertility treatment, IVF, and genetics, during the period between February 2019 to May 2019.

2.2. Number of study cases

In total, there were 60 females undergoing IVF (60 pairs of samples were collected: 60 blood samples + 60 follicular fluid samples from the women participating in the study). Approximately 8 ml of blood on heparin tubes were collected from the women on the day of egg retrieval prior to anesthesia, and 4 ml of follicular fluid collected on dry tubes was obtained during the egg retrieval process. The samples were then centrifuged within a maximum of half an hour after collection using Hettich centrifuge in the hematology laboratory at the hospital, at a speed of 2665 g for 7 min. The supernatant plasma and follicular fluid were divided after centrifugation into Eppendorf tubes, 500 μL per tube. The tubes were labeled and kept in the Faculty of Pharmacy freezer at the University of Damascus/Scientific Research Laboratory at −80 °C until the assay was performed.

2.3. Inclusion and exclusion criteria

Blood and follicular fluid samples were collected from the women undergoing IVF procedure on the same day as egg retrieval.

2.3.1. Inclusion criteria

Cases of Healthy women (in terms of reproductive function) with (i) normal uterus and normal ovaries, according to the ultrasound report performed in the hospital clinic before the preparation procedures for the IVF cycle. (ii) aged between 20 and 40 years and were close in terms of education, nutrition, and social status. (iii) an explicit male factor such as Oligospermia, Azoospermia, Asthenozoospermia, or TESA (Testicular Sperm Aspiration), ensuring that there is no fertility-interfering female factor. (iv) Undergoing long Gonadotropin-releasing hormone (GnRH) agonist down-regulation protocol.

2.3.2. Exclusion criteria

(i) Cases classified by the specialist clinician as a female factor such as PCOs (Polycystic Ovary Syndrome), Uterine Fibroids, Uterine Infections, Uterine Adhesions, and Endometriosis. (ii) Compound cases. (iii) Sex selection cases and those undergoing short GnRH agonist or antagonist protocol. (iv) Women aged under 20 or above 40. (v) Women who took nutritional supplements containing vitamin D, for at least two to three months before the egg retrieval procedure. (vi) Smokers. (vii) Cases with the following medical conditions: Tumors, Diabetes, Multiple sclerosis, Autoimmune Diseases, Liver or Kidney Disorders, Cushing's Syndrome, and women who take chronic medications.

2.4. Study design

Prospective cross-sectional study.

2.5. Vitamin D measurement and method of determination

About the study group, vitamin D levels were measured in the plasma and the follicular fluid using the electrochemiluminescence immunoassay (ECLIA) for the assay of total vitamin D, employing German Roche kit (Catalog Number. 05894913 190).

2.6. Division of the study group

blood and follicular fluid samples were collected from 60 women (fulfilling the required conditions), who were due to undergo IVF operation on the same day as the egg retrieval. The study group was then divided according to vitamin D levels.

In 2011, the IOM (Institute of Medicine) set the cut-off limit for vitamin D in terms of bones health at 20 ng/ml, and values higher are considered normal and sufficient. In the same year, the Society of Endocrine declared that normal values for the vitamin are Between 30 and 60, and less is considered abnormal. The society agreed with the recommendations of IOM that levels below 20 require treatment, but they classified values from 21 to 29 as insufficient (contrary to what IOM found). They recommended that vitamin D blood levels reach up to 30 or above [16,17]. Their recommendations were directed at a healthcare perspective, while the IOM recommendations were directed at community health one. The Endocrine Society considered that the classification of insufficient levels between 20 and 30 is difficult for several reasons related to the differences between laboratories' references and methods of measurement, and the different seasons, exposure to the sun, and dietary intake [18]. Accordingly, the society set the threshold of 20 ng/ml as the threshold for deficiency, and 30 ng/ml as the threshold for sufficiency. Whereas the New England Journal of Medicine recommended that people with vitamin D levels below 20 do not require any treatment, but rather those with levels below 12.5 [19].

The definition of levels as low or insufficient depends on the levels defined as normal, and according to the World Health Organization (WHO), levels below 10 are considered deficient, especially in the countries of the Middle East, whereas levels below 20 are insufficient. On the other hand, people have healthy vitamin D levels between the range of 30–76 [16,18,20].

The setting of the minimum normal as 30 ng/ml can be contributed to research that has concluded that PTH levels rise when levels of 25 (OH)D decrease below 30. Other researches have suggested that active calcium absorption is optimal at levels of 30 and above. The same studies indicate that the correlation between PTH levels and 25(OH)D is not curvilinear, and there is a large variation in PTH levels when 25(OH) D levels are between 20 and 30 ng/ml. Therefore, there is no threshold for blood vitamin D levels beyond which PTH levels rise. According to most studies, peak calcium absorption occurs at levels ranging from 20 to 30 ng/ml [18] (this is concerning bone health and calcium absorption). Whereas, in regard to other diseases, many studies have shown a correlation between low levels of 25(OH)D below 20 ng/ml and an increased risk of metabolic and immune disorders such as Type 1 Diabetes, and Atherosclerosis [18].

In conclusion, there is no international consensus on the definition of vitamin D deficiency and sufficiency. Concentrations of 20 ng/ml have been defined as a threshold for sufficiency but it is not sufficient for bone health, and IOM has concluded that concentrations of 12 ng/ml are the threshold below which symptoms of clinical vitamin D deficiency appear. Levels between 12 and 20 ng/ml have also been defined as inadequate for certain individuals, and this represents an uncertain range that could or could not be sufficient for certain individuals [20].

After this brief presentation of the differences in the classification of vitamin D levels between the various specialized organizations and societies, and given the consensus of all studies on the classification of vitamin D levels below 10–12 ng/ml as a condition of deficiency or even severe deficiency, and considered as eligible for medical treatment. Moreover, their differences in classifying values above 10 ng/ml, as well as according to the WHO, which considered that the cut-off value is 10 ng/ml, and values below are considered as vitamin D deficiency. Fundamentally, the study groups were divided into two according to the levels of vitamin D in the blood, as can be shown in Table 1.

Table 1
Blood 25 (OH)D levels (ng/ml) of the two study groups.

25(OH)D blood levels (ng/ml)	Group	No. of cases	Percentage %
≤ 10	A	11	18
>10	B	49	82

The following demographic criteria were compared between the two study groups: age, body mass index (BMI), and infertility duration. The criteria and results of the IVF cycle were compared between the two study groups, including the number of follicles, endometrial thickness, number of eggs, eggs maturity rate (MR), number of embryos, number of first-degree embryos, fertilization rate (FR), biochemical pregnancy rate, clinical pregnancy rate (CPR).

2.7. Statistical study

To achieve the study objectives and analyze the collected data, we used many appropriate statistical methods of analysis using MS-Excel (Microsoft office excel 2016) for data collection, and a Statistical Package for the Social Sciences (SPSS) software version 22.0 (SPSS) for windows (IBM Corp., Armonk, NY, USA) for data analysis with a confidence level of 95%, and the variables were expressed using the arithmetic mean and standard deviation. Additionally, the differences between all variables within the divisions of vitamin D levels were tested depending on the parametric Student's T-Test and its nonparametric alternative, Mann-Whitney *U* Test. Furthermore, vitamin D levels independence from the incidence rates of biochemical or clinical pregnancy was verified using the Chi-Square Test of independence. Finally, results were considered at a statistically significant p-value ($p < 0.05$).

2.8. Data quality assurance

The data was collected through a face-to-face interview questionnaire for all the target cases of the study (wives and husbands together). Patient files in the clinic were used to complete the data. Informed consent and reliable data were obtained after explaining and clarifying the objectives of the study. In addition, the supervisors Specialists supervising the collection of data and recording it in patients' files on a daily basis.

The study was performed in line with the principles of the Declaration of Helsinki. The ethical review committee at the Faculty of Pharmacy, Damascus University, approved the study design and protocol, the work has been reported in line with the strengthening of the reporting of cohort studies in surgery (STROCSS) criteria [21].

2.9. Ethical approval

Ethical clearance was obtained from Damascus University based on Session No. 1 for the 2016/2017 academic year held by the Scientific Research Ethics Committee at the Faculty of Pharmacy on April 3, 2017 and pursuant to Resolution No. 9.

A support letters was written to all public hospitals where the study was conducted. The study was registered with ClinicalTrials.gov with a unique identifying number NCT05393011.

3. Results

The study group was divided into two groups A and B according to Table 1.

3.1. Correlation between blood and follicular fluid 25 (OH)D

The study showed a strong direct linear correlation (95%) with statistical significance ($p > 0.05$) between the measured 25(OH)D in blood and follicular fluid levels of the study groups, and this was reflected by the Pearson correlation coefficient Fig. 1.

The mean blood 25(OH)D levels of 20.7 ± 11.3 ng/ml were not different from that of the follicular fluid of 18.4 ± 11.4 ng/ml (p =

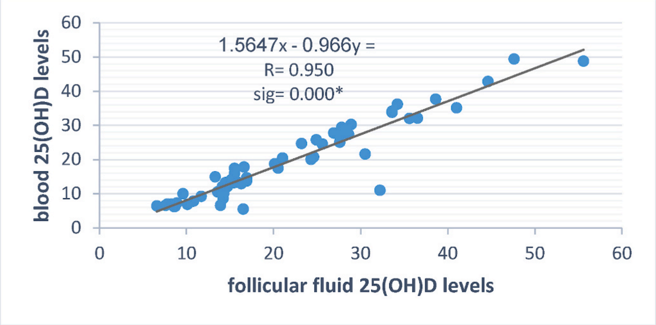

Fig. 1. Correlation between blood and follicular fluid 25(OH)D levels.

0.112) Fig. 2.

3.2. Demographic criteria comparison between the study groups

We compared the average age, BMI, and infertility duration between two groups, the following results are obtained:

The mean age in years for group B of 31.8 ± 5.3 was higher and more statistically significant (p = 0.003) than that of group A of 26 ± 4.7 Fig. 3, the mean BMI in kg/m^2 did not show a statistically significant difference between groups A and B, which were 26.5 ± 3 and 26.4 ± 4.3, respectively Fig. 4, and the mean infertility duration, estimated in years, did not show a statistically significant difference between groups A and B, which was 5.7 ± 3.8 and 8.6 ± 4.2, respectively Fig. 5.

Table 2 summarizes these values for the demographic criteria of the study members.

3.3. IVF outcomes comparison between study groups

3.3.1. Number of follicles

The mean number of follicles in study groups A and B was 12.9 ± 3.2 and 10.6 ± 3.9, respectively, with a non-statistically significant difference Fig. 6.

3.3.2. Endometrial thickness

The mean endometrial thickness in millimeter for the two study groups was 11.3 ± 4.3 and 9.7 ± 1.3, respectively, with a non-statistically significant difference Fig. 7.

3.3.3. Total number of eggs

The total number of eggs in group A, which amounted to 20.3 ± 13.4, was statistically significant (p = 0.003) higher than the total number of eggs in group B, which amounted to 11.6 ± 8.2 Fig. 8, while the average egg MR in group B was higher than that of group A, which amounted to 64.8 ± 16 and 59.8 ± 16.9, respectively, with a non-statistically significant difference Fig. 9.

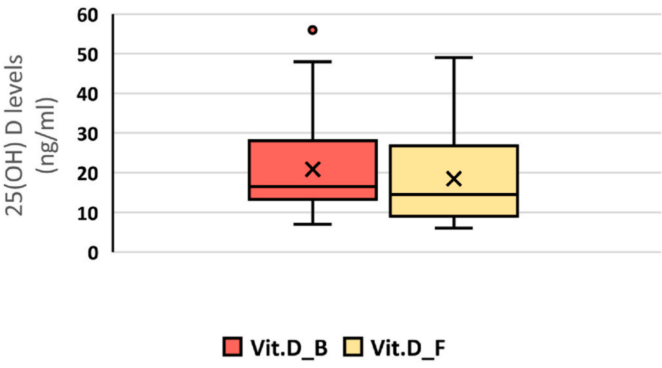

Fig. 2. Blood and follicular fluid 25(OH)D levels.

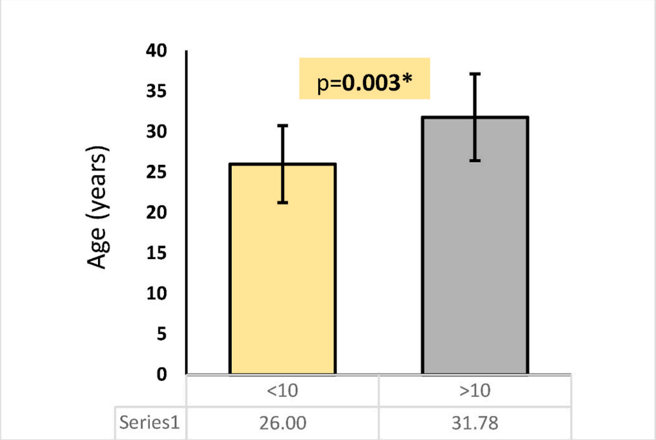

Fig. 3. Mean of age between two study groups.

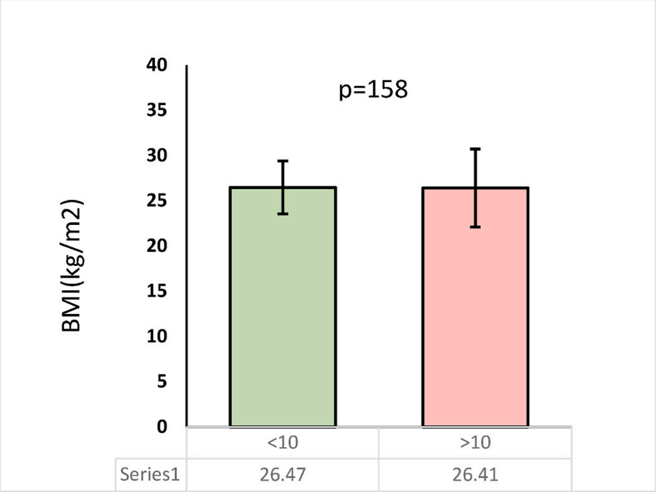

Fig. 4. Mean of BMI between two study groups.

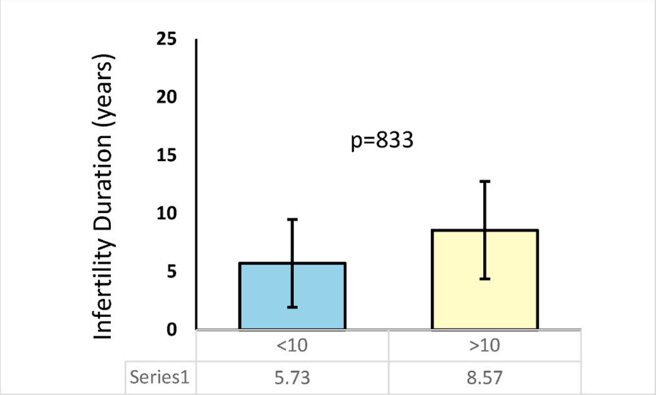

Fig. 5. Mean of infertility duration between two study groups.

3.3.4. Fertility rate (FR)

The FR of group B was higher than that of group A, which was 77.7 ± 20.4 and 62.1 ± 17.4, respectively, with a statistically significant difference (p = 0.009) Fig. 10.

3.3.5. Total number of embryos

The total number of embryos, as well as the number of Grade I

Table 2
Mean of demographic criteria± standard deviation of the tow study groups.

	Group A	Group B	p-Value
No. (%)	11 (18)	49 (82)	
Age (Years)	26 ± 4.7	31.8 ± 5.3	**0.003***
BMI (kg/m^2)	26.5 ± 3	26.4 ± 4.3	0.158
Infertility Duration (Years)	5.7 ± 3.8	8.6 ± 4.2	0.833

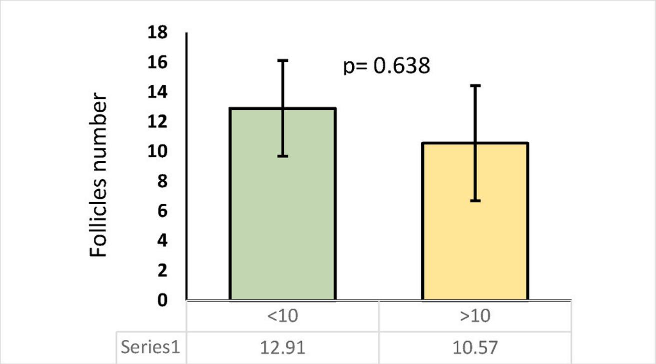

Fig. 6. Mean of follicles number between two study groups.

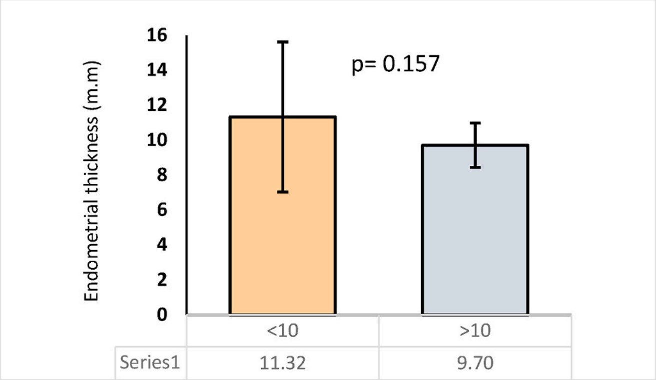

Fig. 7. Mean of endometrial thickness between two study groups.

embryos and Grade II embryos, did not differ between the two study groups. It reached 4.7 ± 1.3 and 4 ± 1.7, 3.6 ± 1.3 and 3 ± 1.8, as well as 1.1 ± 0.7 and 1.02 ± 0.9 respectively Fig. 11, Fig. 12, Fig. 13.

3.3.6. Biochemical pregnancy rate

The biochemical pregnancy rate for the two study groups was 60% and 48%, respectively, with a non-statistically significant difference Fig. 14.

3.3.7. Clinical pregnancy rate (CPR)

The incidence of CPR in the two study groups was 63.6% and 49%, respectively, with a non-statistically significant difference Fig. 15.

According to the Chi-Square Test for independence, the rates of biochemical and clinical pregnancy were independent of 25(OH)D levels (p = 0.487, 0.379), respectively.

The following Table 3 shows and summarizes all previous results.

4. Discussion

The mean value of vitamin D in the blood and the follicular fluid did not differ, and the correlation between them was positive, indicating that the levels of 25(OH)D in the body fluids reflect the body's stores of vitamin D.

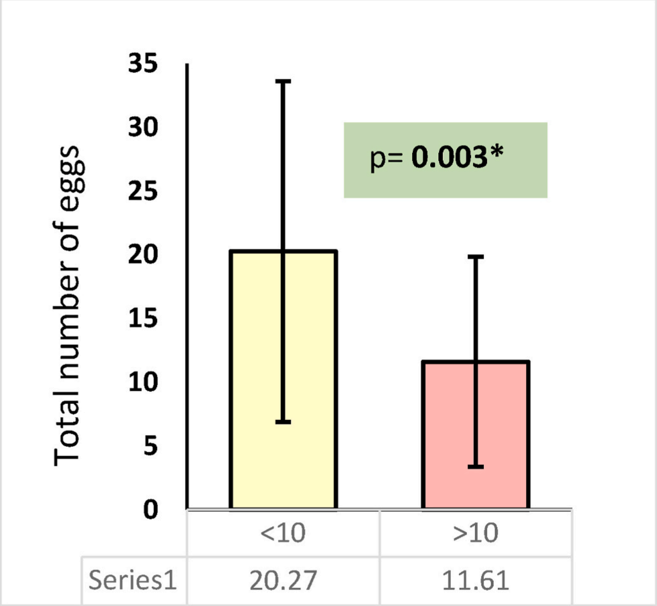

Fig. 8. Mean of total number of eggs between two study groups.

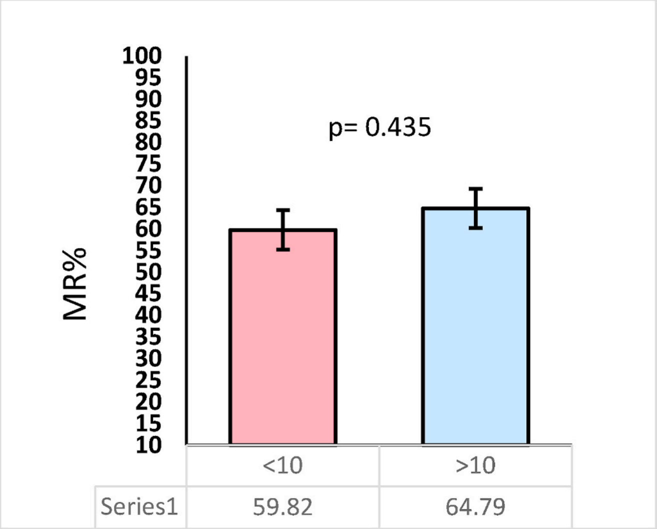

Fig. 9. Mean of MR between two study groups.

Vitamin D deficiency is prevalent as people grow older and requires treatment according to several studies [22]. Nonetheless, our study showed that the mean age of 31.8 of females undergoing the IVF cycle in group B was significantly greater than the average age of females in group A, which reached 26 years old, noting that the age group of the participants in the research ranged from 20 to 40 years. This difference could be elucidated by the increase in the female awareness when she grows older, the increase in the extent to which she takes care of her nutritional status and all sources of vitamins, and the extent of exposure to sunlight, especially whether the female is about to have children, and this is what could be noticed through simple questions that were directed to the participants.

As for BMI, the females in both groups did not show a fundamental difference between them, despite most studies indicating a correlation between obesity and decreased levels of vitamin D in individuals [23]. But our study group is a special group of females who are about to have children and try as much as possible to maintain their health and take care of the quality of food while being careful not to gain weight (the

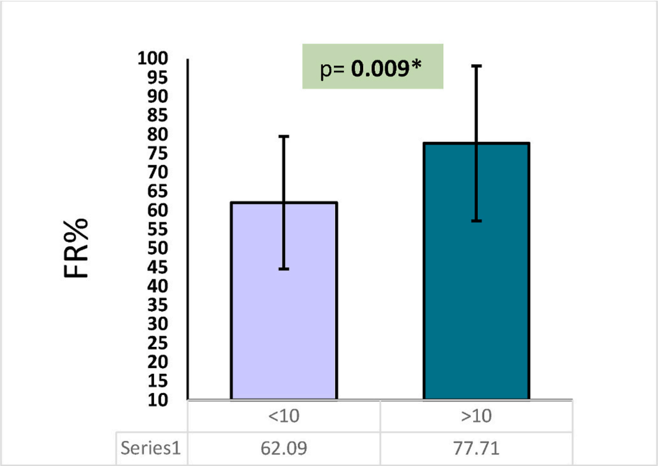

Fig. 10. Mean of FR between two study groups.

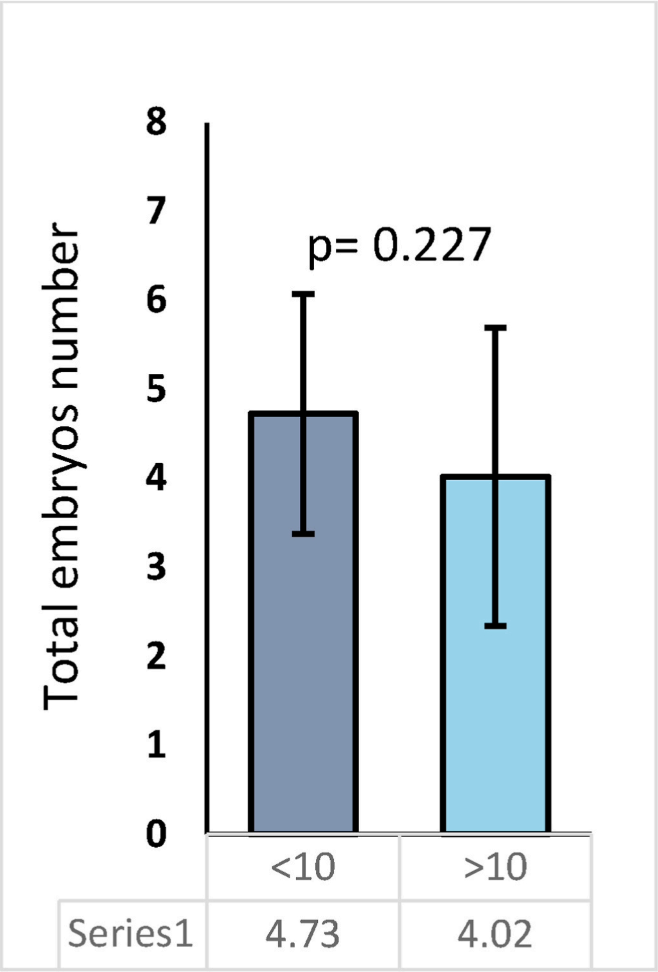

Fig. 11. Mean of total number embryos between two study groups.

highest value of BMI did not exceed 35), and without elevated cholesterol and triglycerides levels, due to their negative effects on the reproductive process in which they already face a problem. The results of our study agreed with several studies [24,25] and also disagreed with other studies as well [14,26].

The number of infertility years was not fundamentally different between the two groups as well, due to the keenness of our society (Middle Eastern countries) on the aspect of reproduction and making it a priority for married couples and the initiative to seek medical advice and use assisted reproductive techniques when childbearing is delayed. Whereas the Middle Eastern countries had the highest FR (number of live births per woman) according to a statistical study conducted in 2018 to compare FR between the countries of the Middle East and North Africa [27], while other studies showed differences in infertility years according to vitamin D levels, such as Italy Study 2014 [14].

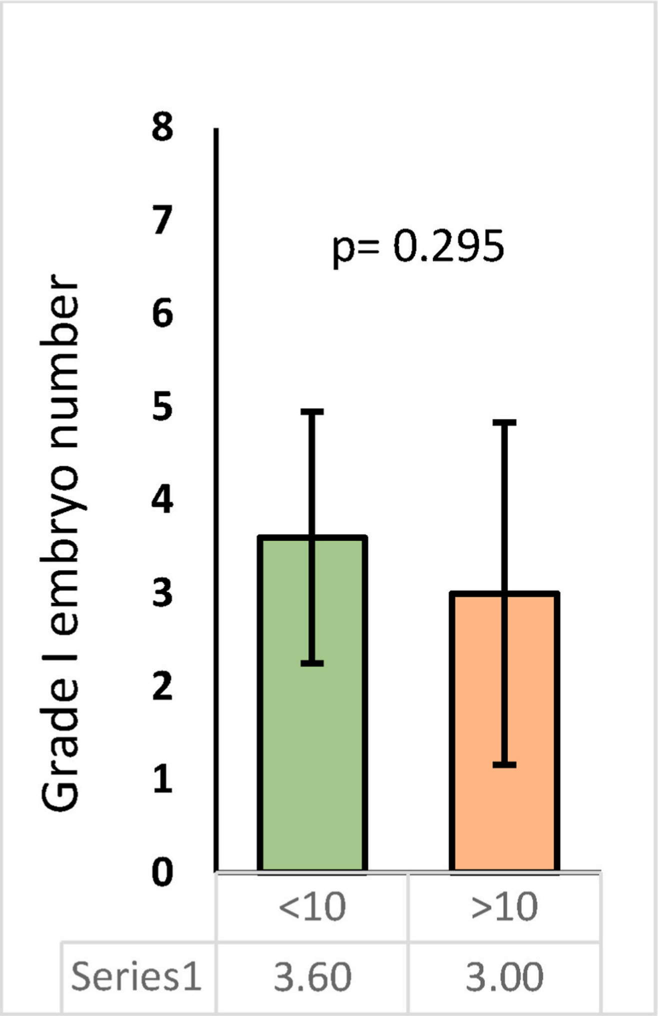

Fig. 12. Mean of Grade I embryo number between two study groups.

The average endometrium thickness in group A was higher than that in group B, but this difference was not statistically significant, as the results of our study matched with the study of Banker et al. in India in 2017 [28]. In this regard, there are studies [29–31] that concluded that adequate levels of vitamin D are associated with the regulation of the HOX gene expression necessary for implantation, which is expressed by the endometrium. Therefore, the study concluded that adequate levels of vitamin D are associated with better endometrial thickness.

The average follicles number did not differ between the two study groups. During the induction period, the doctor aims to obtain mature follicles (with a size of 18 mm or more) through ultrasound follow-up, and the number of follicles between 8 and 15 is considered acceptable [32]. Despite that, the number of follicles is an important indicator of fertility undoubtedly, but the best number cannot be determined, and one number cannot predict the outcome of the IVF cycle. The female may feel disappointed when only one or two follicles are obtained, but here, more is not necessarily better, because every single mature follicle can release one egg, which in turn can be fertilized later [33,34]. Some

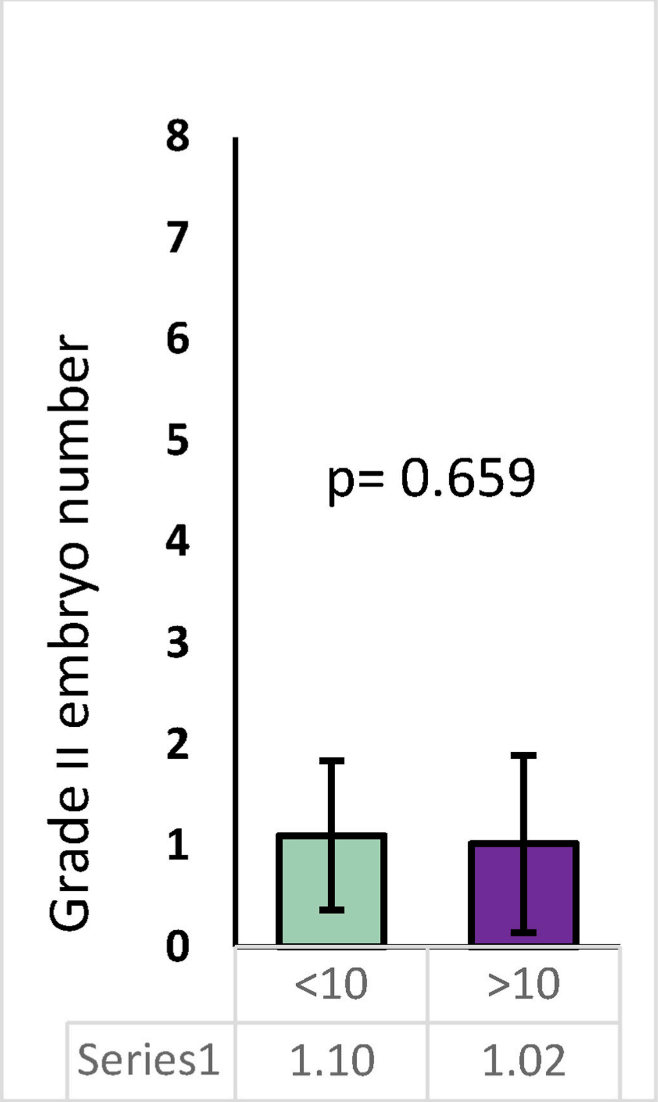

Fig. 13. Mean of Grade II embryo number between two study groups.

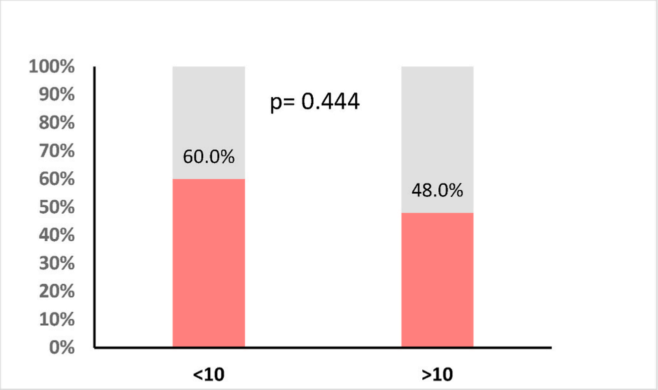

Fig. 14. Biochemical pregnancy rate between two study groups.

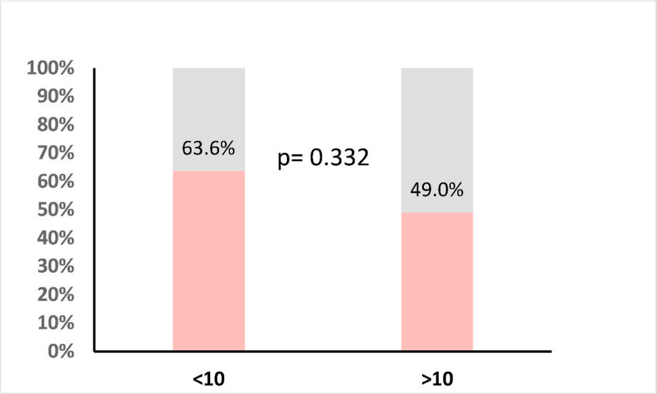

Fig. 15. Clinical pregnancy rate between two study groups.

Table 3
Comparing the IVF outcomes of the two study groups.

	Group A	Group B	p-Value
No. (%)	11 (18)	49 (82)	
Follicles No	12.9 ± 3.2	10.6 ± 3.9	0.638
Endometrial thickness (mm)	11.3 ± 4.3	9.7 ± 1.3	0.157
Eggs No	20.3 ± 13.4	11.6 ± 8.2	**0.003***
MR	59.8 ± 16.9	64.8 ± 16	0.435
FR	62.1 ± 17.4	77.7 ± 20.4	**0.009***
Embryos No	4.7 ± 1.3	4 ± 1.7	0.227
Grade I Embryos No	3.6 ± 1.3	3 ± 1.8	0.295
Grade II Embryos No	1.1 ± 0.7	1.02 ± 0.9	0.659
Biochemical pregnancy rate (%)	60	48	0.444
CPR (%)	63.3	49	0.332

studies [35,36] have concluded that the sufficiency of vitamin D is important for follicular development with improved steroidogenic function and egg maturation. However, it is difficult to study the direct effect of vitamin D on the ovary since the functions and actions of vitamin D as an endocrine and paracrine factor are highly context-dependent, as types and developmental stages of follicles as well as vitamin D metabolite forms and doses must be carefully considered during experimental design. Therefore, there is still a need to conduct experiments on animal models to obtain more information about follicular vitamin D, its metabolism, the expression of VDR, vitamin D-regulated follicular development and egg maturation, and to understand the molecular mechanisms of the effects of vitamin D on the ovary. Clinical studies on the follicular growth associated with vitamin D and the egg MR are still unclear [37]. It should be noted that none of the studies addressed the correlation between vitamin D levels and the number of follicles, but it was added to our study to confirm that there are no significant differences in the average follicles number when vitamin D blood levels changed.

Regarding the effect of vitamin D on the eggs number and their MR, it must be mentioned that all clinical research aimed at evaluating the effect of vitamin D on the ovaries was conducted on females suffering from fertility problems, and most of these researches were in the context of PCOs [35,37]. As for our study, it aimed to select reproductively healthy females. Other studies also demonstrated [38,39] that the metabolism of vitamin D occurs locally in the ovary (besides its metabolism in the liver and kidneys), after the detection of CYP2R1 mRNA, and CYP27B1 mRNA that encode hydroxylase enzyme in ovarian tissues in females subjected to treatment with IVF using RT-PCR and Western Blot technology. This explains the first observation that Potashnik et al. made in 1992 [40] about the presence of vitamin D in the follicular fluid. After that, research continued to prove that vitamin D deficiency causes a decrease in the size of the ovary, as well as the levels of Estradiol (E_2) and Progesterone compared to the control group, and this result is taken as a given [41]. In 2020, Moridi et al. designed a meta-analysis study and collected data from 18 studies on the correlation between vitamin D and levels of the *Anti*-Mullerian Hormone (AMH), which is secreted by ovarian follicle granulosa cells, and is responsible for follicles growth and increase their size to 8 mm [42]. Moridi's study concluded that there is no correlation in most studies between blood levels of vitamin D and AMH. On the other hand, the study of Aramesh et al. in Iran [43] found an important correlation between the number of eggs and the administration of vitamin D supplementation, which supported the possibility of the effect of vitamin D on increasing the expression of AMH through the effect of a promoter in the AMH gene, which led them to deduce that levels of AMH may be increased by vitamin D without affecting the number of follicles (and this is consistent with our result for follicles number). Other research has linked decreased vitamin D status with increased AMH levels, which causes an increase in large follicles production and the number of eggs. Moreover, the levels of follicular vitamin D were inversely correlated with the levels of AMH and its receptor levels in cumulus granulosa cells, and that vitamin D treatment led to a decrease in AMH values [44,45]. Furthermore, the correlation between circulating vitamin D and parameters of follicles and eggs in clinical studies is different and significantly heterogeneous among patients who vary in vitamin D metabolism and signaling, due to physiological and pathological differences between them. Also, one should not forget that vitamin D is transported to the target cells via binding to a high-affinity carrier protein (VBP), which has heterogeneous polymorphisms between humans with different races, which in turn affects vitamin D binding. Another study [35] showed that the correlation between vitamin D deficiency and a decrease in the development of follicles, in addition to a decrease in the number of eggs produced, expression of E_2, and weight of the ovaries. This could explain the increase in the total eggs number in group A of our study, in comparison to group B. Meanwhile, the eggs MR was higher in group B, but this increase did not reach the level of statistical significance. To summarize, it is safe to say that the correlation between vitamin D and reproductive outcomes related to the number of eggs and follicles requires further investigation.

On another note, the mean FR was significantly higher in group B than in group A, this is explained by the previous result that vitamin D contributed to improving the quality of eggs to reach a more mature stage. In contrast, the rate of egg maturation was high in group B. It should also be noted that the fertilization process includes two main points, namely, the interaction of sperms and their penetration into the zona pellucida, and then the merging of sperms with the eggs' membranes, which trigger the egg activation. In our study, the previous stages were bypassed using the IVF-ICSI technique for all participating cases. In the light of our previous discussion that mentioned the role of vitamin D in changing the AMH signal, and thus, shows its role in the development of eggs, consequently, this will certainly reflect positively on its role in the FR. The results of our study matched with a study conducted in China on 848 cases [45] concerning FR, whereas the study conducted in Iran [46] showed high FR rates conjugated with high levels of vitamin D. But these differences did not reach the level of statistical significance.

The average total number of embryos and the average number of Grade I embryos did not differ between the two groups. This indicates that the decrease in vitamin D levels had no effect on the number and maturity of the embryos in our study, and the results of our study matched studies conducted in India and China in this regard [28,45].

The incidence rates of biochemical and clinical pregnancy were completely independent of vitamin D blood levels, and this finding is in fact consistent with the vast majority of studies published in this field. In 2016, Shi Shi et al. performed a meta-analysis [47] of 134 studies of vitamin D levels in relation to IVF outcomes. Ultimately, they concluded that the risk of a low CPR did not increase significantly in the vitamin-deficient group. The results of our study coincided with the results of several studies conducted in India, America, Iran, and China [26,28,31,45,46,48], whereas other studies found a direct positive correlation [14,24,26] and inverse correlation [49,50] between vitamin D levels and the incidence of CPR. Therefore, it is unavoidable to say that the role of vitamin D in the outcome of the IVF process requires a more thorough studying in order to clarify the previous correlation, and the prospective randomized control trial studies may be able to remove biases and provide more well-built answers.

4.1. *Study strengths and limitations*

25-hydroxy vitamin D was assayed in serum and follicular fluid by the golden method, which is an the electrochemiluminescence immunoassay (ECLIA), in order to obtain reliable results. Therefore, due to the high cost of this method, we were limited to a smaller number of samples.

5. Conclusion

Vitamin D is one of the most important vitamins in the body and its intake is recommended for patients with severe deficiency before preparing for the IVF process, in order to improve the eggs FR. But we need more meta-analysis and systematic reviews before we could apply vitamin D as a routine supplement. Moreover, vitamin D levels in the follicular fluid can reflect the body's stores of vitamin D. The prevalence of severe vitamin D deficiency was less common among the patients participating in our study. However, no significant correlation was found between IVF outcomes and 25(OH)D levels, except for the egg FR, which was low in patients with severe vitamin D deficiency.

From the results of our study on eggs MR, higher levels of vitamin D are suggestive of an effect on ovarian steroidogenesis (although this difference did not reach a higher level of significance). Furthermore, vitamin D had a positive correlation with the FR. Having said that, vitamin D did not correlate with the CPR. In the long run, there is scope for more research projects on vitamin D. Future research could include case-control studies of patients on vitamin D supplementation, and the study of its correlation with IVF outcomes.

Ethical approval

Ethical clearance was obtained from Damascus University based on Session No. 1 for the 2016/2017 academic year held by the Scientific Research Ethics Committee at the Faculty of Pharmacy on April 3, 2017 and pursuant to Resolution No. 9.

A support letters was written to all public hospitals where the study was conducted.

Sources of funding

This work is funded by the University of Damascus. The funding includes the purchase of materials needed for the practical stages of research, such as purchasing the necessary assay kits, but it does not include data collection and analysis, writing the manuscript and submitting it for publication.

Author contributions

The main researcher, Raghad Faisal, coordinated the process of designing the study, collecting data, then processing it statistically and writing the paper.

Dr. Marwan Al-Halabi: Supervising the collection of samples in the hospital in terms of their conformity with the appropriate criteria for the study design, and contributing to the interpretation of the results.

Dr. Faizeh Alquobaili: Facilitating work in the college laboratories and reviewing the writing of the paper.

Registration of research studies

Name of the registry: ClinicalTrials.gov, Relationship Between Some Vitamins and Antioxidants With in Vitro Fertilization Outcomes.

Unique Identifying number or registration ID: NCT05393011The research protocol was retrospectively registered with a ClinicalTrials.

gov.

Guarantor

Professor Dr. Faizeh Alquobaili.
Damascus University, faculty of Pharmacy.

Consent

The data was collected through a face-to-face interview questionnaire for all the target cases of the study (wives and husbands together). Patient files in the clinic were used to complete the data. Informed consent and reliable data were obtained after explaining and clarifying the objectives of the study. In addition, the supervisors Specialists supervising the collection of data and recording it in patients' files on a daily basis. All the study participants were informed of the purpose of the study and their right to refuse. Informed voluntary written and signed consent was obtained from all study participants prior to distributing the questionnaires. The respondents were told that the information obtained from them was kept with confidentiality and does not cause any harm to them.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon request.

Provenance and peer review

Not commissioned, externally peer-reviewed.

The aim of the project

This manuscript has been prepared as part of a large original project currently being prepared for a Ph.D. degree in clinical biochemistry.

After we completed the stages of field work in the different hospitals and laboratories, and obtained interesting and related results, we liked to publish them in your esteemed journal, and we hope that publication will be accepted.

Submission declaration

This project has not been previously published, nor is it under publication elsewhere, the publication has been approved by all authors and by the responsible authorities where the work has been carried out. If publication is accepted, it will not be published elsewhere in English or in any other language without the written consent of the copyright holders.

Declaration of competing interest

The authors declare no conflicts of interest.

Abbreviations

UVB	Ultraviolet B rays
VDREs	Vitamin D Response Elements
CPR	Clinical Pregnancy Rate
IVF	Invitro Fertilization
TESA	Testicular Sperm Aspiration
GnRH	Gonadotropin Releasing Hormone
PCOs	Polycystic Ovarian syndrome
ECLIA	Electrochemiluminescence immunoassay
IOM	Institute of Medicine
WHO	World Health Organization
PTH	Parathyroid Hormone
25(OH)D	25 hydroxy vitamin D
BMI	Body Mass Index
MR	Maturity Rate
FR	Fertility Rate
CPR	Clinical Pregnancy Rate
RT-PCR	Reverse transcription Polymerase chain reaction
AMH	Anti-Müllerian Hormone
VBP	vitamin-binding protein

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.amsu.2022.104126.

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