Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v30i18.3334

ASSOCIATION OF SPIRAL ARTERY REMODELING AND VITAMIN D RECEPTORS (VDR) IN PREECLAMPSIA

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Abstract:
Objective: This study is intended to conclude the vitamin D status and VDR gene, with its relation in spiral artery remodeling in normal and pre eclamptic women.
Study Design: Cross-sectional study.
Place and Duration: This study was conducted in Department of Anatomy with collaboration of Department of Gynecology and Obstetrics of Liaquat University Hospital Hyderabad and Jamshoro from August 2021 to July 2022.
Methodology: After ethical approval vide letter (LUMHS/REC/632) total 122 women; of 20-40 years of age with full term, singleton pregnancy, 61from normal-term pregnancies as controls and 61 from preeclampsia were registered at labor room of OBG. After demographic data with septic measures maternal blood sample of 5cc was collected before delivery for assessment of VD and their placentae were collected from labor room immediately after delivery along with 5cm of umbilical cord attached to it. After morphological examination of placenta, sectioning of placenta was performed for microscopic examination. Standard protocol for H &E and immunohistochemistry for VDR were followed performed. Sections were viewed under light microscope and Image J software was used for analysis.
Results: Mean±SD of maternal age, parity and gestational age (in weeks), wall thickness of spiral artery and % of VDR positive cells of study participants were 32.7 years±5.9, 3.9±1.4, 37.3±3.6, 48.11±23.5295 and , 46.49±18.779 respectively. (table1). Mean and SD of vitamin D levels and placental weight (table2) and wall thickness of spiral artery and % of VDR positive stained cells when compared on Image J software between normal and preeclamptic patients is highly significant(table 3).

Conclusions: Abnormal remodeling of spiral artery is significantly associated with low levels of Vitamin D and its receptors, leading to placental diseases such as preeclampsia.

Keywords: Placenta, Preeclampsia, Spiral artery, VDR, Vitamin D deficiency

1. Introduction:

Pre-eclampsia (PE) is a serious pregnancy-related disease that affects both mother and fetus. It complicates around 3-7 percent of pregnancies globally; third leading cause of maternal death. In developing countries, illness and death from PE are far higher than in developed countries. In Pakistan PE contribute 25% deaths due to PE in maternal mortality rate of 186/100,000 (Midhet F 2009, Shamsi U 2013).

This multi-system disease is defined as the emergence of new-onset hypertension with or without proteinuria after 20 weeks of gestation in a formerly normotensive woman. Although, the exact etiology of this complicated disease remains unknown, numerous mechanisms, including aberrant placentation, reduced placental circulation, impaired immunological and gene regulation and environmental factors such as poor calcium and vitamin D intake are hypothesized to be contributed to its etiology. In absence of exact etiology, predictive markers and diagnostic criteria, moreover as it varies from patient to patient, from diverse demographical locales and ethnicities, presents PE as a classical challenge for communities associated with maternal and child health care (Ali AM 2018, Serrano NC 2018).

Vitamin D deficiency (VDD) is reported as an epidemic across the globe with female predisposition of around 50%. VDD in pregnant women is common in several parts of the world and it is coupled with placental insufficiencies like preeclampsia, as evidenced by several studies (Ali AM 2021).

In pregnancy, dietary requirements for vitamin D increase with significant changes in its metabolism, imitated by notable difference in levels when compared with non- pregnant levels. This alteration in pregnancy is in concordance to meet the requirements of mother and developing fetus for ensuring normal development. As PE affects many organ systems, it also affects the homeostasis of vitamin D. Vitamin D supplementation is presumed to avert PE by facilitating implantation and placental development. Currently PE is linked with low activation, enhanced catabolism, and reduced placental uptake of 25(OH) D3 and VDR expression. The conversion of calcidiol or pre vitamin D [25(OH) D] to calcitriol; biologically active form of vitamin D [1, 25(OH) 2D] is raised significantly through twofold in pregnancy. In vitro studies reveal the role of calcitriol and calcidiol in enhancing extravillous trophoblast invasion which facilitate remodeling of spiral artery (Hollis BW 2017, Karras, Wagner et al. 2018).

Placenta contains approximately 80 – 100 spiral arteries (SA) which are musculo-elastic in nature. In normal pregnancy, SA undergoes physiological process of transformation by invasion of trophoblast for placental development. The transformation of lesser diameter and high resistance vessel to wider diameter and low resistance vessel is called as remodeling. These physiological changes are completed by 20 weeks and results in 5-10 times dilated vessel to ensure adequate blood supply for development of the placenta and developing fetus (Bokhari ZH 2010, TW. 2015).

Abnormal spiral artery remodeling is associated with several adverse pregnancy outcomes including PE. Remodeling of SA is not an “all or none” phenomenon; some vessels in normal pregnancy remain un-remolded and in same way in severe preeclampsia some SAs may remolded completely. In abnormal remolded vessel, the mean diameter of SA is less than one third of normal remolded vessels, with microscopic features of vascular dilatation, separation of tunica media, swelling of endothelial cell (EC) and extravillous trophoblast cells migrate within the lumen of the SA with loss of ECs, contributing significantly in uteroplacental insufficiency. Assessment of SA remodeling is often based on an overall impression of histological features (Espinoza, Romero et al. 2006, Dutta A 2017).

Vitamin D receptors (VDR) are expressed in many tissues including female genital organs, decidual and placental tissue. Placenta expresses all vitamin D signaling components. Placental Vitamin D concentration is influenced by epigenetic DNA hypermethylation of VDR gene which decrease expression of placental VDR in preeclampsia. VDD results in disturbance of VDR expression on

endothelium of placental vessels. Cell cultures studies reveals the role of VDR on gene transcription of renin, polymorphism of VDR influences plasma renin activity resulting in prevention of hypertension (Knabl, Vattai et al. 2017).
Despite of multi-centric studies, meticulous association between plasma 25 (OH) D levels and risk of PE is not well established. The results of those studies are difficult to compare because of variable study subjects, demographics and sampling techniques (Bodnar L.M. 2014).
Currently, there is no effective intervention for prevention or diagnosis of PE only available curative treatment is delivery of placenta regardless of fetal wellbeing. Maternal deaths due to preventable factors are about 2/3 of total deaths; VDD is potentially amendable risk factor for PE. There are several negative consequences during pregnancy due to lack of awareness and guidelines/ recommendation for vitamin D intake in our setup. In the light of these facts, this study is intended to conclude the vitamin D status and VDR gene, with its relation in spiral artery remodeling in normal and pre eclamptic women.

2. Material and method:

Study design and subjects:

This cross-sectional study was conducted in Department of Anatomy with collaboration of Department of Gynecology and Obstetrics of Liaquat University Hospital Hyderabad and Jamshoro after ethical approval. In this study, woman of 20-40 years of age with full term, singleton pregnancy was registered prior to normal vaginal/caesarian delivery. A total of 122 placentae, 61from normal-term pregnancies and 61 from pregnancies complicated by PE were studied. Patients with diagnosis of chronic hypertension, pregestational diabetes, smokers, any hormonal or systemic disorders or not willing in study were excluded from the study. Thorough history regarding medical and obstetric wellbeing was taken; demographic data which include gestational age, maternal age, parity, drugs intake, and sun exposure, Vitamin D supplementation, and maternal systolic and diastolic blood pressure was recorded on predesigned proforma (Akshara VR 2018).

Blood sampling and assessment of Vitamin D:

After all septic measures maternal blood sample of 5cc was collected before delivery in vacutinar by trained staff nurse. Serum was separated via centrifugation at 5000 rpm for 10 minutes and stored at -80°C immediately. Vitamin D was assessed on Advance technique of chemiluminescence micro particle immunoassay (CMIA) and analysis was done on Architect i2000 (Abbott Drag USA) (Hutchinson, Healy et al. 2017).

Placental tissue sampling:

Placentae were collected immediately after delivery; morphometric examination of each placenta was done after washing with PBS, sectioned and preserved for further studies with methodology described previously (Goswami.,P et al. 2012). Two sections of full thickness of placental samples (2x2x1 cm) approximately 05 gram from placental septa near the center of the basal plate section (BPS) excised from macroscopically normal looking maternal surface from one half of excised placenta randomly. For Hematoxylin and Eosin(H&E) and immunohistochemistry (IHC) section is collected in cassettes preserved in plastic jars containing (10%) formalin were fixed for 48 hours at room temperature after labeling with code number and processed in Diagnostic and Research Laboratory (DRC) Liaquat University of Medical & Health Sciences, Jamshoro, Sindh, Pakistan at Liaquat University Hospital (LUH), Hyderabad (Roberts, Gaffney et al. 2019).

Tissue processing for routine staining and IHC:

Formalin fixed tissue is dehydrated in alcohol, cleared by xylene, embedded in paraffin, section of 5 µm on rotatory microtome and placed over coated glass slides for staining. All procedures were performed using Automatic tissue processor, (Leica TP1020).

Hematoxylin and Eosin staining (H&E):
Slides were first deparaffinized in xylene and rehydrated with graded alcohol (100, 90, and 70%), and stained with hematoxylin (Carl Roth, Germany) for 3 minutes and washed thoroughly with distilled water followed by eosin staining for 30 sec. Then, slides were washed and dehydrated with graded alcohol (70, 90, and 100%) and mounted with DPX (Dibutylphthalate Polystyrene Xylene) (Dako A/S, Glostrup, Denmark) mounting media (Feldman and Wolfe 2014).

Immuno-histochemical (IHC) staining of VDR expression:
The 5 μm thick paraffin sections were processed for dewaxing, rehydration and endogenous peroxidase quenching with 3% hydrogen peroxide (H_2O_2) in methanol for 15 minutes. Antigen retrieval was done in Tris HCl (PH 9.0) in steamer for 35 minutes followed by washing with phosphate saline buffer (PBS) thrice. Once cooled for 30 minutes, primary antibody [VDR mouse monoclonal, Thermofisher Scientific USA (1:100 dilutions in PBS)] was applied; sections were incubated for 1 hour at room temperature. Sections were washed with PBS (3X) before being stained with secondary antibody [Horseradish peroxidase (Dako A/S, Glostrup, Denmark)] for 30 min at room temperature in dark. Mounting with diaminobenzidine (DAB) was done for 5 minutes once, washed with PBS 3X. After washing with distilled water, sections were counterstained with hematoxylin (Dako ready-to-use) and dried in oven and mounted with DPX (Merck KGaA Germany) for staining of nuclei before examined under microscope (Pospechova, Rozehnal et al. 2009).

Microscopy and image acquisition:
Sections were viewed under light microscope (Nikon Eclipse 50i Japan) at 20X and 40X magnification, images were taken with attached camera (Nikon-Digital sight DS L1) and saved as jpeg files. Digital data analysis was performed using Image J software. Slides/images were blindly analyzed by blind observer and observations were made from randomly selected areas in each slide, 3-5 spiral arteries were observed in every placenta under a magnification of 200X. The wall thickness of SA was calculated as the ratio of the difference between the external diameter (ED) and internal (ID) to the external diameter of the SA in all cases as (ED-ID) x100/ED obtained results were saved in Microsoft excel sheets. For cell counting**, c**ell Counter plug-in platform to count VDR positive DAB stained brown and VDR negative hematoxylin stained blue nuclei under a magnification of 400X obtained results were saved in Microsoft excel sheets (Fulawka L 2016).

3. Statistical Analysis:
Statistical analyses were performed using SPSS 22.0 software. Numerical variables were expressed as mean ± standard deviation; $P < 0.05$ was considered significant. Student's t-test was used to assess normally distributed variables and Mann Whitney was applied on the non-parametric variables.

3. Results:
Mean±SD of maternal age, parity and gestational age (in weeks), wall thickness of spiral artery and % of VDR positive cells of study participants were 32.7 years ±5.9, 3.9±1.4, 37.3±3.6, 48.11±23.5295 and , 46.49±18.779 respectively. **(table1).** Mean and SD of vitamin D levels and placental weight compared between healthy pregnant women and the preeclampsia patients by applying independent test and revealed p values (p value<0.01) and (p value < 0.01) respectively**. (table2).** Comparison of wall thickness of spiral artery and % of VDR positive stained cells between normal and preeclamptic by Mann Whitney test which reveals highly significant p value (**table 3 and 4**).

Table No.1: Maternal age, parity, gestational age, wall thickness of spiral artery and % of VDR positive cells)

	Minimum	Maximum	Mean	Std. Deviation	Std. Error Mean
Maternal age(in years)	21.00	47.00	32.7	5.9	0.53
Parity	2.00	8.00	3.9	1.4	0.13
Gestational Age(in weeks)	33.0	42.00	37.3	3.6	0.33
Wall thickness of Spiral artery	3..90	87.28	48.1138	23.52955	2.13027
% of VDR positive cells	16.70	80.00	46.4975	18.77973	1.70024

Table No. 2: Comparison of Vitamin D levels and placental weight

		Mean	Std. Deviation	Std. Error Mean	Sig.
Vitamin D levels (ng/ml)	Normal	25.4718	6.13	0.78	<0.01**
	Preeclamptic	8.7056	4.17	0.53	
Placenta weight	Normal	5.1164	33.2	4.25	<0.01**
	Preeclamptic	3.8046	79.2	10.14	

Table No. 3: Comparison of wall thickness of SA and VDR positive cells

		Mean	Std. Deviation	Std. Error Mean	Sig.
Wall thickness of spiral artery	Normal	29.1730	16.50308	2.11300	<0.01**
	Preeclamptic	67.0547	10.70912	1.37116	
% of VDR positive stained cells	Normal	61.5443	14.43525	1.84824	<0.01**
	Preeclamptic	31.4508	6.51950	.83474	

Table No. 4 Test Statistics

	Wall thickness of spiral artery	% of VDR positive stained cells
Mann-Whitney U	153.000	210.500
Wilcoxon W	2044.000	2101.500
Z	-8.747	-8.451
Asymp. Sig. (2-tailed)	.000	.000

4. Discussion:

In last two decades emerging role of Vitamin D in cure and prevention of diseases is significantly highlighted across the globe. Maternal VDD is closely related to pregnancy related co-morbidities such as abortion, preeclampsia, gestational diabetes and intra uterine growth retardation. VDD in pregnancy is well known observation and supplementation of VD reduces the risk of PE. Although the exact mechanism in prevention of PE is still obscure needs further research. But unfortunately recommendations for vitamin D supplementation in pregnancy for feto-maternal well -being are not yet properly addressed in our practice (Palacios, De-Regil et al. 2016, Heyden and Wimalawansa 2018).

Therefore this study is designed to address this issue which is not well supported due to scarcity of local data in developing, low economic countries including Pakistan.

In this study the mean and SD of vitamin D level in normal and preeclamptic was 25.4718 ±6.13 and 8.7056 ±4.17 ($p<0.01$) which is comparable with study conducted by Aisha Mansoor who found same for normal, while in preeclamptic parturient this study reveals more lower levels, while near similar results were observed in study conducted by Abedi in Iran, reflecting dire situation similar to our setup. While study conducted by Naima Umar doesn't find any significant difference in normal and preeclamptic, but overall lower levels was definitely observed in both groups (Abedi, Mohaghegh et al. 2014, Umar, Tauseef et al. 2016, Ali AM 2021).

Regarding placental weight current study reveals marked difference in normal when compared with preeclamptic which is in line with other studies as well. We observe mean of 5.1164±33.2 and 3.8046±79.2 (<0.01**) in normal and preeclamptic, similar is reported by Wubale from Africa, Vijayalakshmi and Shevade et al from India, similar results in all are thought to be same health and literacy status as all belong to low income per capita states (Shevade S 2015, Vijayalakshmi B 2015, Wubale Y 2017).

The positive correlation of VDD with preeclampsia was observed by wall thickness of spiral artery in normally remolded and non-remolded vessel and percentage of VDR positive cells in normal and preeclamptic placentae. Both parameters for the first time to best of our knowledge in Pakistan were measure on Image J software.

In this study we calculated the wall thickness by difference between external diameter and internal diameter and find mean and SD 29.1730±16.50308 and 67.0547±10.70912 in normal and preeclamptic placentae respectively with significant difference of <0.01. Study conducted by Hamza in Baghdad on Morphometry of spiral artery on image j reveals more significant results than ours probably because of difference in measurements. Another study conducted in Department of Anatomy of Ziauddin Medical University reveals spiral artery in preeclampsia with thick wall and narrow lumen by scanning electron microscopy with significant difference on Nikon NIS-elements D software when compared with normal (Baloch GKR 2021, SS. 2021).

Previously immuno-studies were conducted on semi quantitative, immune reactive scores, which are subjected to observer bias. Therefore this study was conducted on Fiji image j software which is more reliable and easier. We observe VDR positive cells with mean and SD in normal 61.5443±14.43525and preeclamptic ±6.51950with significant difference of <0.01. Other studies also observed placental expression of VDR lower in preeclampsia than normal but present results on scoring as there analysis methodology is differ than current study (Hutabarat, Wibowo et al. 2018, Cao, Jia et al. 2021).

5. Conclusion

Exact etiology of preeclampsia is still unrevealed, association of low levels of Vitamin D with it is significantly found in many studies including this but gaps need to be filled by further research in context of mechanism from implantation to spiral artery remodeling, facilitating uteroplacental circulation.

Declaration of Competing Interest:

The authors declare no conflict of any interest.

Acknowledgement:

The researcher is thankful to the faculty and duty staff of Labor room and operation theatre of Gynecology and Obstetrics Department of Liaquat University of Medical and Health sciences, for their help in sample collection and to parturient for allowing their placentae for study.

Funding Resources:

Funding for this study was granted from Liaquat University of Medical and Health Sciences vide letter no. LUMHS/DF/1860 dated 26-06-2018

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