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The Relation of Serum and Follicular Fluid Vitamin D Levels and the Oocyte Maturation, Embryo Grade and Pregnancy Rate in In Vitro Fertilization/Intracytoplasmic Injection Cycles

ABSTRACT

The clinical pregnancy rate in In Vitro Fertilization and intracytoplasmic sperm injection (ICSI) remains low in spite of many evolutions in assisted reproduction. The ovulatory infertility is the main causes of female infertility related to oocyte problems. Maternal age, oocyte competence with its follicular fluid affect oocyte quality and pregnancy rate. So many studies were done for the follicular fluid and its content to study the effect of its environment and their relation to pregnancy success. The aim of the study is found a relation between vitamin D blood level and the oocyte maturation, embryo grade and pregnancy rate in In Vitro Fertilization/Intracytoplasmic injection cycles

Materials and Methods

A prospective observational study which was connected at the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies. Infertile women (75) who admitted to in vitro fertilization programs in the institute for the In Vitro Fertilization/Intracytoplasmic injection. Controlled ovarian stimulation was done to each patient and at the day of ova pick up, follicular fluid aspiration was done and Vitamin D analysis by Eliza was done. Level of Vitamin D was assessed to each patient.

Results:

Follicular fluid matching to successfully mature good quality oocyte to achieve pregnancy. In this study the common causes of infertility are: ovarian 13(21%), unexplained 12(19.4%), PCOS 10(16.1%), male factor 10(16.1%), tubal factor 10(16.1%), and mixed 7(11.3%). Serum level of vitamin D was divided into three groups: Group (1) or normal group (≥30 ng/ml), group (2) or insufficient group (21-29.9 ng/ml) and group (3) or deficient group (≤ 20.9 ng/ml) is found among 28 (43.5%),15(25.8%) 19(30.6%), of patients respectively. According to the serum level of vitamin D in three groups, the mean number of oocyte MII or embryo grades number was not significantly different .Positive chemical pregnancy is not significantly more among those with sufficient group (1) as compared with deficient group (3) . The vitamin D in follicular fluid level demonstrated a not significant positive correlation between its level and retrieved MI ,MII oocyte number. The results demonstrate a non-significant positive correlation between follicular fluid vitamin D level and grade (I) embryo. There is no significant difference between pregnancy rate and follicular fluid of vitamin D.

Conclusion:

There is a non-significant correlation between oocyte maturity, embryo grade and chemical pregnancy rate with both serum and follicular vitamin D level.

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Introduction:

Childlessness is one of the common form of life stress. Infertility has been found to influence 10–15 % of couples. In a research carried out between (2006-2010, greater than 1.5 million females in United State, or 6% of the couple of age between 15-44 years of age, reported infertility, and 6.7 females reported failure of being pregnant or carry a baby till term. Between females aged 15-44 years, almost 7 million have utilized infertility facilities at certain point.(1) .

In Iraq, infertility elevated, particularly in the last 16 years (2000–2016) and it is associated with a lot of factors, like; (as stress, war, lifestyle, smoking, occupation, hereditary, eating behavior. (2)

Ovulatory causes of female infertility play a major cause of them. It is affected by many local and hormonal factors which affect oocyte quality. Oocyte competence is affected mainly by follicular fluid that has microenvironment of oocytes development significantly. Secretory activity of both of the granulosa and theca cells with blood plasma transfer ingredient that cross the blood follicular barrier are the main follicular fluid production sources. (3)

Calcitriol or bioactive Vit. D is a steroid hormone that has been recognized for its substantial role in regulating serum levels of phosphorus and calcium, and in bone mineralization, cell differentiation and proliferation. Vit. D receptors are existing in a broad diversity of cells. This hormone has biologic influences that expand far beyond control of metabolism of mineral. (4) Two forms of Vit. D occurs in two forms: vitamin D2 (ergocalciferol) and D3 (cholecalciferol). In animals, D3 is molded in the skin from its derivate (7-dehydrocholesterol) with ultraviolet B radiation (UVB) influence, while D2 is made in fungi, green vegetable and yeast. (4)

The liver metabolizes Vit,D to 25(OH) D and then transformed it to 1,25(OH)2 D to keep calcium and

phosphate in serum by its action on intestine, bone, kidney and the parathyroid. In the small intestine (primarily in the duodenum),1,25(OH)2 D motivates calcium and phosphate absorption by the jejunum and ileum.(5)

In the ovarian tissue, 1,25-$(OH)_2D_3$ motivates the production of progesterone, estradiol, and estrogen. It regulates expression and secretion of human chorionic gonadotropin (hCG) in syncytiotrophoblast. It encourages the manufacture of placental sex steroid. Also,it is required for the satisfactory uterine endometrial growth, permitting uterine receptivity for implantation.(6)

The vitamin D receptor (VDR) presence in a lot of tissues over the female reproductive axis, including the ovary, pituitary, placenta and uterus, proposes that vitamin D is an energetic regulator of the female fertility. Active form of vitamin D (1,25 dihydroxy vitamin D3 or calcitriol) binding to its receptor, helps a transcription factor to regulate the expression of the CYP19 gene, which encodes aromatase, an vital enzyme in the production of estrogen. (5,7)

Material and Methods:

The study was connected at the High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University. Written informed consent was taking from every patient in the study, which was approved by the Ethics Committee.

Only (62) women finished the study from (75) infertile women, because either lost to follow up or failure of fertilization or ovarian hyperstimulation (OHSS). Age of enrolled women was between 20-39 years old. (8)

Women undergoing IVF / ICSI at the day of ova pick up who take 75IU of follitropin alpha (Gonal f®, Merck, Serono) for ovarian stimulation and followed by the GnRH antagonist (Cetrorelix acetate for injection 0.1 mg: Cetrotide®, Merk, Switzerland), administration of either fixed or flexible treatment. At least 3 follicles larger than 17mm were obtained and serum estradiol level was more than 500, ovulation was activated by injection of

intramuscular hCG 10000 IU (Pregnyl®, Organon, Holland) .

Endometrioses, women age ≥ 40 years old, congenital anomalies of female reproductive system, systemic disease or endocrinal disorder (thyroid and adrenal disorder), moderate male factor infertility (concentrations 5 million–10 million sperms/ml) or severe male factor infertility. (concentrations less than 5 million sperms/ml) ,(9) history of female pelvic surgery, AMH ≤ 1 ng/ml and AFC ≤ three follicles in ultrasound were exclusion criteria.(10)

Full medical, surgical and obstetrical history was taken. measurement of BMI and clinical and gynecological examinations was done. BMI was done by dividing weight in kilograms by the square of height in meters (kg/m2). World Health Organization definition of BMI is: <18.50 kg/m2 (underweight) ,18.50– 24.99 kg/m2 (normal),25.00–29.99 kg/m2 (overweight), 30.00–34.99kg/m2 (obese Class-I),35.00–39.99kg/m2 (obese class-II),and ≥40.00 kg/m2 (obese class-III).(11)

WHO (2010) for male seminal fluid analysis assessment was used during semen preparation for intracytoplasmic injection.

Blood sampling was done by taken venous blood samples of 3–5 ml. It was drawn from the antecubital vein from each woman for vitamin D level estimation at the day of ova pick up. Blood samples were centrifuged at 1,000 g for 15 min, and serum was stored at -20°C until analyzed.

After 34-36 hours of HCG injection oocytes retrieval was performed using a transvaginal probe. Follicular fluid was taken from each women in separate tubes for each side from the left and right ovary and centrifuged (800 g for 10 min). Storage of follicular fluid was at -20°C until analyzed.

Oocyte grading either germinal vesicle (GV) oocyte, which is the most immature oocyte. Metaphase I (MI) oocyte that is immature egg with the absence of a polar body or a germinal vesicle, which is an intermediate stage between the GV and MII (mature)

stages . Metaphase II oocyte (MII) is mature oocyte . (12) **S**perm extraction from semen sample was prepared according to WHO preparation of 2010.

After oocytes denudation and grading at Laminar Flow Cabinet and insemination, observation of zygotes was done after 18 - 20 hours to look for the two pronuclei development. After 25 - 29 hours to confirm early cleavage. Then embryos were evaluated at day 2 (43 - 45 hours after insemination) and day 3 (67 - 69 hours after insemination) to see the cells number, proportion of fragmentation and blastomere resemblance. Less than 10% of cytoplasmic fragments considered good quality embryo. (13)

Embryo transfer was done when fertilization took place and the patient with a partially full bladder was placed into the uterine cavity under pelvic ultrasound guidance and by an intrauterine catheter. Progesterone (Cyclogest®400mg twice: Avantis, UK) was taken daily as luteal phase support. Serum ß-HCG assay was done on day 14 after the embryo transfer.

Laboratory method of assessment of vitamin D was done as This ELISA kit applies to the in vitro quantitative determination of vitamin D concentrations in serum, plasma and other biological fluids.

The ELISA kit uses the Competitive-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with vitamin D. Vitamin D in the sample or standard competes with a fixed amount of vitamin D on the solid phase supporter for sites on the Biotinylated Detection Ab specific to vitamin D. Avidin conjugated to Horseradish Peroxidase (HRP) are added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme-substrate reaction is terminated by the addition of stop solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of vitamin D in the samples is then determined by comparing the OD of the samples to the standard curve.

When serum β hCG was estimated14

days from embryo transfer rates and ≥ 10 miu/l to measure biochemical pregnancy as a primary outcome.

Statistical analysis:

Data entry and analysis were done by statistical package for social sciences (SPSS, version 25), And Excel. Chi (χ^2) square test of association was used to compare proportions of different parameters between study groups. One way ANOVA, and student t tests used to compare quantitate data. Statistically significant when P value ≤ 0.05 was considered as. Bar charts was used to represent data.

Results:

1. Demographic features:

The mean age of females was 29.7±6.0. The mean age of males is37.0±7.8. Mean BMI was 28.6±4.3. Most of females are overweight (25-29.99) 25(40.3%), followed by obese class(I) was 17(27.4%), and obese class (II) was 5(8.1%). The mean duration of infertility is shown in table (1).

Table (1) The Demographic features of study sample

General characteristics	Frequency	%(62)patients
Age of female (Mean ±SD)	29.7±6.0	
Age of male (Mean ±SD)	37.0±7.8	
BMI	28.6±4.3	
18.5-24.99(normal)	15	24.2%
25-29.99(over weight)	25	40.3%
30-34.99(Obesity class I)	17	27.4%
35-39.99(Obesity class II)	5	8.1%
Duration of infertility	7±4.6	
Infertility type		
Primary infertility	42	67.7%
Secondary infertility	20	32.3%

Sample size =62 pt.

The analysis of (62) patients involved in the study revealed that the rate of pregnancy rate (PT) was 15(24.2%).

2.Serum and follicular fluid(FF) vitamin D:

Group (1) or normal group (≥30 ng/ml), group (2) or insufficient group (21-29.9

ng/ml) and group (3) or deficient group (≤ 20.9 ng/ml) is found among 28 (43.5%),15(25.8%) 19(30.6%), in patients respectively.

Those with sufficient group (1), insufficient group (2) and deficient group (3) Vit. D is non significantly differ regarding the mean number of oocyte MII (4±2.7), (4.07±3.4), (4.26±1.69), respectively, as shown in table (2).

Table (2) The correlation between serum vitamin D and retrieved oocytes maturity

	Group (I) 1(≥30 ng/ml)	Group (II) (21-29.9 ng/ml)	Group3 (≤ 20.9 ng/ml)	P value
	Mean ±SD	Mean ±SD	Mean ±SD	
MI oocyte	2.35±1.9	3±3.4	1.92±0.9	0.514*
MII oocyte	4±2.7	4.07±3.4	4.26±1.69	0.945*
GV oocyte	2.6±2.02	2.25±1.3	3.43±2.27	0.507*
Abnormal	1.92±1.6	2.83±1.17	1.8±0.79	0.277*

*Not significant, sample size =62 pt.

There is no real difference in the embryo grade according to serum vitamin D level; the mean number embryo grade (1) was (2.7±1.5) (2.6±1.3) (2.4±1.2) for groups (1), (2),(3) this relation is statistically not significant. The mean number embryo grade (2) is (1.8±1.1), (2.0±1.1), (1.3±0.5) for groups (1),(2),(3) respectively, this relation is statistically not significant. The mean number embryo grade (3) is (1.0±0.0) and (2.5±2.1) for groups (1) and (2), this relation is statistically not significant.as shown in table (3).

Table (3) The relation of serum vitamin D with the embryo grade

Embryo grade	Group (1) (≥30 ng/ml)	Group (2) (21-29.9 g/ml)	Group(3) (≤ 20.9 ng/ml)	
	Mean	Mean	Mean	ANOVA test
Grade1 embryo	2.7±1.5	2.6±1.3	2.4±1.2	0.81*
Grade 2 embryo	1.8±1.1	2.0±1.1	1.3±0.5	0.27*
Grade3 embryo	1.0±0.0	2.5±2.1		0.12*
Grade4 embryo	1	1±0	2	

*Not significant, sample size =62 pt.

Positive chemical pregnancy is more among those with sufficient group (1) vitamin D 7(25.9%), as compared with those with deficient group (3) vitamin D 4(21.1%), this relation is statistically not significant as shown in table (4).

Table (4) The relation between serum vitamin D with chemical pregnancy

Serum vitamin D	Pregnancy test		Total
	Positive	Negative	
Group (1) (≥30 ng/ml)	7	20	27
	25.9%	74.1%	100%
Group (2) (21-29.9 ng/ml)	4	12	16
	25%	75%	100%
Group (3) (≤ 20.9 ng/ml)	4	15	19
	21.1%	78.9%	100%
Total	15	47	62
	24.2%	75.8%	100%

X2=1.15, df=2, p value = 0.9 not significant, sample sizes=62 pt.

3.Follicular fluid (FF) vitamin D:

The mean of FF vitamin D is (29.6 ±10.97), as shown in table (5).

Table (5) The mean FF vitamin D levels

	Mean	SD
Right follicular fluid vitamin D	30.1	11.5
Left follicular fluid vitamin D	29.2	12.01
Mean follicular fluid vitamin D	29.6	10.97

sample sizes=62 pt. .

Table (6) shows the correlation between FF vitamin D and number of oocytes and quality(maturation) .Their results demonstrate a non-significant positive correlation between follicular fluid vitamin D level and retrieved oocytes (r= 0.5 , P=0.249), MI oocyte number (r= 0.11 , P=0.503) and MII oocyte number (r=0.201 and p= 0.128), but there is a negative non-significant correlation with GV number , and abnormal oocyte number (r= -0.022 , P=0.916), (r = -0.181 , P=0.346) respectively.

Table (6) The correlation of FF vitamin D level with oocyte number and maturation.

	Retrieve oocytes number	MI oocyte	MII oocyte	GV Oocyte	Abnormal oocyte
Pearson Correlation	0.5	0.11	0.201	-0.022	-0.181
P value	0.249*	0.503*	0.128*	0.916*	0.346*

*P value not significant, sample size =62pt.

Table (7) shows the correlation between FF fluid vitamin D and embryos grading. The results demonstrate a non-significant positive correlation between FF vitamin D level and grade (I) embryo (r=0.231, p= 0.07) , but there is no significant correlation with grade (II) (r= 0.148, P= 0.252) and grade (III) (r = 0.024, P= 0.853) embryos.

Table (7) The correlation between FF vitamin D level and embryo grade

	embryo grade 1	embryo grade 2	embryo grade 3	embryo grade 4
Pearson Correlation	0.231	0.148	0.024	0.058
P value	0.070*	0.252*	0.853*	0.653*

*P value is not significant, sample size =62 pt.

There is non-significant difference between pregnancy rate and follicular fluid vitamin D. Table (8)

Table (8) The mean FF vitamin D according to pregnancy rate

Follicular fluid vitamin D(ng/ml)	Pregnancy		P value
	pregnant	Non pregnant	
Follicular fluid vitamin D (right side)	30.5±10.2	29.9±12	0.87*
Follicular fluid vitamin D (left side)	30.02±11.7	28.9±12.2	0.70*
Mean follicular fluid vitamin D	30.3±9.9	29.4±11.4	0.80*

*P value is not significant. sample size 62 pt.

Discussion:

Age is the determinant factor in each IVF cycle as its one of the belonga criteria in definition of poor responder that affect quality of oocyte. (14;15) Also BMI has a complex relation to IVF success. BMI when it was abnormal would be an independent negative prognostic factor for numerous outcomes, like cycle cancellation, oocyte and embryo totals, and pregnancy success especially obesity of class-II/III, PCOS or ovulatory dysfunction. (14,15,16)

The influence of deficient level of vitamin D on fertility has been examined since the 1970s. (14) Table (4). Positive chemical pregnancy is more among those with sufficient group (1) vitamin D 7(25%), as compared with those with deficient group (3) vitamin D 4(21.1%), this relation is statistically not significant. These result agreed with many studies which discussed in Garbedian et al study (2013) which concluded from these studies that no considerable variations between females in the insufficient and sufficient 25(OH)D series with regard to parameters of the IVF cycle including human chorionic gonadotropin injection day , dose of gonadotropin , level of estradiol, endometrial thickness, oocytes retrieved number, or the number of embryos transferred. Thus, it is improbable that the noticed variation in rates of clinical pregnancy was due to variations in ovarian steroidogenesis. (16)

Several researches considered that vitamin D deficiency may have injurious influence on the female's infertility and IVF pregnant outcomes. However, other researchers demonstrated that deficiency of vitamin D did not function a crucial action in the ART outcome. These conflicts may be due to variations in cases' mean age, race, ethnicity, BMI, social and financial status, countries, the season, study design, exposure classification and analysis systems. Although with these conflicts, the combined findings demonstrated that deficiency of vitamin D may create negative pregnancy outcome. The cause may be effect on

the growth of follicle and embryo. In mice, in that the VDR genes have been removed, demonstrated uterine underdevelopment and damaged folliculogenesis. Vitamin D have advantageous influence on the manufacture of Insulin-like growth factor-binding protein-1 (IGFBP-1) in ovary and ovarian steroidogenesis. Vitamin D enhance the of estradiol, estrone, and progesterone production. (17)

Receptor of Vitamin D was establishing in the endometrium of mice, while vitamin D receptor malformed female mice have an hypoplastic uterus and was infertile. The 1,25(OH) 2D3 management might increase the expression of HOXA10 mRNA and protein by joining with its receptor in uterus .So HOXA10 is vital for female fecundity and pregnancy. In vitro representing early gestation ,the 1a–hydroxylase enzyme increases in the endometrial stromal cells . (18,19)

In another study done by Ciepiela etal (2018) the implantation rate was non significantly higher among women with sufficient 25(OH)D levels ($p = 0.6$); however, the rate of clinical pregnancy per embryo transfer was significantly more among females with adequate levels ($p < 0.001$). (16) These results agrees with our results regarding pregnancy rate but not number of oocyte (MI,MII,GV) .

The fate of the follicle, oocyte quality , embryo quality and subsequently on IVF outcome depends on the communication between extra-ovarian and intra-ovarian issues . (18,21)

In this study there is a non-significant positive correlation between follicular fluid vitamin D level and retrieved oocytes , MI and MII oocytes number. Also there is a negative non-significant correlation between it and GV number and abnormal oocyte number and number of oocytes maturation .The correlation between follicular vitamin D and embryos grading showed a non-significant positive correlation between them especially grade (I) embryo but there is no significant correlation with grade (II) and grade(III) embryo.

The level of 25(OH)D in follicular fluid correlates negatively with the oocytes' ability to undergo fertilization and subsequent preimplantation embryo development. Oocytes matured in it with low 25(OH)D concentration are more likely to produce top quality embryos and are associated with higher pregnancy and delivery rates. On the other hand, low serum vitamin D concentration is associated with higher miscarriage rates. (20)

Vitamin D play role in implantation and pregnancy because of immune effect by suppressing the activity of decidual T-cell and decrease levels of nearly cytokines like interleukin 1 (IL-1), IL-6 and TNF-a, that needed for implantation and uterine receptivity. It changes T helper 1(TH-1) to the more accepting TH-2. Endometrial cells yield 1a hydroxylase, which makes 25-OH vitamin D and is up-regulated by IL-1β that had been made by the blastocyst. Low level of it, increases the percentage of B cells, INF-a manufacturing T cells and NK cytotoxicity, all of the above were factors for pregnancy loss. (17)

Zhoa *et al*.,2018 concluded in their meta-analysis of multiple studies indicated that deficient vitamin D was connected with decreased casual live birth of IVF/ICSI and bad pregnancy outcome. (17) These results agreed with our results.

Conclusion:

According to the result of the study, there is no significant correlation between oocyte maturity, embryo grade and chemical pregnancy with serum and follicular Vitamin D levels.

Acknowledgment:

The researchers are thankful to all patients for their cooperation in the study. Also We acknowledged to the staff of High Institute of Infertility Diagnosis and Assisted Reproductive Technologies, embryology department at Al-Nahrain University.

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Author contribution:

The research was done by Dr.Enas Mahmood Yaseen as part of her High

Diploma (equivalent to Master degree) in Clinical Infertility and Assisted Reproductive Technologies thesis under the supervision of Assisst. Prof .Dr. Huda Al- Hussani and Assisst. Prof. Dr. Wasen Al-Jobori.

Conflict of interest:

Conflict of interest declared none.

Ethical clearance:

The study was approved by the ethical approved committee.

Conclusion:

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