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ORIGINAL ARTICLE

Disrupted placental vitamin D metabolism and calcium signaling in gestational diabetes and pre-eclampsia patients

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Abstract

Introduction Gestational diabetes (GDM) and pre-eclampsia (PE) represents the unrecognized risk factors for reduced bone content in neonates. The present study is planned to explore the components of vitamin D metabolism and calcium transport in placenta of GDM and PE cases and its effect on the neonatal bone mass determination using bone densitometry system.

Methods We have collected serum and placenta tissues from GDM ($n = 20$), PE ($n = 20$), and healthy pregnancies ($n = 20$). In the present study, we found mRNA expression of oxidative stress markers, vitamin D metabolic components and calcium channels, calcium channel binding proteins, plasma membrane calcium ATPase, ATP synthase and Ca2+ release genes; Ryanodine receptors genes were assessed by quantitative real-time PCR (qRT-PCR) in placental tissue of GDM, PE, and healthy pregnancies.

Results We observed high level of oxidative stress in both GDM and PE placenta compared to normal pregnancies. CYP2R1 and VDR mRNA expression was significantly downregulated and upregulation of CYP27B1 and CYP24A1 in GDM and PE compared with healthy cases. Similarly, calcium transporters were downregulated in GDM and PE placental tissues. In addition, CYP24A1, VDR, CaBP28K, TRPV5 and PMCA3 mRNA expression were correlated with BMC of neonates.

Discussion Oxidative stress is probably relevant to disrupted vitamin D homeostasis and calcium transport in the placenta of GDM and PE cases. The altered regulatory mechanism of CYP24A1 and VDR could indicates more pronounced serum 25(OH)D reduction. Additionally, reduced BMC in the neonates of these cases might be as consequences of modified CYP24A1, VDR, CaBP28K, TRPV5 and PMCA3 mRNA expression.

Keywords Gestational diabetes · Pre-eclampsia · Vitamin D · Calcium · Bone mineral content · Placenta

Introduction

Vitamin D signaling is important to maintain calcium homeostasis and bone mineralization to ensure fetal growth [1]. During pregnancy, the placenta is the major site for vitamin D metabolism to meet the requirement of fetal bone mineralization. Vitamin D deficiency as reported in both GDM [2, 3] and PE [4, 5] cases, may be a consequence of placental dysfunction [6]. Association of vitamin D deficiency and impaired femur growth and reduced bone density is observed in the neonates of GDM and PE cases [7]. As of placenta supports optimal fetal growth, mechanisms of transplacental proteins regulating vitamin D metabolism and calcium transport remain an area of research.

In general, vitamin D levels are maintained by the enzyme 1a-hydroxylase, a product of CYP27B1 gene. It converts 25(OH)D to active $1,25(OH)_2$ D and binds with

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vitamin D receptor (VDR) for most of the physiological actions. $1,25(OH)_2D$ is further metabolized to an inactive metabolite 24,25(OH)D by enzyme 24-hydroxylase, a product of CYP24A1 gene [8]. During the period of fetal development, maternal vitamin D [$1,25(OH)_2D$] levels are elevated due to uncoupled physiological feedback regulation [9]. Transplacental calcium transfer increases with increasing gestational age and diffuses from maternal circulation to the cytoplasm of syncytiotrophoblasts via specialized channels, 'transient receptor potential vanilloid' (TRPV). Using electrochemical gradient, these channels diffuse calcium from maternal circulation to cytoplasmic compartment of placental cells. calcium binding proteins (CaBPs) in the cytoplasm bind calcium, and move it to fetal side of placenta where plasma membrane calcium ATPase pumps (PMCAs) actively expel the calcium and thus maintain calcium transport in the placental cells [10, 11]. In the placenta, transcription of these transport proteins regulated by $1,25(OH)_2D$ in association with VDR activity [12]. In vitro studies have shown addition of syncytiotrophoblasts with $1,25(OH)_2D$ increased calcium absorption by the cell [13, 14].

Since vitamin D metabolism may affect calcium transport to support optimal fetal growth, we explored the expression pattern of these two pathways simultaneously in the placental tissue and their effect on neonatal skeletal formation. Placental gene expression analysis may provide better information to delineate vitamin D metabolism and calcium transport directly at the maternal–fetal interface. Furthermore, we estimated oxidative stress in the tissue, as a hypoxia condition is a key event in placental pathologies.

Materials and methods

Subject details

Study subjects were enrolled from antenatal clinic of Obstetrics & Gynecology department from January 2016 and June 2018. After approval by the Institutional Ethics Committee, informed written consent was obtained from each subject. GDM cases were diagnosed on basis of IADPSG criteria (Any one of three values greater than recommended cut-offs after 75 g OGTT, fasting = 92 mg%; 1 h = 180 mg%; 2 h = 153 mg%. Diagnosis of PE was based on blood pressure >140/90 mm of Hg with at least two separate readings after 20 weeks of gestation with proteinuria >300 mg/24-h urine or >1+ in dipstick. All included subjects carried a singleton pregnancy. Any subject with pre-pregnancy diabetes mellitus, untreated hypo/hyperthyroidism, chronic liver/renal disease, or any systemic illness was excluded from the study. Similarly, pregnancies with congenital malformations in fetuses were also excluded. After a detailed history and tailored clinical examination, blood samples from mothers were collected while they were admitted for delivery and cord blood samples were collected during delivery. Placental weight was measured by a standardized table-top digital weighing scale.

Placental tissue collection

Placental tissues were recovered immediately after delivery. After removals of maternal and fetal membranes, small pieces of tissue were obtained from the central part of the placenta, close to the umbilical cord. These pieces were collected immediately in RNA-Later (Qiagen, Germany) and subsequently stored at –80 °C until isolation of total RNA.

Whole body DXA assessment of the newborn

After milk-feed, neonates were swaddled in a soft warm cloth and placed on the platform in the supine position without sedation. DXA scans were performed within 48 h of birth by Hologic Discovery A 84023, QDR, USA scanner using pediatric software (Apex System software, version 4.5.2.1). The radiation exposure from a total body scan was <0.5 mrem/scan (acceptable ranges for pediatric populations). The instrument underwent daily quality assessment and was calibrated against a water phantom weekly. All scans were performed by the same technician and repeated if the baby movement was encountered during the measurement.

Serum biochemical analysis

Serum calcium (adjusted for serum albumin), phosphate and alkaline phosphatase were measured by an automated chemistry analyzer (Roche Hitachi 912 Chemistry Analyzer, GMI Inc., USA). Serum 25(OH)D levels were measured by DiaSorin autoanalyzer ('LIASON' DiaSorin Inc, Stillwater, USA) using a chemiluminescent tracer-based immunoassay.

Total RNA extraction and cDNA synthesis

Briefly, total RNA was extracted by TRIZOLE method. A nanoDrop spectrometer (Thermo Fisher Scientific, Wilmington, USA) was used for RNA quantification and quality was determined by optical density ratio at 260:280 nm, which is between 1.9–2.1. The integrity was confirmed by denaturing gel electrophoresis, whereby sharp 28S and 18S bands were demonstrated. The cDNA was synthesized from total RNA by a cDNA synthesis Kit (Qiagen, Germany).

Table 1 Primers and conditions used for amplification of target genes

S.No.		Gene	Primer Sequence (5′ → 3′)	Annealing temperature (°C)	Product size (bp)	Accession number
1	Oxidative stress marker	hOGG1	F-5′TGGAAGAACAGGGCGGGCTA3′ R-5′ATGGACATCCACGGGCACAG3′	63	164	NM_002542.5
2	Vitamin D Metabolizing Enzymes	CYP2R1	F-5′TGAGGCAGTTTTGCATGAAG3′ R-5′AATCGCTCAGGATGGAACAC3′	61	192	NM_024514.4
3		CYP27B1	F-5′GGAAGGCGAAGAATGGCAAAGG3′ R-5′TCGCAGACTACGTTGTTCAGGGTTC3′	65	105	NM_000785.3
4		CYP24A1	F-5′TCTGGAAAGGGGGTCTCAAGAAACA3′ R-5′ACCGACTCAAAGGAACCCAACTTCA3′	56	100	NM_000782.4
5		VDR	F-5′GGACCTGTGGCAACCAAGACTACAA3′ R-5′TTCAGTCCCACCTGGAACTTGATGA3′	55	106	NM_000376.2
6	Calcium transporter and Calcium release genes	TRPV5	F-5′AGACAAGGAGGATGACCAGGA3′ R-5′CCCAGGGTGTTTTGACGAAG3′	57	168	NM_019841.6
7		TRPV6	F-5′TCTGCGGACGGGAGTATGG3′ R-5′CCTGTGCGTAGCGTTGGAT3′	56	96	NM_018646.5
8		CaBP-9k	F-5′ATATGCAGCCAAAGAAGGTG3′ R-5′TGGACCTTTGAGTAAACTGGG′3	56	94	NM_004057.2
9		CaBP28k	F-5′TCAGGACGGCAATGGATACA3′ R-5′AAGAGCAAGATCCGTTCGGT′3	56	166	NM_004929.3
10		PMCA1	F-5′CAGCAGGAGAACCAGAACCA3′ R-5′ ATTCCAGCCCTCTGACACTT3′	57	139	NM_001001323.2
11		PMCA2	F-5′GGCTCACACAGAAGGAGGAG3′ R-5′GGATGGAGGTTCGAGATTCA3′	60	204	NM_001001331.3
12		PMCA3	F-5′CCGTGGTCCAGTCCTACCTA3′ R-5′CTTCAGGTCCAAGACGAAGC3′	56	215	NM_021949.3
13		PMCA4	F- 5′TCAGGAATCCCAACGGTG3′ R- 5′TCGATGACAGTGCGTACC3′	53	148	NM_001001396.2
14		ATPase	F-5′TCTGGGTGAGGCAGCTCTAT3′ R-5′TCTCTGATCCAGGCTGTGTG3′	61	169	NM_001206927.1
15		IP3R1	F-5′GGTTAATGCCAAGCCAAAAA3′ R-5′CCGGTAGTCTCTGGAAGCTG3′	54	224	NM_001099952.2
16		IP3R2	F-5′ACCCAGAGAACTGGTGGATG3′ R-5′ TGCAGTGTGGTTGGCATAAT3′	54	235	NM_002223.3
17		IP3R3	F-5′AACTACCTGGCTGCTGAGGA3′ R-5′CGAAAGAGTCGGTTTTCTGC3′	56	205	NM_002224.3
18		RyR1	F-5′GGGACGAGTTCTCTGTGCTC3′ R-5′AGTTGTGGGACTTGGACCAG3′	59	168	NM_000540.2
19		RyR2	F-5′CAACCGGACTCGTCGTATTT3′ R-5′TTGGCTTTCTCTTTGGCTGT3′	60	249	NM_001035.2
20		RyR3	F-5′TCTGCTGTCTGGGTCTATCTC3′ R-5′CCAAATGTCTTATGCGTCAC3′	56	262	NM_001036.4
21	Housekeeping gene	β-Actin	F-5′CATCCTCACCCTGAAGTACCCCATC3′ R-5′AGCCACACGCAGCTCATTGTAGA3′	63	103	NM_001101.4

Gene expression analysis

Placental mRNA expression of vitamin D metabolizing genes (i.e., CYP2R1, CYP27B1, CYP24A1, VDR) and calcium channel binding proteins (i.e., CaBP-9K, CaBP-28K), plasma membrane calcium ATPase pumps (i.e., PMCAs 1,2,3,4), ATP synthase and Ca2+ release genes (i.e., inositol-1,4,5-triphosphate receptors (i.e., IP3R1,2,3; and Ryanodine receptors (i.e., RyR1,2,3) were assessed by qRT-PCR using SYBR Green I dye method (Kapa Biosystems, South Africa) on CFX96^{TM} Real-Time System (Bio-Rad, USA). The primer sequences and amplification conditions are listed in Table 1. All samples were amplified in duplicate; non-template reactions were included as a negative control. Relative quantification of gene expression was done by the $2^{-\Delta\Delta Cp}$ method. The Cp values of both the calibrator (Control) and the sample

Table 2 Clinical and biochemical characteristics of maternal study population

Variables	All ($n = 63$)	Control ($n = 20$)	GDM ($n = 20$)	PE ($n = 20$)	P value
Maternal age (years)	27.59 ± 3.29	26.85 ± 3.44	28.90 ± 3.40	27 ± 2.78	0.0779
Gestational age at delivery (days)	255.73 ± 22.82	271 ± 6.82	260 ± 9.16	237 ± 28.11	**<0.001**
BP-Systolic (mm Hg)	126 ± 19.34	110 ± 9.34	118 ± 9.30	149 ± 10.66	**<0.001**
BP-Diastolic (mm Hg)	80 ± 13.66	69 ± 6.93	75 ± 6.60	96 ± 6.15	**<0.001**
BMI (kg/m2) (Early pregnancy)	23.81 ± 3.83	22.22 ± 3.06	25.02 ± 3.80	24 ± 4.13	0.0563
Weight gain during pregnancies (kg)	10.59 ± 4.10	12.44 ± 4.17	9.32 ± 3.44	10.13 ± 4.19	0.3315
Placenta weight (g)	493.75 ± 140.68	536 ± 68.87	564 ± 132	389 ± 137.41	**<0.001**
Neonatal birth weight (g)	2696 ± 721.03	2965 ± 406	3049 ± 295	2136 ± 885	**<0.001**
Fasting Blood Glucose (mg/dl)	81 (75.5–91.5)	77.0 (72.0–80)	93.0 (83.0–99)	79.5 (74.2–88.2)	**<0.001**
Serum calcium (mg/dL)	8.77 ± 0.54	8.73 ± 0.50	8.90 ± 0.44	8.67 ± 0.64	0.3516
Phosphate (mg/dL)	4.13 ± 1.10	4.13 ± 5.9	4.75 ± 1.40	3.53 ± 0.64	**<0.001**
Alkaline phosphatase (IU/l)	326.0 (259.3–407.0)	321.0 (232.0–384.3)	341.5 (290.3–413.8)	322.5 (258.3–477.0)	0.4939
Serum 25(OH)D (ng/ml)	20.0 (12.0–24.5)	20.0 (12.0–34.4)	19.0 (13.5–22.5)	17.0 (8.4–23.6)	0.2235

Data are presented in mean (SD) and Median (25th–75th interval) for normally distributed & non-normally distributed variables, respectively. Significant values were shown in bold letters

BP blood pressure, *BMI* body mass index, *IGF1* insulin-like growth factor 1, *RR* reference range, *BA* whole body bone area, *BMC* whole body bone mineral content, *BMD* whole body areal bone mineral density

Table 3 Clinical and biochemical characteristics of neonates

Variables	All ($n = 63$)	Control ($n = 20$)	GDM ($n = 20$)	PE ($n = 20$)	P value
Neonatal birth weight (g)	2696 ± 721.03	2965 ± 406	3049 ± 295	2136 ± 885	<0.001
Serum calcium (mg/dL)	9.09 ± 1.02	10.17 ± 0.60	9.06 ± 0.72	8.31 ± 0.76	<0.001
Phosphate (mg/dL)	5.84 ± 1.5	5.9 ± 1.05	6.67 ± 1.37	4.98 ± 1.52	<0.001
Alkaline phosphatase (IU/l)	382.0 (324.0–505.0)	342.0 (284.8–411.0)	358.5 (302.5–479.5)	480.0 (348.5–593.0)	**0.0089**
Serum 25(OH)D (ng/ml)	18.0 (11.5–23.5)	22.0 (16.0–46.2)	18.0 (12.0–22.0)	17.0 (8.3–21.2)	0.0771
Neonatal IGF1 (ng/ml)	76.0 (49.6–108.5)	79.0 (53.5–114.5)	72.0 (49.0–118.0)	80.9 (49.8–105.0)	0.5941
Femur length (cm) at 18–20 weeks	3.07 ± 0.62	3.03 ± 0.27	3 ± 0.63	3.17 ± 0.83	0.7064
Bone Area (cm^2)	209.96 ± 28.61	224 ± 19.86	212 ± 23.47	192 ± 33.90	**<0.001**
BMC (g)	42.67 ± 10.15	48.53 ± 6.76	42.62 ± 7.80	36.88 ± 12.28	**<0.001**
BMD (g/cm^2)	0.2 ± 0.03	0.215 ± 0.01	0.19 ± 0.02	0.16 ± 0.07	**<0.001**
Fat mass (g)	267.7 (157.4–36.7)	307.8 (220.9–521.3)	268.6 (169.1–348.1)	202.8 (142.7–335.2)	**0.0386**

Data are presented in mean (SD) and Median (25th–75th interval) for normally distributed & non-normally distributed variables, respectively. Significant values were shown in bold letters

BP blood pressure, *BMI* body mass index, *IGF1* insulin-like growth factor 1, *RR* reference range, *BA* whole body bone area, *BMC* whole body bone mineral content, *BMD* whole body areal bone mineral density

of interest were normalized to the corresponding mean value of an endogenous housekeeping gene, β-Actin.

Statistical analyses

All analyses were performed using STATA V14 Stata Corp LLC, TX, USA. Descriptive statistics of non-normally distributed data were reported as median (interquartile range), while normally distributed data were represented as mean ± SD and categorical variables as numbers (percentages). Students t-test and ANOVA were performed for parametric data. Otherwise, Mann Whitney U test and Kruskal Wallis followed by Dunnett's multiple comparison test were used for non-parametric data. The Pearson and

Spearman's correlation test was applied for parametric and non-parametric data respectively. A P value of < 0.05 was considered statistically significant.

Results

Clinical and biochemical characteristics of mothers and neonates

A total of 63 subjects (GDM = 21; PE = 22 and healthy controls = 20) were included in the study. Maternal and neonatal clinical and biochemical characteristics were presented in Tables 2 and 3 respectively. Systolic and diastolic blood pressure levels were increased in the pre-eclampsia group as compared to the normal pregnancy and gestation diabetes group ($p < 0.05$). Fasting blood glucose levels were increased in the gestational diabetes group as compared to the healthy pregnant and pre-eclampsia group ($p < 0.05$) (Table 2). Further to this fat mass is significantly decreased in gestational diabetes and pre-eclampsia groups ($p < 0.05$).

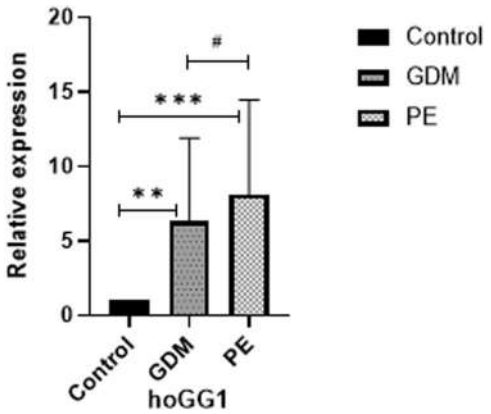

Fig. 1 mRNA expression of the hOGG1 gene in healthy pregnant (control), GDM and PE placentas. Data were normalized according to β-actin housekeeping gene. For mRNA expression. $^{\#,*}p < 0.05$, **<0.01,***<0.001

Neonatal bone mineral density (BMD) and bone mineral content (BMC) levels were significantly decreed in gestation diabetes and pre-eclampsia groups as compared to the healthy pregnant group ($p < 0.05$). No significant difference was observed in chronological age among the three groups. Similarly, no significant difference was seen in any of the biochemical parameters (Table 3).

Serum 25(OH)D levels in study group mothers and neonates

Circulatory vitamin D levels of maternal in the study groups i.e., Gestational diabetes and Pre-eclampsia mothers and healthy pregnant subjects were identified as vitamin D deficient (<20 ng/ml) as per the definition criteria (8) and no significant difference was observed between the maternal study groups (Table 2). Similarly, we observed vitamin D deficiency in neonates of the study groups and no significant differences were observed among the neonatal study groups (Table 3).

Oxidative stress maker hoGG1 expression levels in study groups

Increased oxidative stress marker i.e., hOGG1 mRNA expression was observed in GDM and PE placentas (GDM-6.33 fold; PE- 8.08-fold; Fig. 1). Surprisingly, we observed further increased hOGG1 levels in the PE group as compared to the gestational diabetes group.

Placental gene expression of vitamin D metabolism

There was a significant difference in the placental expression of vitamin D metabolizing enzymes in GDM and PE compared to healthy cases. CYP2R1 mRNA expression was 86% lower expression in GDM group with a mean fold change of 0.51 and 87% lower in PE cases with mean fold change of 0.42 (Fig. 2A) as compared to the healthy

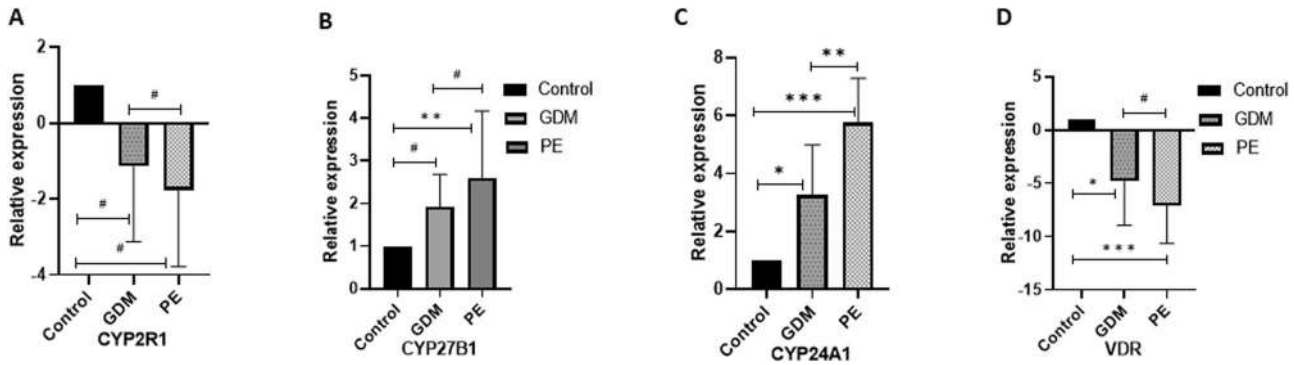

Fig. 2 mRNA expression of the vitamin D regulated gene in normal, GDM and PE placentas. **A** CYP2R1, **B** CYP27B1, **C** CYP24A1, **D** VDR. Data were normalized according to β-actin housekeeping gene. $^{*\#}p < 0.05$, **<0.01, ***<0.001

pregnant group. However, an increased expression of CYP27B1 mRNA was seen in more than 75% cases of GDM and PE cases. The mean CYP27B1 mRNA fold change was 1.77 and 2.27 in GDM and PE cases, respectively (Fig. 2B). Similar but at a higher magnitude, an increased expression of CYP24A1 mRNA was observed in GDM and PE cases with mean fold of change 3.26 and 5.75 respectively compared to healthy cases (Fig. 2C). Reduced expression of VDR mRNA was seen in the 76% GDM group with a mean fold change of 0.76 and 86% of PE subjects with a mean fold change of 0.44 (Fig. 2D) as compared healthy pregnant group.

Placental gene expression of calcium transporters

The TRPV5 (Fig. 3A) and TRPV6 (Fig. 3B) mRNA expressions were 90% lower expression in GDM and PE groups as compared to the healthy subjects group (TRPV5-0.50-fold in GDM and 0.39-fold in PE; TRPV6 −0.28 fold in GDM and 0.24 fold in PE). The mRNA expression of calcium binding/chaprone proteins, CABP-9K (Fig. 3C); and CABP-28K (Fig. 3D) were 90% lower expression in GDM group (CABP 9K-0.31 fold; CABP 28K-0.44 fold), as well as with PE group (CABP 9K-0.36 fold; CABP 28K-0.28 fold) as compared with healthy subjects group.

Similarly, PMCA mRNA isoforms (PMCA1, PMCA2, PMCA3, PMCA4; Fig. 3E–H) expression was significantly lower expression in 90% of subjects with GDM (PMCA1-0.16 fold; PMCA2-0.65 fold; PMCA3-0.36 fold, PMCA4-0.22 fold) and PE (PMCA1-0.30 fold; PMCA2-0.57 fold; PMCA3-0.61 fold, PMCA4-0.25 fold) as compared with the healthy pregnant group. Reduced expression of ATPase mRNA was observed in 62% subjects with GDM while 77% subjects with PE (GDM-0.79 fold; PE-0.65 fold; Fig. 3I) as compared with the healthy pregnant group.

Placental calcium release IP3R (IP3R1, IP3R2, IP3R3; Fig. 3J–L) and RyR (RyR1, RyR2, RyR3; Fig. 3M–O) isoforms mRNA expressions were 60% lower expression in subjects with GDM (IP3R1-0.45 fold; IP3R2-0.21 fold; IP3R3- 0.44 fold, RyR1-0.79 fold; RyR2-0.54 fold; RyR3-0.27 fold) and 75% in PE Group (IP3R1-0.34 fold; IP3R2-0.33 fold; IP3R3- 0.36 fold; RyR1-0.75 fold; RyR2-0.31 fold; RyR3-0.37 fold) as compared with the healthy pregnant group.

Correlation analysis

Maternal serum 25(OH)D was positively correlated with neonatal serum 25(OH)D in all subjects ($r = 0.858$, $p < 0.001$; Fig. 4A) .However, mother serum calcium levels were not correlated with neonatal serum calcium levels.

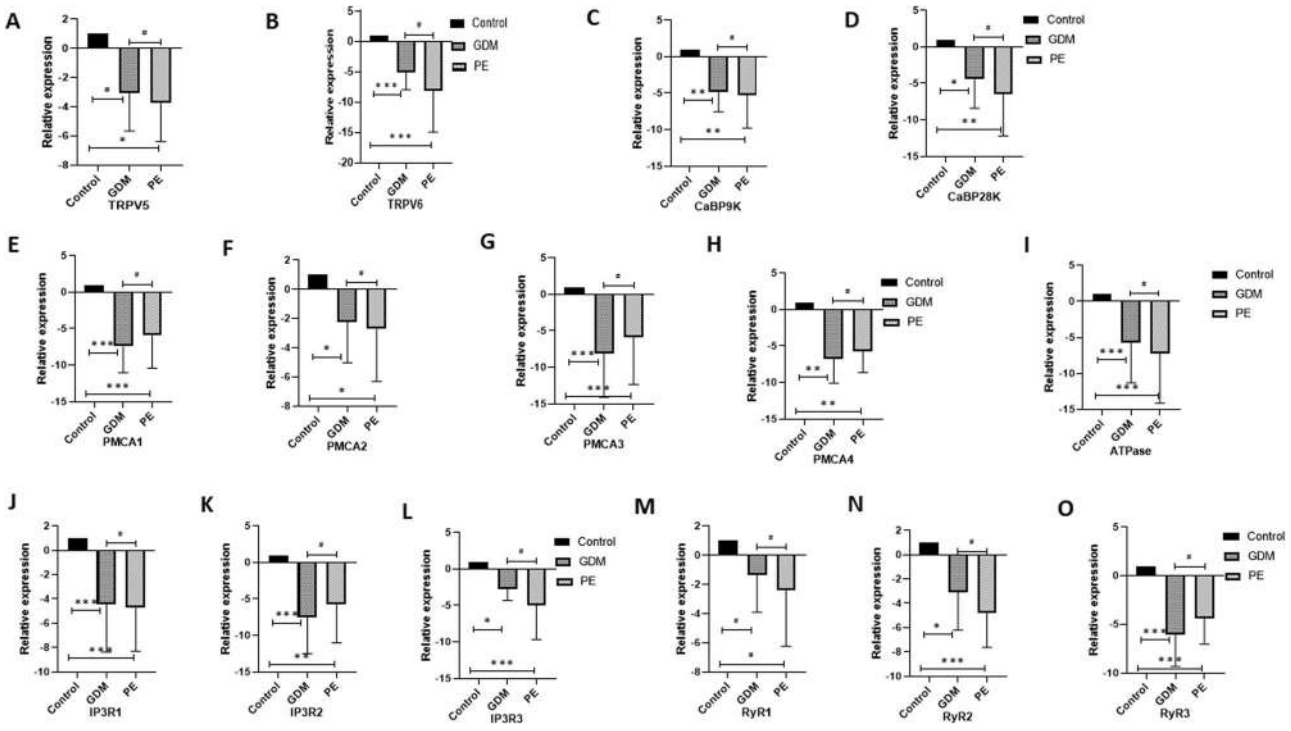

Fig. 3 mRNA expression of Ca2 handling genes **A** TRPV5 and **B** TRPV6, **C** CaBP-9K, **D** CaBP-28K and **E** PMCA1 **F** PMCA2, **G** PMCA3, **H** PMCA4, **I** ATPase, **J** IP3R1, **K** IP3R2, **L** IP3R3, **M** RyR1 **N** RyR2 **O** RyR3 in placentas coming from normal women compared to women with GDM and PE. Data were normalized according to β-actin housekeeping gene. $*^{,\#}p < 0.05$, $**p < 0.01$ and $***p < 0.001$

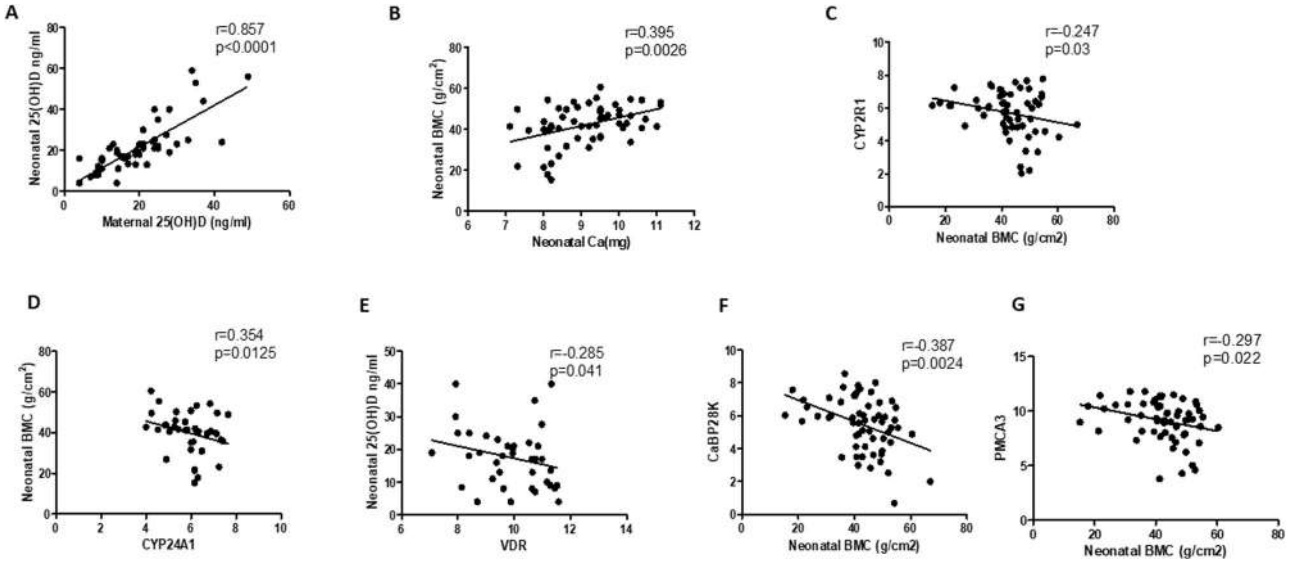

Fig. 4 Scatter plot representing correlation analysis: **A** neonatal 25 (OH) D and maternal 25(OH)D **B** neonatal Calcium and Neonatal BMC **C** Placental CYP2R1 and neonatal calcium **D** neonatal BMC and CYP24A1 **E** neonatal 25(OH)D and placental VDR **F** Placental CaBP28K and Neonatal BMC **G** placental PMCA3 and neonatal BMC

Neonatal whole body BMC was positively correlated with neonatal serum calcium levels ($r = 0.395$, $p = 0.0026$; Fig. 4B). However, neonatal whole body BMC is not correlated with maternal serum calcium levels.

The relative expression of CYP2R1 and CYP24A1 mRNA expression was negatively correlated with whole body BMC in all the cases ($r = -0.354$, $p = 0.012$, Fig. 4C) and ($r = -0.350$, $p = 0.005$; Fig. 4D) respectively. Similarly, VDR expression was negatively correlated with neonatal serum 25(OH)D levels ($r = 0.314$, $p = 0.019$; Fig. 4E).

The relative expression of placental CaBP28K mRNA expression was negatively correlated with neonatal BMC levels ($r = -0.387$, $p = 0.0028$; Fig. 4F) and placental PMCA3 expression was correlated with neonatal whole body BMC ($r = 0.297$, $p = 0.022$; Fig. 4G) in all the cases.

Discussion

Here, we are reporting high levels of oxidative stress in both, PE and GDM placental pathologies with higher levels in PE as compared to GDM cases. Vitamin D metabolism and calcium transportation were affected by disrupted (or altered) mRNA expression of the genes involved in these pathways. In addition, we also demonstrated a significant correlation of CYP24A1, VDR, CaBP28K, TRPV5 and PMCA3 mRNA expression with whole body BMC of neonates.

In our study, we observed an increased oxidative stress marker i.e., hOGG1 expression in the placentas of GDM and PE it is clearly demonstrated that hypoxia condition could damage the component of calcium and vitamin D pathways, which may affect fetal bone mineralization. Placental functioning could be impaired due to abnormal remodeling of spiral arteries in pregnancy related disorders, which may result in hypoxia and fetal growth restriction. The hypoxic condition can lead to oxidative stress and thereby damaging DNA and its protein products due to antioxidants and reactive oxygen species [15]. Decreased CYP2R1 and increased CYP27B1 expression in the placenta of GDM and PE would be consistent with less efficient control of serum $25(OH)D_3$ and $1,25(OH)_2D_3$ levels. Although we could not measure serum $1,25(OH)_2D_3$, it is expected to have lower $1,25(OH)_2D_3$ levels in hypertensive disorders in pregnancy cases as reported previously [16]. The exact mechanisms of deregulation of these enzymes are not known, we hypothesize that oxidative stress developed in the tissue might have led to disruption in expression. Enzyme CYP24A1, responsible for converting serum $25(OH)D_3$ into an inactive form of vitamin D (serum $24,25(OH)_2D_3$) [17]. Similarly, in our study we observed an active vitamin D form is over expressed in GDM and PE placentas compared to healthy pregnancies. This shift of vitamin D metabolism towards inactive form may also be responsible for reduced serum vitamin D levels in both, PE and GDM subjects. A previous study has reported that promoter methylation in CYP24A1 gene in placental cells might result in modulation of gene expression [9]. Reduced DNA methyl transferase activity may result in increased expression of CYP24A1 [17]. Noteworthy, neonatal BMC at birth was negatively correlated with CYP24A1 expression, hence this can play a significant role in neonatal BMC determination in our study. However, correlation can't address the causal relationship between the neonatal BMC and CYP24A1 expression.

The biological action of $1,25(OH)_2D$ is mediated by binding to cell surface receptors to mediate downstream signaling [18]. The maximum effect of this enzyme is mediated by VDR. We reported reduced expression of the VDR gene in GDM, as well as PE cases and the magnitude of reduction, was similar to the previous report on PE [17]. It seems more likely that reduced VDR expression could be due to less availability of the ligand $1,25(OH)_2D$. Although we have reported increased expression of CYP24A1, increased degradation of the ligand may result in less availability. Post-translational modification or gene silencing by promoter region methylation could be another possibility for reduced expression of the VDR gene [18].

In this study, that we reported reduced VDR expression affected BMC of newborns. A relation between fetal femur length and VDR expression shows a role of VDR in bone formation, most probably due to transplacental calcium transport [19]. $1,25(OH)_2D$/VDR activity up-regulates genes involved in placental calcium transport namely, TRPV6 [20], CaBP9K and CaBP28K [21, 22]. Transepithelial calcium transfer through placental cells requires various channels/transporters. Reduced expression of these transporters may result into impaired calcium transport [23]. Gatekeepers, TRPVs and TRPV6 have demonstrated calcium dependent channel inactivation to prevent intracellular calcium toxicity [24]. An animal study with TRPV5 and TRPV6 knockout mice has shown down-regulation of CaBP28K [25] and CaBP9K [26], respectively in the placenta. Noteworthy, in low calcium concentration, CaBP28K interacts with TRPV5 and CaBP8K with TRPV6 to maintain calcium influx at the apical region of placental cells [23, 27]. Our results demonstrated decreased expression of TRPV5/6 accompanied by decreased CaBP9K/28K mRNA expression in both GDM as well as PE placentas, compared to healthy subjects similar to a previous study in primary pre-eclamptic syncytiotrophoblasts [23]. We found the magnitude of TRPV5 and CaBP28K expression in the placenta is correlated with neonatal BMC, suggesting more important role of TRPV5/CaBP28k in calcium transport regulation to the fetus. Previously, TRPV5 knockout mice have shown reduced CaBP28K mRNA expression and disruptive calcium transfer to the fetus [28].

Further, PMCA pumps on the cell membrane are important proteins implicated in calcium extrusion to the fetal side to maintain cytosolic calcium concentration [29]. In our study, the magnitude of reduction in PMCAs expression was similar to a previously published study [23]. PMCAs functioning depends on ATPs, synthesized by the ATP synthase (ATPase) gene [30]. We further demonstrated that PMCA3 expression was correlated with ATPse expression as observed in a rodent study [31]. Reduced expression of ATPase could be responsible for the reduced supply of ATPs to PMCA and thus, affecting the activity of these pumps [23]. Oxidative stress, by promoting the synthesis of lipid peroxides, may be another possibility that might have resulted in reduced expression of PMCAs in GDM and PE placentas [32]. Our data shows that PMCA3 expression influences BMC of newborns. Martin et al. have also reported [11] that PMCA3 could be the principal calcium transporter in the placenta to determine fetal skeletal outcomes. Similarly, published data from animal study also suggest that increased PMCA expression stimulates placental calcium transport [33]. Further to this, we have not observed a correlation between maternal and neonatal serum calcium levels, this might be due to the no differences in the serum calcium levels in maternal and neonatal samples.

According to the available literature, diabetes during pregnancy promotes fetal lipid transfer by increasing the maternal–fetal gradient, which contributes to an increased body fat mass in newborns of diabetic mothers [34]. However, in our study, we observed a decreased fat mass in GDM neonates than in controls. This may be due to the impaired nutritional transfer across the placenta in our study population. In our study, the neonatal fat mass is reduced in PE neonates than in controls, which may be because of the intrauterine fetal growth retardation associated with PE [35]. The results are inconsistent with the existing literature.

Apart from CaBPs, IP3Rs and RyRs on the endoplasmic reticulum (ER) are known to maintain calcium buffering in the cell [36, 37]. Reduced expression of these genes in our study suggests impairment of intracellular concentration and inefficiency for calcium extrusion from ER.

Limitations of the study

The present study has a few limitations. The main limitation of the study, it was a single-center study and designed with a small sample size. Future studies are needed with a large number of samples. Another major limitation of the study an observational study does not address causative relationship. Future studies need to be focused on interventional studies to correct the vitamin D and calcium imbalance in preeclampsia and gestational diabetes patients.

Conclusion

In conclusion, the important finding of perturbed vitamin D homeostasis and calcium transport in placental tissue of GDM and PE cases, might be due to increased oxidative stress. Lowered serum 25(OH)D levels in these pregnancies might be a consequence of increased activation of CYP24A1 gene and reduced expression of VDR. In addition, ATP depletion affected PMCAs and subsequently leads to reduced activity of CaBPs and TRPVs.

We also demonstrated the reduced expression of IP3Rs and RyRs on ER. All these components might affect calcium transport to the fetus in pregnancies affected with GDM and PE, thereby might affect neonatal BMC at birth. Noteworthy, CYP24A1, VDR, CaBP28K, TRPV5 and PMCA3 genes expression remarkably correlated with reduced BMC. This analysis could help to understand the placental transfer process to develop strategies for better fetal bone mineralization. Although, we took age matched subjects in the groups, at delivery their gestational age was not matched to healthy cases. Nevertheless, the strength of our study is the simultaneous analysis of these pathways in the placenta along with their effect on neonatal skeletal growth at birth.

Data availability

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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Author contributions S.V. and R.Adela contributed to study designing, sample collection, gene expression analysis, clinical data collection, data analysis, interpretation, and manuscript writing. G.K., V.K., and R.Kumari contributed to the study designing, interpretation, gynecological review. R.D. contributed to study designing, molecular data interpretation, and review. R.Agarwal contributed to the study designing, interpretation, and neonatal opinion. R.Khadgawat contributed to the study designing, interpretation, endocrine review. All authors critically revised the manuscript, agree to be fully accountable for ensuring the integrity and accuracy of the work, and read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval The study was reviewed and approved by the institutional ethics committee of the All India Institute of Medical Sciences-New Delhi (IEC/414/8/2016). The clinical study was conducted in accordance with principles outlined in the Declaration of Helsinki and institution and ethical standards. The individuals supplied written informed consent.

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