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Placenta - Immunomodulatory Organ

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ABSTRACT

Introduction: vitamin D is a steroid based vitamin with various functions. It is a hormone that helps in pregnancy adaptations in vitamin D metabolism.

Objectives: to study the serum levels of 25 hydroxycholicalciferol levels and correlate it with expression of vitamin D receptor, CD4, CD8 and Foxp3 cell expression in full term placental tissue.

Methods: 50 Pregnant women with mean age 29.5±2 south Indian population were included in the study. Serum was collected for their routine pregnancy screening was utilized for estimation of 25 hydroxycholicalciferol during 33±4 weeks. The placental tissue of the same patient was collected after getting prior consent. CD4, CD8, Foxp3, VDR expression was studied by immunohistochemistry.Control group included 25 samples.

Results: there was CD4 expression observed without any evident change in the normal and vitamin D deficient placenta. While cytotoxic CD8 cell expression was increased in high risk pregnancies like, preeclampsia, gestational diabetes mellitus and sparsely observed in normal pregnancies.Foxp3 cells were sparsely expressed in vitamin D deficient placenta when compared to the normal placenta. Vitamin D receptor protein was also sparsely distributed in vitamin D deficient placenta when compared to the normal placenta.

Conclusion: maternal Serum 25 (OH) D was low in high-risk cases and it was within normal range for other pregnant cases. Vitamin D was directly proportional to the expression of FOxP3 cells (T regulatory cell expression).

Introduction

Pregnancy is an altered physiological adaptation in calcium homeostasis and vitamin D metabolism. Vitamin D is known for its calcium metabolism. The demand for vitamin D also varies in pregnancy. The synthesis and activation of vitamin D is utilized for maternal demand. Fetus utilizes 25 hydroxycholicalciferol from maternal sources and activates it to 1,25 dihydroxycholecalciferol in the fetal kidneys. Pregnancy adaptations in vitamin D is less understood. Most of the studies reveal that storage form of 25 dihydroxycholecalciferol remains stable. Vitamin D binding protein expression increases in deficient cases[1]. Intestinal calcium absorption doubles during pregnancy[2]. The increase in demand is due to fetal utilization. Studies reveal that 1,25, (OH)D does not contribute to the gestational increase in calcium absorption, PTH which is the co factor in maintaining 1,25, (OH)D during pregnancy. PTH has been identified as a marker for pregnancy. PTH has also shows the vitamin D status during pregnancy. This study focuses on the correlation between the serum 25 dihydroxhycholicalciferol and its role in expression of CD4, CD8, Foxp3 on the placental tissue. The study assesses the serum 25 (OH)D levels throughout pregnancy with the vitamin D receptor expression on the placental tissue.

Materials and Methods

The study involves serum cholecalciferol levels and placenta of the mother from whom serum cholecalciferol levels were measured and utilized for immunohistochemistry. The mean age of the study participants includes 25±2. The study involves two groups: a control group and an experimental group. 25 serum samples in the third trimester pregnancy with normal cholecalciferol levels were included in the control group and 50 samples of mothers in third trimester with cholecalciferol deficiency were included in the experimental group. Serum samples were analyzed to estimate the 25 hydroxy cholecalciferol levels. They were included in the control group if the values were normal and included in the experimental group if the cholecalciferol values were deficient.

Placental examination

Placenta of the medical termination of pregnancy was collected with prior consent and approval from ethical clearance board. Placenta from high-risk pregnancies like Gestational diabetes mellitus, pre-eclampsia 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, numbers, accessory lobes, haemorrhage, tumours, nodules to be noted.

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Umbilical Cord

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

Gestational age,Weight, Shape, Number and size of the cotyledons, Vascular pattern, Location of umbilical cord and variations in its placement. IHC of the placental tissue with antibodies CD3, CD4, CD8, Foxp3, CD20, VDR.

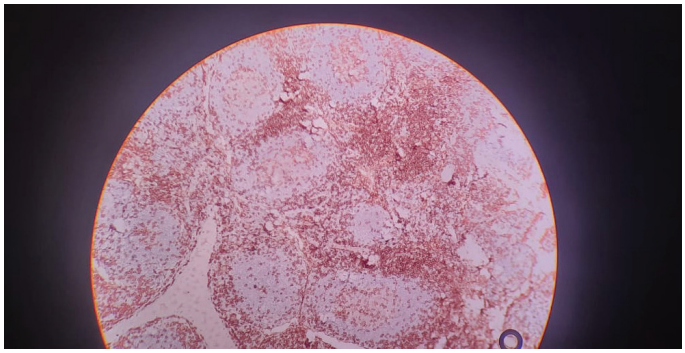

Figure 1

Table 1. Parameters studied in the placental tissue.

Placental Tissue	Umbilical Cord	Immunohistochemistry
⇨ Size ⇨ Shape ⇨ Consistency ⇨ Completeness of the placenta should be determined, and ⇨ The presence of accessory lobes, ⇨ Placental infarcts, ⇨ Haemorrhage, tumours and nodules should be noted.	⇨ Length ⇨ Insertion ⇨ Number of vessels, ⇨ Knots ⇨ Presence of Wharton's jelly. ⇨ The colour, luster and odour of the foetal membranes examined	⇨ Antibodies used: CD4, (T cell panel) ⇨ CD8 -Cytotoxic T cells ⇨ Foxp3 (T regulatory panel) ⇨ Vitamin D Receptor

Methods

Venous blood was used (with clot ac tivator) for 25 (OH) D. These were sent to the biochemistry laboratory of the hospital after labelling the vials with the patient's name and (IP) number.After allowing the blood to settle for 10-15 minutes, it was centrifuged, serum was separated and used for vitamin D estimation. The samples were stored at 40 C until analyzed if it had to be preserved for a few days. Vitamin D levels were estimated by the chemiluminescence immunoassay (CLIA) method. The method had been fully automated, high throughput immunoassay system. The machine used was SIEMENS ADVIA Centaur® XP. Serum levels of vitamin D were classified according to severity.Based on the serum cholicalciferol levels the placental tissue was collected. Placental specimens were collected, washed in sterile water fixed in 10% formalin and section were taken in the fetal end, maternal end and umbilical cord region. Paraffin-embedded, 3-µm tissue sections were mounted onto SuperFrost slides (Dako Denmark), deparaffinized in xylene and ethanol of graded concentrations. For antigen retrieval, the slides were treated in a 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 receptor 1:200 dilution

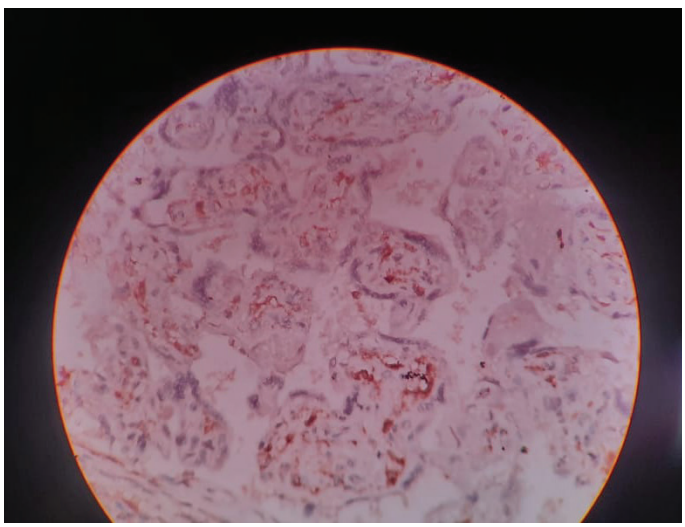

Figure 2

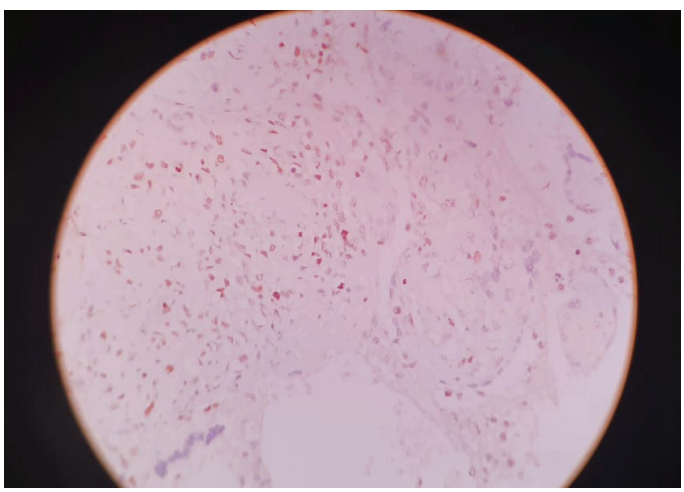

Figure 3

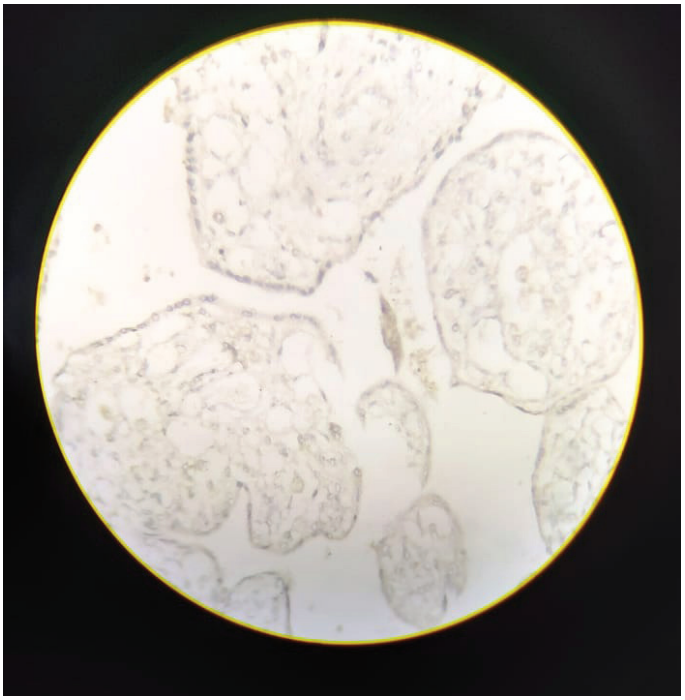

Figure 4

Observation

The study groups were divided into the control group and the experimental group. Placental tissue of participants with normal serum 25 dihydroxycholecalciferol was taken as control for placental examination. Placental tissue collected from mothers with deficient serum cholecalciferol levels were included in the experimental group.

Placental morphometry

Mean serum cholecalciferol levels in control group was mean serum vitamin D levels: 59.75nmol/L.

Immunohistochemistry

The placental tissue collected from the control group mother and vitamin D deficient mothers were sectioned, fixed and paraffin blocks were prepared. The slides were stained immunohistochemically with CD4, CD8, FoxP3 antibodies and Vitamin D receptors. The CD 4 antibody staining was seen strongly positive in both the control group and experimental group placental sections. The CD4 cells are T helper cells which are present in the placental villous. The slides are view under 1000x magnification. 20 field areas were observed. The cells cytoplasm was stained dark brown and nucleus from pale blue to dark blue.

CD 8

The section of control group and experimental group showed sparsely stained cells or less number of postive cells in normal placenta while the CD8 cells were strongly postive in vitamin D deficient placenta in the experimental group. The Pre eccamplsia and gestational diabetes mellitus speciemens showed CD8 strongly positive.

FOXp3

T regulatory cells are specific types of immunosuppressant cells present in the endometrium of the gravid utreus. These cells are required for implantation and sustaining of pregnancy. These cells were reduced in the vitamin D deficient placenta (experimental group). While it was strongly expressed in normal placenta.

Vitamin D receptor

Vitamin D receptor distribution was increased in normal placental tissue when compared to the

Table 2. Placental morphometry of normal and vitamin D deficient placenta.

S.nº	Placental Morphometry	Normal	Vitamin D Deficient Placenta
1	Shape	Normal	Normal
2	Size	No variation	No variation
3	Consistency	normal	Normal
4	Completeness of placenta	noted	noted
5	Acessory lobe	Not seen	Noted in preecclamptic cases
6	Placental hemorrhage, syntial knots	Not seen	Noted in preeclamptic cases

Table 3. Umbilical Cord morphometry of normal and vitamin D deficient palcenta.

S.nº	Umbilical Cord Morphometry	Normal	Vitamin D Deficient Placenta
1	Length	50-60 cm	Normal length 50 –60cm
2	Number of vessels in the cord	3	3
3	Arrangement of the blood vessels	Normal	Normal
4	Whartons jelly	Normal	Normal
5	Colour, odour	Normal	Normal

Table 4. Serum Cholicalciferol Levels in Third Trimester pregnancy tabulated.

Pt.id*	Age	Serum Calcium	Vitamin D 25(OH)D	Gestational Week
1	24/F	7mg/dl	56.25ng/ml	37 weeks
2	21/f	6.5mg/dl	59.5ng/ml	36 weeks 4 days
3	28/f	10mg/dl	52ng/ml	40 weeks 2 days
4	28/F	8.2mg/dl	44.25ng/ml	39 weeks 5 days
5	24/F	7mg/dl	34.75ng/ml	38 weeks
6.	25/F	7.8mg/dl	55.5ng/ml	36 weeks 6 days
7	25/f	8.9mg/dl	51ng/ml	35weeks 3 days
8	22/F	8.3mg/dl	65.75ng/ml	32 weeks
9	22/F	9.4mg/dl	54ng/ml	29 weeks 6 days
10	23/F	9.2mg/dl	36ng/ml	31 weeks 3 weeks
11	39/F	8.6mg/dl	40ng/ml	30 weeks 3 days
12	27/F	9.2mg/dl	46ng/ml	34 weeks
13	22/F	8.5mg/dl	62ng/ml	34 weeks
14	22/F	8.8mg/dl	42ng/ml	30 weeks
15	27/F	9.1mg/dl	64ng/ml	34 weeks
16	23/F	9.7mg/dl	55ng/ml	33 weeks
17	24/F	9.9mg/dl	65ng/ml	34 weeks 6 days
18	24/f	9.8mg/dl	56ng/ml	33 weeks 6 days
19	26/f	8mg/dl	49.75ng/ml	35 weeks 6 days
20	23/f	9.8mg/dl	54.25ng/ml	36 weeks 5 days
21	27/f	9mg/dl	51ng/ml	33 weeks 6 days
22	25/f	8.9mg/dl	51ng/ml	36 weeks 5 days
23	22/F	8.3mg/dl	65.75ng/ml	36 weeks 4 days
24	22/F	9.4mg/dl	54ng/ml	35 weeks 3 days
25	23/F	9.2mg/dl	36ng/ml	36 weeks 4 days
26	39/F	8.6mg/dl	40ng/ml	30 weeks 5 days
27	27/F	9.2mg/dl	46ng/ml	34 weeks 53 days
28	22/F	8.5mg/dl	62ng/ml	33 weeks
29	22/F	8.8mg/dl	42ng/ml	34 weeks
30	27/F	9.1mg/dl	64ng/ml	32 weeks
31	28/F	8.9mg/dl	15.25ng/ml	35weeks 3 days
32	20/f	9mg/dl	59.9ng/ml	32 weeks
33	28/f	7.8mg/dl	29ng/ml	29 weeks 6 days
34	22/F	10.4mg/dl	42ng/ml	31 weeks 3 weeks
35	27/F	11mg/dl	64ng/ml	30 weeks 3 days
36	20/F	8.7mg/dl	72ng/ml	34 weeks
37	26/F	8.2mg/dl	63.25ng/ml	34 weeks
38	21/F	7.3mg/dl	86.5ng/ml	30 weeks
39	26/F	9.4mg/dl	56.25ng/ml	34 weeks
40	20/F	10.5mg/dl	72.75ng/ml	33 weeks

41	39/F	11.2mg/dl	101.25ng/ml	34 weeks 6 days
42	22/F	9.5mg	43.5ng/ml	33 weeks 4 days
43	24/F	9.3mg/dl	55ng/ml	35 weeks
44	26/F	8.2mg/dl	78ng/ml	34 weeks
45	28/F	9.4mg/dl	80ng/ml	34 week 5 days
46	27/F	9.7mg/dl	50ng/ml	34 weeks 4 days
47	30/F	9.6mg/dl	60ng/ml	35 weeks
48	33/F	8.6mg/dl	70ng/ml	36 weeks
49	34/F	8.2mg/dl	72ng/ml	37 weeks 5 days
50	27/F	9mg/dl	60ng/ml	35 weeks

experimental placental tissue. The Vitamin D receptor stained strongly positive in the normal placenta. Vitamin D binding protein is attached to the calcitriol than 25 (OH) cholecalciferol.

Discussion

Pregnancy is an altered physiological state. Vitamin D is steroid pro hormone that has many functional roles other than bone health. Maternal immune system is monitored by vitamin D during gestation. Vitamin D has immunomodulatory functions which proves it to be a crucial marker in the gestation period[4]. Recent studies have proved that calcitriol modulates activation, proliferation and differentiation of immune and inflammatory cells through the vitmain D binding protein[5,6]. The T panel of cells act to supress the immnue responses by other T cells and essential for suppressing the maternal immune responses againt the fetus promoting tolerence. These T cells also help in controlling inflammation and promoting tolerance to allergens. The CD4+FOXP3+ natural regulatory T cells (nTreg) mediate tolerance to self-antigens. The average of maternal serum cholicalciferol levels were measured in the third trimester pregnancy. Then it was compared with the CD4, CD8 and FoxP3 antibody distribution in the placental tissue by immunohistochemistry. The 25 oh cholicalciferol levels were maintained insufficient levels while calcitriol which is the active form of vitmain D was maintained in normal level. There may also be naive T regulatory cells formed along with combination with Transforming Growth Factor (TGF)-beta exposed to antigens[8]. (nTreg) and iTreg, play a key role in maintaining peripheral tolerance. Vitamin D has identified as a crucial factor to monitor the immune tolerance. High levels of 1,25(OH)2D have been shown to induce the lineage-specific FOXP3 transcription factor, which is essential for the development and functioning of Treg [69,70] by enhancing the number and activity of circulating CD4+[10,11,12]. There are animal in vitro studies showing higher 25(OH)D levels are associated with higher Treg/total T-cell ratios and a more immunosuppressive phenotype[13,14,15].CD 8 cytotoxic cells were also a marker for high risk pregnancies with deficient vitamin D levels. The concentration of CD8 cells were increased in the pre ecclamptic cases and gestational diabetes mellitus case pacental tissues. The ratio of CD8 cells were increased in the preterm aborted specimen when compared to the term placenta.

References

1. Brannon PM, Picciano MF. Vitamin D in pregnancy and lactation in humans. Annu Rev Nutr. 2011 Aug 21;31:89-115. doi: 10.1146/annurev.nutr.012809.104807. PMID: 21756132.
2. Heaney RP, Skillman TG. Calcium metabolism in normal human pregnancy. J Clin Endocrinol Metab. 1971 Oct;33(4):661-70. doi: 10.1210/jcem-33-4-661. PMID: 5093770.
3. Kovacs CS. Calcium and bone metabolism during pregnancy and lactation. J Mammary Gland Biol Neoplasia. 2005 Apr;10(2):105-18. doi: 10.1007/s10911-005-5394-0. PMID: 16025218.
4. Rueter K, Siafarikas A, Palmer DJ, Prescott SL. Pre- and Postnatal Vitamin D Status and Allergy Outcomes in Early Childhood. Biomedicines. 2022 Apr 19;10(5):933. doi: 10.3390/biomedicines10050933. PMID: 35625670; PMCID: PMC9139153.
5. Chun, R.F.; Liu, P.T.; Modlin, R.L.; Adams, J.S.; Hewison, M. Impact of vitamin D on immune function: Lessons learned from genome-wide analysis. Front. Physiol. 2014, 5, 151
6. Cantorna, M.T. Mechanisms underlying the effect of vitamin D on the immune system. Proc. Nutr. Soc. 2010, 69, 286–289.
7. Lio, C.W.; Hsieh, C.S. Becoming self-aware: The thymic education of regulatory T cells. Curr. Opin. Immunol. 2011, 23, 213–219.
8. Mucida, D.; Kutchukhidze, N.; Erazo, A.; Russo, M.; Lafaille, J.J.; Curotto de Lafaille, M.A. Oral tolerance in the absence of naturally occurring Tregs. J. Clin. Investig. 2005, 115, 1923–1933
9. Dimeloe, S.; Nanzer, A.; Ryanna, K.; Hawrylowicz, C. Regulatory T cells, inflammation and the allergic response—The role of glucocorticoids and Vitamin D. J. Steroid Biochem. Mol. Biol. 2010, 120, 86–95.
10. Christakos, S.; Dhawan, P.; Verstuyf, A.; Verlinden, L.; Carmeliet, G. Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. Physiol. Rev. 2016, 96, 365–408.
11. van Halteren, A.G.; Tysma, O.M.; van Etten, E.; Mathieu, C.; Roep, B.O. 1alpha,25-dihydroxyvitamin D3 or analogue treated dendritic cells modulate human autoreactive T cells via the selective induction of apoptosis. J. Autoimmun. 2004, 23, 233–239.

[CrossRef] [PubMed].
12. Van Belle, T.L.; Vanherwegen, A.S.; Feyaerts, D.; De Clercq, P.; Verstuyf, A.; Korf, H.; Gysemans, C.; Mathieu, C. 1,25-Dihydroxyvitamin D3 and its analog TX527 promote a stable regulatory T cell phenotype in T cells from type 1 diabetes patients. PLoS ONE 2014, 9, e109194.
13. Khoo, A.L.; Koenen, H.J.; Chai, L.Y.; Sweep, F.C.; Netea, M.G.; van der Ven, A.J.; Joosten, I. Seasonal variation in vitamin D3 levels is paralleled by changes in the peripheral blood human T cell compartment. PLoS ONE 2012, 7, e29250.
14. Prietl, B.; Treiber, G.; Mader, J.K.; Hoeller, E.; Wolf, M.; Pilz, S.; Graninger, W.B.; Obermayer-Pietsch, B.M.; Pieber, T.R. High-dose cholecalciferol supplementation significantly increases peripheral CD4(+) Tregs in healthy adults without negatively affecting the frequency of other immune cells. Eur. J. Nutr. 2014, 53, 751–759.
15. Maalmi, H.; Sassi, F.H.; Berraies, A.; Ammar, J.; Hamzaoui, K.; Hamzaoui, A. Association of vitamin D receptor gene polymorphisms with susceptibility to asthma in Tunisian children: A case control study. Hum. Immunol. 2013, 74, 234–24.

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