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Vitamin D as an Immunomodulator in Pregnancy - an Observational Cross-Sectional Study

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ABSTRACT

Introduction: Serum cholecalciferol levels are known to be insufficient in 89% of the women during gestation. Serum 25 dihydroxycholecalciferol has been a key marker for sustaining pregnancy, it has been noted that the levels are highly deficient in first trimester while it reaches sufficient limits in second and third trimester. Placentalsecretion compensates for the deficient serum cholecalciferol level with its secreting placental receptors. Serum 25 dihydroxycholecalciferol levels have been noted to reduce considerably in high-risk pregnancies like gestational diabetes mellitus and preeclampsia. Placenta is also the greatest immunomodulatory organ that prevents the effect of maternal immune responses on the semi-allogeneic foetus. The normal placental tissue has been compared with the vitamin D deficient placenta in high-risk cases like preeclampsia and gestational diabetes mellitus Immunohistochemically. The expression of CD4, (Cluster of Differentiation 4), CD8(Cluster of differentiation 8), FoxP3 (forkhead box P3) and VDR (Vitamin D Receptor) has been studied immunohistochemically. The study reports VDR (Vitamin D Receptor) directly proportional to the expression of CD8 while indirectly proportional to FoxP3 positive (T regulatory cells). There is no significant relationship observed with VDR and CD4 positive cells. The results prove the relationship of Vitamin D Receptor (VDR) and T regulatory cells (foxp3) immunomodulatory antibodies. High risk Pregnancies show considerable deficiency in serum cholecalciferol with increased vitamin D receptor expression, increased CD8 expression and reduced FoxP3 expression (T regulatory cells).

Keywords: Calcifediol; Vitamin D binding protein; Placenta; Fork head box P3 protein.

Introduction

Serum 25 Dihydroxy cholecalciferol is a fat based steroid secretion that is produced by the body from external resources. Serum 25 dihydroxycholecalciferol is the storage form of vitamin D, this gets activated to 1, 25 dihydroxycholecalciferols (calcitriol) in kidneys by the enzyme 1 alpha hydroxylase. 1, 25 dihydroxycholecalciferol levels are maintained within normal limits even while there is deficiency in storage form. Hence measurement of 25 hydrochycholiclaciferol gives the clear vitamin D status of a person[1,2]. In pregnancy the conversion or activation of 25 dihydroxycholecalciferol occurs in placental tissue also. Vitamin D has proved play a crucial role in implantation, cytokinine productions, prevention of infections as well as foetal rejection[3].

Foetus absorbs the 2 dihydroxycholecalciferol (storage form of vitamin D) and activates it in the foetal kidney[4]. The foetal bone mineralisation and calcium homeostasis depends on the circulating storage from of Maternal 25 dihydroxycholecalciferol levels[5]. Serum 25 dihydroxycholecalciferol level has been associated with low birth weight and various complications. Since it is s a steroid hormone that plays a major role in calcium metabolism as well as non-bone health functions. Ultraviolet light from the sunlight acts on the subcutaneous fat converting it into 7 hydroxycholecalciferols, this is stored in liver as 25 hydroxycholecalciferols. The 25 hydroxycholecalciferol is storage form of vitamin D, this is activated into 1,25 dihydroxycholecalciferol in the kidneys[6] by the 1 alpha hydroxylase in the kidney. Assessment of 25 dihydroxycholecalciferol (storage form) gives the vitamin D status of a person[7,8]. This study focuses on the serum 25 dihydroxycholecalciferol levels in various trimesters. Serum Cholecalciferol levels are correlated with the placental immunohistochemistry showing the distribution of Immune cells (CD4, CD8, FoxP3) cell mediated immune cells.

Methods

The study was certified by Institutional review board and Institutional ethical committee (Approval no, IEC 2020/590, Ref, IRC/005/2019). It was conducted between 2016-2020. The participants were in the reproductive age 20-40 years; singleton pregnancy was included for observation. Prior consent was obtained from the participants. The study was an observational cross-sectional study hence the sample size was calculated based on the formula $Z_{1-\alpha/2^2}$ (1-p)/$d^{2.9}$.

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Sample Calculation
$N=4pq/L^2$
P is 46% (serum vitamin D)
L is Precision error (15%)
Q= is 100-46=54
N=4*46*54/15*15
N=9936/225
4+16+10% non- response
44.16+4.16= 48.58
N=50

There were 150 participants 50 belonging to the control group and 100 belonging to the experimental group. Women of the reproductive age group from 20-40 years were included in the control group. The experimental group included 33 participants from first trimester, 33 participants from second trimester and 34 participants from third trimester. 50 participants belonging to the control group were non pregnant women of the same age group. The serum was analyzed for 25 dihydroxycholecalciferol level during different weeks of gestation. If the participant was found deficient, they were given counseling on diet supplements and treated with vitamin D and were excluded from the study. This was done to analyze the exact serum level of 25 dihydroxycholecalciferol in gestation. When the deficiency was noted, the participant was supplemented and serum cholecalciferol levels after supplementation was not included in the analysis. Placenta from the third trimester participants were collected after prior patient consent.

25 dihydroxycholecalciferol level deficient placentas were included in the experimental group and normal vitamin d level placenta was considered control group for the placental study.

Blood samples from participants were collected and serum 25 dihydroxycholecalciferol, serum magnesium, serum phosphorus and serum calcium were determined.

The participants were supplemented if there was acute deficiency. Those supplemented participants were not included further in evaluation.

Venous blood was used (with clot activator) for 25 estimated by the Chemiluminescence ImmunoAssay (CLIA) method. The method had been fully automated, high throughout immunoassay system. The machine used was SIEMENS ADVIA Centaur® XP. Serum levels of vitamin D were classified according to severity[10].

Placenta Morphometric and Immunohistochemistry

Placenta from high-risk pregnancies like Gestational Diabetes Mellitus, Pre-Ecclampsia were also included after checking their serum 25 dihydroxycholecalciferol levels. Placental tissue was examined for the size, weight, odour, completeness, vascular pattern, umbilical cord position. Cotyledon size, number, accessory lobes, haemorrhage, tumours, nodules were noted.

Umbilical cord

Length, vessels number, Wharton's jelly, knots, colour, lustre, odour was noted and recorded. These parameters were compared in a serum 25 dihydroxycholecalciferol deficient placenta and normal placenta.

Immunohistochemistry

Placental specimens were collected, washed in sterile water fixed in 10% formalin and section were taken in the foetal end, maternal end and umbilical cord region.

Paraffin-embedded, 3-µm tissue sections were mounted onto Super Frost slides (Dako Denmark), deparaffinised in xylene and ethanol of graded concentrations.

For antigen retrieval, the slides were prepared using microwave oven in a solution of TRS (Target Retrieval Solution, High pH, Dako, Denmark) for 30 min (2 × 6 minutes 360 W, 2 × 5 180 W, 2 × 4 minutes 90 W). After cooling down at room temperature, they were transferred to 0.3% hydrogen peroxide in methanol, for 30 min, to block endogenous peroxidase activities.

Sections were rinsed with Tris-buffered saline (TBS, Dako, Denmark) and incubated from 30 to 60 min with monoclonal mouse primary antibodies against, CD4 (Dako; clone, 4B12, dilution 1,40), CD8 (Dako; clone, C8/144B, dilution 1,50), Foxp3 (Abcam; clone, 236A/E7, dilution 1,50) and VDR (Vitamin D Receptor) receptor 1,200 dilution (table 1).

Table 1. Ultrasound observations.

BPD	Normal	Vitamin D Deficient
Occipitofrontal diameter (mm)	73.5±2.12	69±7
Head circumference (mm)	214.5±4.94	210±25
Abdominal circumference (mm)	194.5±19.09	180±30
Femur length (mm)	42±0.001	39±4
Occipitofrontal diameter (mm)	73.5±2.12	69±7
Head circumference (mm)	214.5±4.94	210±25

Ultrasound

Ultrasound measurements were collected from the participants. The fetal anthropometric measurements were collected from the ultrasound findings. Serum cholecalciferol levels were correlated with Bi parietal diameter, head circumference abdominal circumference and femur length was measured.

Observations

The mean serum 25 dihydroxycholecalciferol levels in first trimester were 50.87ng/ml.

There was 40% deficiency noted in the first trimester. 60% of the participants in first trimester showed

insufficiency of vitamin D while standard deviation of 9.9. None of the participants in first trimester had vitamin D within sufficient limits. While in second trimester the mean serum vitamin D level was 54.05 ng/ml. 33.3% of the participants were in the deficient limits, 60% of the participants in the insufficient levels while 6.7% were in the sufficient limits. Standard deviation in second trimester was 13.18.

During third trimester the mean serum vitamin D in third trimester was 61.24ng/ml. 27% participants were in deficient levels. 46% insufficient levels while 27% are in the sufficient level. Standard Deviation was 22.44.

It was observed that the mean serum vitamin D was observed to be low in the first trimester and gradually increased in the second and third trimester. While serum calcium, magnesium and phosphorus levels were within normal limits. Participants with deficient serum dihydroxy cholecalciferol levels were seen in higher in first trimester rather than second and third trimester. While percentage of sufficient vitamin D values for increased in third trimester. The participants with normal serum 25 dihydroxycholecalciferol levels placenta was studied and compared with placenta from deficient 25 dihydroxycholecalciferol levels.

Calculation of Standard deviation
First trimester

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2},$$

Standard Deviation, s, 9.99

Count, N,	15
Sum, Σx,	763
Mean, x̄,	50.86
Variance, s^2,	99.92

Second Trimester

Standard Deviation, s, 13.18

Count, N,	15
Sum, Σx,	810.85
Mean, x̄,	54.05
Variance, s^2,	173.79

Third Trimester

Standard Deviation, s, 22.44

Count, N,	15
Sum, Σx,	918.15
Mean, x̄,	61.21
Variance, s^2,	503.90

Ultrasound findings

The ultrasound findings were recorded to study the foetal biometry. Serum 25 dihydroxycholecalciferol levels were correlated with foetal biometry to assess the foetal skeletal growth. Placental VDR was a predictor for Femur length and can be positively correlated with maternal to foetal transfer. Biparietal width of the foetus was directly proportional to the vitamin D status of the mother. Abdominal circumference was also directly proportional. Head circumference and femur length was not significantly correlated with serum 25 dihydroxycholecalciferol.

Placenta

Placental tissue was examined for the size, weight, odour, completeness, vascular pattern, umbilical cord position. Cotyledon size, number, accessory lobes, haemorrhage, tumours, nodules to be noted. Placental VDR(vitamin D Receptor) expression was inversely associated with serum 25 OH vitamin D levels (table 2).

Umbilical cord

Length, vessels number, Wharton's jelly, knots, colour, lustre, odour was noted and recorded. These parameters were compared in a serum 25 dihydroxycholecalciferol deficient placenta and normal placenta (table 3).

Table 2. Morphology of placenta in vitamin D deficient and normal cases.

S. N°	Placental Morphology	Observations
1	Shape	No variations noted
2	Size	No considerable variation
3	Consistency	Same as normal placenta
4	Completeness of placenta	Completeness noted
5	Accessory lobe	Noted in a preeclampsia case
6	Placental infarcts, haemorrhage, synctial knots	synctial knots noted in preeclampsia term case placenta

Table 3. Measurements of umbilical cord.

S. N°	Umbilical Cord	Vitamin D Deficient Cases	Control Group
1.	Length and diameter	50 – 55 cm in length and 12 mm in diameter.	60 cm in length and 12 mm in diameter.
2	Insertion	Normal	normal
3	Number of Vessels	Number normal, narrowed vessels in severe vitamin D deficient	Normal pattern
4	Wharton's Jelly	Normal	Normal
5	Colour	Normal	Normal
6	Lustre	Normal	Normal
7	Odour	No changes in odour	No changes in odour

Control Group

The serum 25 dihydroxycholecalciferol levels of control group were also in the insufficient levels. The control group were participants in the reproductive age group who were not pregnant.

The commonest morphological changes involved includes Villous immaturity, Villous fibrinoid necrosis, Improper vascular maturity –Pre ecclampsia cases, Syncytial Knots etc (table 4).

Table 4. Foetal Biometry measurements

BPD	Head Circumference	Abdominal Circumference
Occipitofrontal diameter (mm)	73.5±2.12	69±7
Head circumference (mm)	214.5±4.94	210±25
Abdominal circumference (mm)	194.5±19.09	180±30
Femur length (mm)	42±0.001	39±4
Occipitofrontal diameter (mm)	73.5±2.12	69±7
Head circumference (mm)	214.5±4.94	210±25

Results

Serum 25 dihydroxycholecalciferol levels of blood was reported to be 88.8% insufficient in pregnancy (south Indian population). Measuring 25 dihydroxycholecalciferol level gives the true vitamin D status of a person. It was reported to be lowest in the first trimester (less than 20nmol/L) gradually increasing in the second and third trimester. Third Trimester the serum 25 dihydroxycholecalciferol levels reach sufficient levels (50-70nmol/L). But the active form of vitamin D 1,25 dihydroxycholecalciferol levels was maintained normal throughout pregnancy. Low level of 25 hydroxycholecalciferol level did not disturb the serum calcium, phosphorus and magnesium levels.

Week wise observation

Serum Cholecalciferol levels were lowest in the week 6-12. Gradually increased from 13 - 24th week. From 25th - 40th week it is maintained in sufficient limits. Vitamin D is secreted by the placental tissue during the second and third trimester by the placental tissue itself. Serum alkaline phosphate, a surrogate marker of vitamin D deficiency, cannot be used as such in pregnancy, because of the placental secretion of this enzyme (figure 1).

Immunohistochemistry

- Immunohistochemistry staining is based on IRS score (Immunoreactive Score) calculated by multiplying staining intensity (grading of staining intensity.
- None
- Weak - 1 10% cell
- 2 – up to 50% of cells
- 3 – up to 80% of cells
- 4 – up to 100%

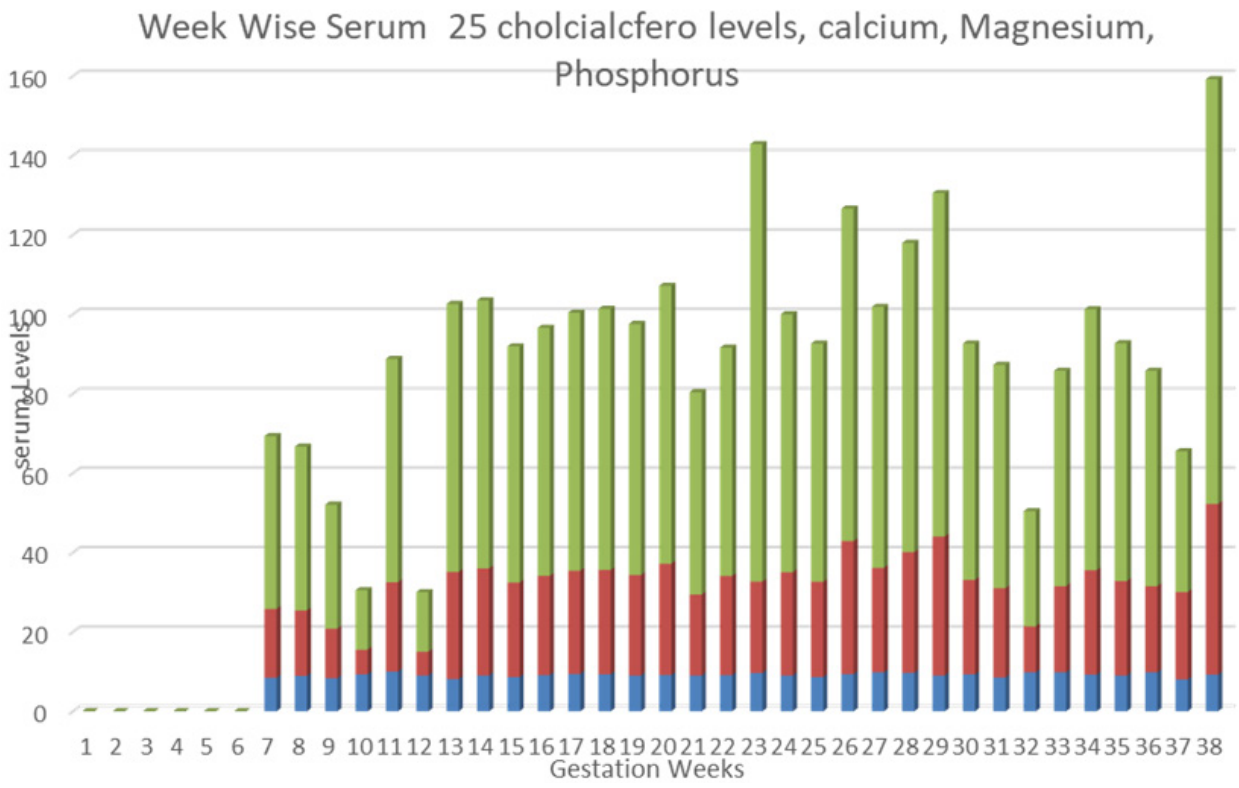

Figure 1. Week wise serum cholicalciferol levels observed during the entire gestation.
Blue: Serum Phosphorus; Orange: Serum Calcium; Green: Serum 25 hydroxy Cholecalciferol.

CD4 Expression

Immunohistochemically changes include expression of CD 4, CD8 and FoxP3 expression in the placental tissue. CD 4 positive cells are inconspicuous in all cases with greater positivity for Hofbauer cells. These cells are protective against infections the develop to natural killer cells. Its mandatory for maternal and fetal protection against infections, pathogens. The strength of these cells areincreased in first trimester placenta. While shows strong positivity in Gestational diabetes mellitus placenta as well as Pre Ecclampsia placenta (figure 2a and 2b) (table 5).

CD8 Expression

CD 8 expression is usually weak in normal pregnancy. While it is strongly expressed in vitamin D deficient Gestational Diabetes Mellitus or preeclampsia. CD 8 Expression Is Mild In First Trimester Aborted Specimen. CD 8 positive cytotoxic cells when increase can induce abortion in seen increased in number (figure 3a,3b).

FoxP3 Expression

Fork head box P3, also known as scurfin, is a protein involved in immune system responses. A member of the FOX protein family, FOXP3 appears to function as a master regulator immune cell. It is a key nuclear stain for T regulatory cells. T regulatory cell count has been an effective marker for vitamin D deficiency pregnancy. FoxP3 cells decrease in high risk pregnancies like gestational diabetes mellitus and Preeclampsia with vitamin D deficiency. Hence Foxp3 positive cells can used as an effective marker for sustaining pregnancy along with progesterone (figure 4).

Vitamin D receptor Expression

Vitamin D receptor protein expression in increasingly found in vitamin D deficient cases, while it has been found with mild expression in normal placenta (Figure 5).

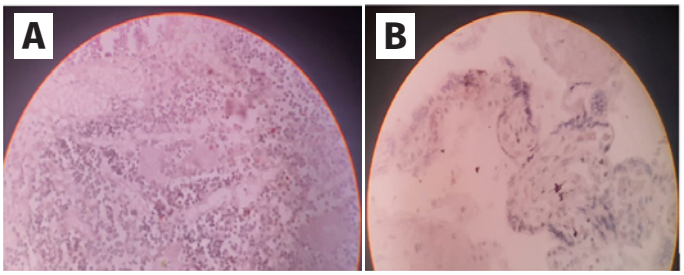

Figure 3. A) Strong Expression of CD 8 in gestational diabetes mellitus. B) Weak Expression of CD8 in normal placenta.

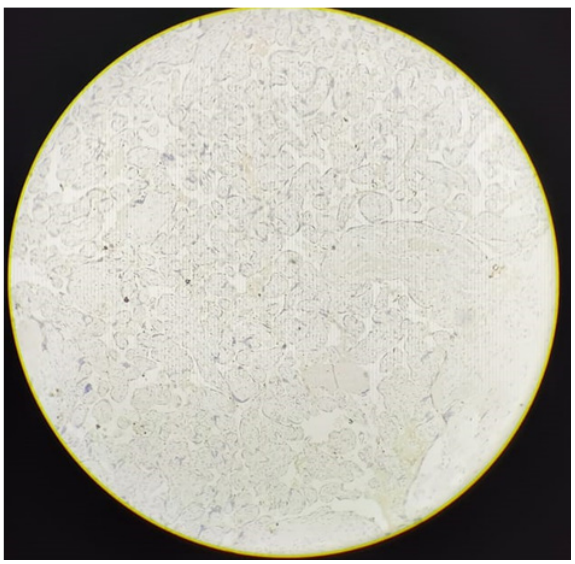

Figure 5. A VDR expression in normal placenta

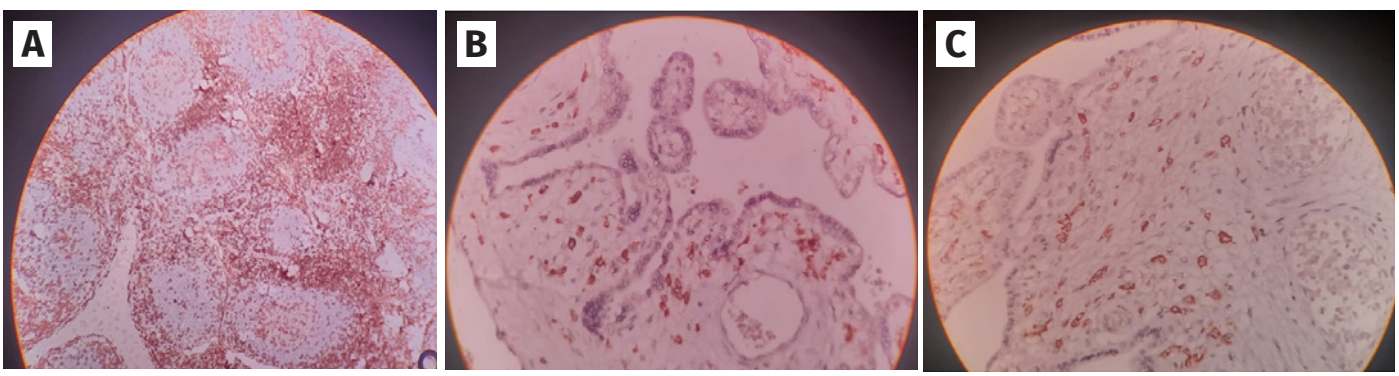

Figure 2. A) CD4 10-week aborted placenta (first trimester). B) CD 4 aborted placenta 12 weeks. C) Expression of CD4 in Vitamin D deficient Gestational diabetes full term placenta

Table 5. Immunohistochemistry of placenta, expression of CD4, CD8 and Foxp3 antibodies.

VDR(vitamin D Receptor)	Vitamin D receptor protein is weakly expressed in normal placenta while its strongly expressed in vitamin D deficient placenta
CD4 (Cluster of Differentiation)	Strongly positive in normal and vitamin D deficient placenta
CD8 (Cluster of Differentiation)	Less positive in normal pregnancy and increased expression in vitamin D deficient gestational diabetes mellitus and preeclampsia
FoxP3	FoxP3 positive cells are reduced in high-risk pregnancies while strongly expressed in normal placenta

Discussion

Pregnancy is an altered physiological state. The semi allogenic fetus is tolerated by the mother's womb due to the alterations in the decidua and placental immune tolerance[11]. The study has established the relationship of 25 hydrochycholiclaciferol on immunotolerance. Serum 25 hydroxycholecalciferol not only maintains serum calcium level but also sustains pregnancy. Serum 25 dihydroxycholecalciferol levels differ in various trimesters. It has been reported very low in the first trimester than second trimester and third trimester[12]. The vitamin D levels was decreased in high-risk pregnancies like pre-Ecclampsia, gestational diabetes mellitus etc. Protection against maternal immunity was regulated by vitamin D, there by acting against maternal micro chimerism after delivery[13,14]. Sperm entry into the female genital tract is prevented by female immune system while the immune rejection is masked by SLEX (N-glycans terminated with multivalent Sialyl-Lewis^x sequences)is also a ligand for, an immunoglobulin-like lectin that carries an immunoreceptor tyrosine-based inhibitory motif (ITIM) that generates an inhibitory signal in several immune cell populations[15]. There by fertilization and implantation occurs.The maternal Decidua contains CD4 and CD 8 positive immune cells. Their ratio remains less in the decidual plate when compared to the peripheral blood[16]. Balance between CD4 and CD8 is reduced during implantation. This is because of activation of Dendritic Cells to produce T regulatory type of cell with anti-inflammatory role th2 cells (T helper cell type-2). The Th2 cells are anti-inflammatory cells with strong ability for foetal tolerance[17]. T effector Cell (CD8+) belonging to maternal immune system is masked.

Placenta

Placenta is the greatest immunomodulator organ. Its complete development is in 12th week of gestation. The trophoblastic layer is the layer that comes into contact with the maternal immune system at the feto-maternal interphase. Syncytotrophoblasts inhibits maternal immune rejection[18]. The placenta promotes tolerance of the semi-allogeneic fetus while protecting against vertical transmission of infections[19]. The maternal-fetal interface is composed of the maternally derived decidua and the foetally derived placenta. The placenta develops from the trophectoderm of the blastocyst. Time is critical for pregnancy to occur.

Implantation occurs successfully if it occurs before pre-decidualization of endometrium. Maternal leucocytes remodel and accommodate the implantation of fetus[20]. Cross signaling between the fetal trophoblasts and maternal leucocytes dilate the spiral arteries increasing maternal blood volume in the villi. Placental barrier is formed by the Syncytotrophoblasts which is continuous multinucleated cell layer that forms the first layer. Cytotrophoblasts are developed from cytotrophoblast progenitor layer into extravillious trophoblasts and cytotrophoblasts. First trimester, the human placenta is hemochorial, it has two layers of trophoblasts separating the fetal and maternal bloodstreams (the syncytotrophoblasts and cytotrophoblasts)[21]. Second and third trimester placenta contain only single layer of Syncytotrophoblasts[22]. Maternal immune system is masked or tolerance is induced by the change in the balance between Th1 (T helper type 1 cell) and Th2(T helper type 2 cell) cells. Serum vitamin D is an important factor for immunomodulator for sustaining pregnancy and fetus rejection.

Vitamin D as an immunomodulator

Vitamin D is activated by the immune cell panel including the macrophages and T lymphocytes. The levels of 25 dihydroxycholecalciferol levels have been associated with disturbances in fertility. It has evolved to be a crucial marker for infertility. Serum cholecalciferol levels in third trimester have been correlated with the expression of CD4, CD8 and Foxp3 antibodies expression. The Vitamin D receptor protein distribution was also studied. There is clear evidence that CD 4 expression appears same in the normal and vitamin D deficient placenta. While the Effector cell expression (CD8 expression) is reduced in normal placenta while increased in vitamin D deficient placenta. The Gestational diabetes and Preeclampsia placenta showed considerable increase in the CD 8 expression. The key marker for immunomodulation is the Foxp3 expression in placenta which increased in normal placenta while it decreased in high-risk group with vitamin D deficiency. Foxp3 is a marker for T regulatory cell which sustains pregnancy. The vitamin D receptor protein is inversely proportional to the serum levels of 25 dihydroxycholecalciferol. Trophoblasts serve as first line of defense preventing infections from invading the placenta[21,22]. Seventy percent of decidual leukocytes are NK(natural killer cells), 20–25% are macrophages and 1.7% are dendritic cell[23].

Vitamin D supports placental development and its functions. It is known to support decidualization, blastocyst implantation, modulate immune cells and inhibit Th1 cell activity at the site of implantation [23].

Progesterone is a natural immune suppressor (sustains pregnancy). Serum cholecalciferol is also a natural immunosuppressor that sustains pregnancy especially in first trimester. Deficiency in first trimester can lead to improper development of placenta leading to conditions like preeclampsia. T regulatory cell which is a type of th2 type of anti-inflammatory cell that masks the maternal immune system from rejecting the fetus. This cell is directly proportional to the maternal serum 25 dihydroxycholecalciferol levels.

The signals originated in the placenta will modulate the way the maternal immune system will behave in

the presence of potentially dangerous signals. The definition of pregnancy as a Th-2 or anti-inflammatory state was enthusiastically embraced, and numerous studies attempted to prove and support this hypothesis. This theory postulates that pregnancy is an anti-inflammatory condition.

Conclusion

The T regulatory cell expression is reduced in vitamin d deficient high-risk cases like gestational diabetes mellitus and preeclampsia. Serum Cholecalciferol levels are in the deficient limits in 30 percent of the antenatal cases while insufficient in 89% of the cases. Very low levels on 25 dihydroxycholecalciferol levels are associated with defective placental development as well as congenital anomalies. T regulatory cells are an effective marker to identify vitamin D deficiency as well as high risk pregnancy.

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