

Does Vitamin D supplementation always play a beneficial role in human reproduction? Steps towards an evidence-based management

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ABSTRACT. *From the chemical point of view, Vitamin D is a fat-soluble compound that resembles steroid hormones. Indeed, it is classified as secosteroid hormone and regulates several metabolic pathways: on one hand, it concurs to maintain calcium, magnesium and phosphate homeostasis; on the other hand, it has immunomodulatory, anti-proliferative and anti-inflammatory effects in different microenvironments of the body. Despite the accumulating attention about Vitamin D supplementation in human reproduction, data published so far still remain elusive. Nevertheless, available evidence suggests that Vitamin D excess plays a detrimental role both on female and male fertility: in women, it disturbs the physiological process of oocyte maturation and embryo quality; in men, it decreases spermatozoa count, their progressive movement and increases morphology abnormalities. These data urge us to recommend avoiding "empiric" supplementation of Vitamin D without a clear indication. In addition, our critical appraisal allow us to highlight that Vitamin D should be supplemented only during the luteal phase of the menstrual cycle; considering that this Vitamin has a clear progesterone-like activity, it may play a beneficial role on endometrial receptivity and support embryo implantation during early pregnan-*

cy. On the other side, its supplementation during the follicular phase does not have any specific indication and has a negative impact on fertility outcomes.

KEYWORDS

Vitamin D, Infertility, In Vitro Fertilization, Endometrium, Ovary, Sex steroid hormone receptors.

VITAMIN D BIOCHEMISTRY: SOURCES AND METABOLISM

From the chemical point of view, Vitamin D is a fat-soluble compound that resembles steroid hormones. Indeed, it is classified as secosteroid hormone and regulates several metabolic pathways: on one hand, it concurs to maintain calcium, magnesium and phosphate homeostasis^{1,2}; on the other hand, it has immunomodulatory, anti-proliferative and anti-inflammatory effects in different microenvironments of the body³. Although Vitamin D exists in different forms, cholecalciferol (D3) and ergocalciferol (D2) are the most important in humans. In details, Vitamin D3 is produced in the skin from 7-dehydrocholesterol, in response to sunlight (UVB radiation) exposure⁴ and absorption from the gastrointestinal tract⁵; subsequently, it is transported to

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the liver via the serum Vitamin-D binding protein (VDBP), where it is hydroxylated to the pre-hormone 25-hydroxycholecalciferol [25(OH)D] by 25-hydroxylase; finally, the physiologically active form, known as 1,25(OH)₂D₃ (calcitriol), is produced within the kidneys⁶ by enzymatic hydroxylation (1 α -hydroxylase - CYP27B1) of 25-hydroxycholecalciferol. In addition, a small amount of Vitamin D can also be supplemented by the diet. Vitamin D₃ is contained especially in cod liver oil and sea fish fat, while Vitamin D₂ in plants and mushrooms. Considering that Vitamin D synthesized in the skin and its dietary and supplemental intake does not affect the regulation of hepatic 25-hydroxylase, the plasma concentration of 25-hydroxycholecalciferol is the most reliable indicator of Vitamin D body storage⁷. Conversely, both parathyroid hormone and low blood levels of phosphate are able to stimulate the expression and activity of renal 1- α -hydroxylase and, consequently, act as checkpoint for the production of the active hormone⁸. Based on these elements, it is crystal clear that the supplementation of Vitamin D may affect several biological pathways besides the "classic" regulation of calcium/magnesium levels. Indeed, data published so far about Vitamin D supplementation in human reproduction still remain elusive. For this reason, the aim of the current work is to gather all the pieces of evidence about the topic and offer a clear, evidence-based point of view. Particular attention will be paid to unhinge the current dichotomous approach that considers beneficial or not the Vitamin D supplementation, trying to elucidate the selected condition(s) in which it may play a positive effect on reproductive outcomes.

CROSS-TALK BETWEEN VITAMIN D AND HORMONAL REGULATION

1,25(OH)₂D₃ acts at nuclear level through both genomic and non-genomic fashions^{9,10}, due to its chemical structure closely similar to that of steroid hormones, and exerts its different biological functions by binding and activating the nuclear Vitamin D receptor (VDR), which belongs to the group of the steroid/thyroid/retinoid receptors¹¹ and is widely expressed in the majority of cells¹². Once activated by binding to the Vitamin D, VDR forms a heterodimeric complex with retinoid-X receptor (RXR), successively binds to Vitamin D response elements (VDREs) in the promoter regions of Vitamin D-responsive genes¹³, and finally regulates the tissue-specific expression of several genes^{10, 14-17}. As already mentioned above, VDR is also able to trigger rapid non-genomic signaling on the cell membrane and/or cytoplasm; activation of signaling molecules, such as phospholipase C and phospholipase A2 (PLA2), phosphatidylinositol-3 kinase (PI3K) and p21ras, and

the rapid generation of second messengers (Ca²⁺, cyclic AMP, fatty acids and 3-phosphoinositides such as phosphatidylinositol 3,4,5 trisphosphate), accompanied by the activation of protein kinases, such as protein kinase A, src, mitogen-activated protein (MAP) kinases, protein kinase C (PKC) and Ca²⁺-calmodulin kinase II¹⁸. Afterwards, the degradation of 1,25(OH)₂D₃ to inactive metabolites is catalyzed by VDR-dependent induction of Vitamin D-24-hydroxylase (CYP24A1)^{19,20}, one of the most important enzyme to maintain 1,25(OH)₂D₃ homeostatic levels²¹. Despite it is widely known how Vitamin D plays a pivotal role in calcium absorption as well as in maintaining adequate serum calcium and phosphate levels, the recent challenge moved to the fine characterization of its effects on cell proliferation, differentiation and apoptosis, angiogenesis, cancer invasion and modulation of immune system^{4, 22-24}. In this regard, several studies showed that Vitamin D has paramount importance in human reproduction; indeed, accumulating evidence suggests that active Vitamin D metabolites, including 1,25(OH)₂D₃, are produced by placenta and decidua²⁵; furthermore, robust results confirmed the expression of 1 α -hydroxylase and VDR receptors in the endometrium²⁶, ovary (above all in granulosa cells)²⁷, placenta²⁸ and pituitary gland²⁹. Last but not least, recent data found not only that Vitamin D can influence hormonal production of estrogens, progesterone and insulin-like growth factor binding protein 1 (IGFBP-1) in cultured human ovarian cells, but also that a hormonal-dependent regulation³⁰ is involved in VDR expression which changes during all the phases of the menstrual cycle in both human endometrium and myometrium. Taken altogether, these elements seem to underline that Vitamin D can be considered a connecting link between hormonal and metabolic cross-talk; in particular, it was already showed that Vitamin D, in association with insulin, is able to increase inhibition of IGFBP-1 production in human ovarian cells. Interestingly, IGFBP-1 production in these cells can be enhanced by Vitamin D alone²⁷. In addition, Vitamin D is able to regulate insulin receptor expression³¹ and, in this way, modulates the tissue-specific insulin sensitivity. 1,25(OH)₂D₃ has also been associated to enhanced estrogen and progesterone production in human placenta^{28,32} in a dose-dependent manner; its pivotal role for physiological pregnancy is also highlighted by the modulation of human chorionic gonadotropin (hCG) expression and secretion in cultured syncytiotrophoblasts³³. Vitamin D also seems important in preventing uterine contractions, possibly reducing the risk of preterm delivery, since VDR regulates the transfer of calcium between trophoblast and the endometrial decidua³⁴. From a molecular point of view, Vitamin D treatment leads to an increase of HOXA10 mRNA and protein expression, which is known to be funda-

mental to allow the correct embryo implantation³⁵ together with WNT/ β -catenin³⁶, in endometrium, decidua and placenta³⁷. In this regard, Vitamin D may significantly affect the outcomes of human reproduction by both regulation of calcium homeostasis and modulation of the expression of aromatase gene; starting from this background, it was recently showed that CYP19 gene (that encodes aromatase enzyme) is directly regulated by VDR-bound $1,25(\text{OH})_2\text{D}_3$ in a dose-dependent fashion³⁸. This Vitamin D regulatory mechanism on aromatase expression may be involved in estrogen homeostasis. However, another study found that Vitamin D is able to induce only minimal changes on steroidogenic acute regulatory protein (StAR), 3- β -hydroxysteroid dehydrogenase (3- β HSD) and aromatase mRNA expression²⁷, underlining the clear necessity of further investigations about this point. $1,25(\text{OH})_2\text{D}_3$ is also able to reduce significantly estrogen receptor (ER)- α , progesterone receptor (PR)-A and -B and steroid receptor coactivator (SRC) expression in human uterine leiomyoma cells³⁹. Although this antagonistic effect on sex steroid hormone receptors was regarded as potential therapeutic target in endometrial^{40,41}, ovarian²⁰, breast^{42,43} and other⁴⁴⁻⁴⁷ cancers, it can severely impair the hormonal and metabolic homeostasis in several reproductive microenvironments. Besides the increasing attention of Vitamin D's effects on female reproduction, several studies also demonstrated its role in human male reproductive tract; indeed, VDR is expressed in testicles and spermatozoa⁴⁸, where there is also an enhanced activity of CYP27B1, the enzyme necessary to form active $1,25(\text{OH})_2\text{D}_3$ ^{49,50}. The latter is able to increase the concentration of Ca^{+2} ions within the cells and it also seems to have a central role for acrosome reaction during fertilization of the oocyte, due to the increase of acrosin activity in spermatozoa in a paracrine/autocrine way⁵¹. Finally, Vitamin D stimulates calcium uptake in Sertoli cells⁵², whose secretory activities are ion channel-dependent. Considering Vitamin D as regulatory gatekeeper of the abovementioned pathways, every element that reduces or increases its quantity and/or activity may cause severe disturbance of body homeostasis. Reflecting this cornerstone on human reproduction, Vitamin D supplementation without a clear indication may alter not only the calcium/phosphorus balance, but also the fine-regulated hormonal and metabolic pathways. Despite there is still the need of further human-based investigations, recent data suggest that progesterone concentration and its biosynthesis was significantly decreased in porcine granulosa cells in response to $1,25(\text{OH})_2\text{D}_3$ ⁵³. These results should be carefully evaluated, considering that progesterone is one of the most important hormones that allow adequate endometrial preparation for embryo implant, support the development of placental framework during ear-

ly pregnancy and prevent the rejection of fetal "semi-allograft"⁵⁴. In addition, Vitamin D also seems to have a dynamic interplay with the Anti-Müllerian Hormone (AMH), one of the most reliable and accurate markers of ovarian reserve currently available^{55,56}. Recent evidence suggests that the promoter region of human AMH gene includes a VDRE⁵⁷; corroborating this point, $1,25(\text{OH})_2\text{D}_3$ seems to be able to increase the mRNA expression of AMH in human prostate cancer cell line⁵⁸. Interestingly, Wojtusik and Johnson⁵⁹ studied granulosa cells from hen and found a dose-dependent decrease in AMH mRNA levels, from 3-5 mm to 6-8 mm follicles, after treatment with Vitamin D; in addition, they observed increased FSH mRNA expression levels in response to Vitamin D. Furthermore, a human-based *in vitro* study found an inverse correlation between 25OH-D status in follicular fluid and AMH receptor-II (AMHR-II) mRNA gene expression; in particular, women with insufficient/deficient 25OH-D levels (25OH-D <30 ng/ml in follicular fluid) showed a 2-fold increase in AMHR-II mRNA expression levels in granulosa cells of small follicles (<14 mm) compared to those with 25OH-D levels ≥ 30 ng/ml in follicular fluid⁶⁰. Considering that the binding of AMH to AMHR-II suppresses follicular maturation, these data suggest that Vitamin D alters AMH production patterns and FSH sensitivity in ovarian granulosa cells⁶¹. Finally, several studies found that parathyroid hormone-related protein (PTH-rP) gene is repressed by $1,25(\text{OH})_2\text{D}_3$ ⁶²⁻⁶⁹. Considering that PTH-rP has a potent vasorelaxant activity, this element may have at least two important consequences: on one hand, high doses of Vitamin D may reduce the contribution of PTH-rP to the necessary remodeling of spiral artery during early pregnancy; on the other hand, the reduced vasorelaxant activity may play a detrimental role in preventing the onset of preterm labor. Although we should wait for more robust investigations, these data may address to speculate about a possible danger of "empirical" Vitamin D supplementation.

VITAMIN D SUPPLEMENTATION IN HUMAN REPRODUCTION: HOW MUCH AND WHEN

Based on the evidence from *in vitro* experiments and data from animal models, the challenge of understanding how Vitamin D modulates reproductive outcomes moved to human-based clinical trials and observational studies. In this regard, the most important data were obtained from *in vitro* fertilization (IVF) studies. Recently, Anifandis et al.⁷⁰ showed that women with sufficient levels of 25(OH)D in follicular fluid (>30 ng/ml) produced poorer-quality embryos and had a lower chance of achieving clinical pregnancy compared to women with insufficient (20-30 ng/ml) or deficient

(<20 ng/ml) 25(OH)D levels. Nevertheless, other authors^{71,72} failed to demonstrate a significant association between serum and follicular fluid 25(OH)D levels with IVF outcomes. It is possible that different concentrations of Vitamin D may affect the physiological process of oocyte maturation and, consequently, of embryo quality, due to the modulation of several hormonal pathways; in particular, *in vitro* Vitamin D treatment may selectively increase the progesterone production by human granulosa cells in the presence of the precursor substrate pregnenolone⁶⁰. In addition, human granulosa cells cultured with 1,25(OH)₂D₃ show a drastic and significant decrease (32%) in AMH Receptor-II mRNA levels⁶⁰; since it was already demonstrated that Vitamin D down-regulates AMH gene and up-regulates FSH receptor gene expression^{59,73}, we could likely hypothesize a strong influence on the ovarian reserve. Apart from these important cornerstones, accumulating evidence seems to suggest a selective and pivotal cross-link between glucose metabolism and Vitamin D; as reported by Anifandis et al.⁷⁰, the excess of Vitamin D in serum and follicular fluid in combination with decreased follicular fluid glucose levels have a detrimental impact on IVF outcomes. These results are partially confirmed by several investigations both on animal models^{74,75} and humans⁷⁶, showing altered oocyte cytoplasmic maturation when the glucose metabolism is severely altered in the follicular fluid. Nevertheless, all the data reported above should be carefully evaluated considering that several different conditions may influence Vitamin D actions at both ovarian and endometrial levels. It is known, for example, that Vitamin D supplementation in a cohort of patients affected by PCOS was not associated with significant changes in AMH serum levels⁷⁷. In addition, Vitamin D reserve seems to be higher in women with endometriosis, a condition characterized in most of the cases by infertility⁷⁸. Finally, physical characteristics may also severely affect Vitamin D actions: on one hand, 25(OH)D deficiency measured in single follicles was associated with a high BMI⁷⁹, which could be considered an independent risk factor for infertility^{80,81}; on the other hand, pregnancy rates were found higher in Asian women with lower serum 25OH-D levels⁸², but not among non-Hispanic white women, suggesting that these outcomes may depend by ethnicity and/or (epi)genetic background. If we consider together these strong pieces of evidence, we may hypothesize that Vitamin D actions in human reproduction run in two different rails; whereas IVF trials clearly suggest that an excess of this Vitamin severely impairs oocyte development and embryo quality, less is known about its action on endometrial receptivity. Although data are still elusive, it is particularly interesting that Vitamin D deficiency and insufficiency was associated with lower pregnancy rates even in recipients of

egg donation⁸³; considering that during egg donation all the follicles should be of good quality, this element allows us to assume that Vitamin D deficiency exerts a detrimental action on endometrial receptivity, which accounts for the observed lower pregnancy rate. On this basis we may speculate that Vitamin D supplementation should be carefully planned during the menstrual cycle (i.e. carried out only during the luteal phase and not during the follicular phase). The alteration in Vitamin D concentrations is also likely to have a dangerous effect on male fertility; indeed, it was demonstrated that both low (<50 nmol/L) and high (>125 nmol/L) Vitamin D serum concentrations decrease not only spermatozoa count but also their progressive movement, and increase morphology abnormalities⁸⁴⁻⁸⁶. In addition, the molecular similarity between VDBP and antisperm antibodies⁸⁷ allows us to raise further concerns about this point. Finally, experiments from animal models found that Vitamin D is transferred through the placental barrier; in case of excess this Vitamin reduces the total skeletal calcium store and causes severe consequences in the offspring⁸⁸⁻⁹⁰.

CONCLUSIONS

Despite the continuous efforts, there is still a lack of robust evidence about the role of Vitamin D supplementation to support human reproduction. This foggy scenario urges us to solicit further studies about the topic and, most important, to avoid “empiric” supplementation of Vitamin D without a clear indication. Based on the available evidence, Vitamin D excess plays a detrimental role both on female and male fertility. In this regard, we recommend to follow international consensus guidelines and supplement Vitamin D only if serum concentration falls below 50 ng/ml (equivalent to 125 nmol/L)⁹¹.

In addition, our critical appraisal allow us to highlight that Vitamin D should be supplemented only during the luteal phase of the menstrual cycle; considering that this Vitamin has a clear progesterone-like activity, it may play a beneficial role on endometrial receptivity and support embryo implantation during early pregnancy. On the other side, the supplementation during the follicular phase does not have any specific indication and seems to have a detrimental effect on oocyte quality and fertility outcomes.

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KEY POINTS:

- “Empiric” supplementation of Vitamin D without a clear indication should be avoided.
- Vitamin D excess plays a detrimental role both on female and male fertility.
- Robust data about Vitamin D supplementation during IVF suggest an opposite effect on the follicular and luteal phase of the menstrual cycle.
- Vitamin D seems to provide positive effects when supplemented in condition of luteal deficiency, improving endometrial receptivity.
- An excess of Vitamin D impairs oocyte development and embryo quality.

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