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Effect of maternal omega-3 fatty acids and vitamin E supplementation on placental apoptotic markers in rat model of early and late onset preeclampsia

Vaishali Kasture, Anvita Kale, Karuna Randhir, Deepali Sundrani, Sadhana Joshi*

Mother and Child Health, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth (Deemed to Be University), Pune-Satara Road, Pune, 411043, India

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ABSTRACT

Aim: Disturbed placentation results in pregnancy complications like preeclampsia. Placental development is influenced by apoptosis during trophoblast differentiation and proliferation. Increased oxidative stress upregulates placental apoptosis. We have earlier reported increased oxidative stress, lower omega-3 fatty acids and vitamin E levels in women with preeclampsia. Current study examines effect of maternal omega-3 fatty acids and vitamin E supplementation on apoptotic markers across gestation in a rat model of preeclampsia.

Main methods: Pregnant Wistar rats were randomly assigned to control; early onset preeclampsia (EOP); late onset preeclampsia (LOP); early onset preeclampsia + omega-3 fatty acid + vitamin E supplementation (EOP + O + E) and late onset preeclampsia + omega-3 fatty acid + vitamin E supplementation (LOP + O + E) groups. Animals (Control, EOP, EOP + O + E) were sacrificed at d14 and d20 of gestation while animals (LOP, LOP + O + E) were sacrificed at d20 to collect blood and placentae. Protein and mRNA levels of apoptotic markers were analyzed by ELISA and RT-PCR respectively.

Key findings: Protein levels of proapoptotic markers like Bcl-2 associated X-protein (BAX) ($p < 0.05$), caspase-8 and 3 ($p < 0.01$ for both) and malondialdehyde ($p < 0.01$) were higher only in the EOP group as compared to control. However, the antiapoptotic marker, B cell lymphoma 2 (Bcl-2) protein levels were lower in both the subtypes of preeclampsia ($p < 0.01$ for both).

Significance: Our findings suggest that supplementation was beneficial in reducing the caspase-8 and 3 in early onset preeclampsia but did not normalize BAX and Bcl-2 levels. This has implications for reducing placental apoptosis in preeclampsia.

1. Introduction

Programmed cell death referred to as apoptosis, is a cellular mechanism for cell deletion and is vital for cell morphogenesis, embryonic development and maintenance of tissue homeostasis [1]. Apoptosis influences trophoblast differentiation and proliferation and therefore plays a critical role in placental development [2]. Increased levels of villous trophoblast apoptosis has been identified in placental pathologies, including early pregnancy loss and preeclampsia [3]. The etiology of preeclampsia is unknown but aberrant placentation appears to be crucial [4]. This abnormal placental development could be a result of altered apoptotic and angiogenic mechanisms [5]. Apoptosis is mediated by various apoptotic markers, which are of two types proapoptotic and antiapoptotic [6]. A dynamic balance between proapoptotic markers (Bcl-2 associated X-protein (BAX), caspase-8, caspase-3) and antiapoptotic markers B cell lymphoma 2 (Bcl-2) is important for cell survival and proliferation [7].

During the first trimester the placenta develops in a low oxygen environment wherein angiogenesis and vascularization starts occurring for the establishment of maternal and fetal vasculatures. Once vascularization is complete, the onset of maternal blood flow in the intervillous space increases the oxygen tension which is counteracted by the activity of antioxidants in the placenta. Any defect in the antioxidant defense mechanism will result in increased oxidative stress [8,9]. Our earlier studies have reported higher oxidative stress, lower maternal and placental angiogenesis in women with preeclampsia as compared to normotensive women [10–12]. Reports indicate that increased oxidative stress upregulates apopototic markers in the placenta [13] resulting in aberrant placentation and impaired angiogenesis [14]. A review summarises that endothelial cell apoptosis is associated with vascular

* Corresponding author. Mother and Child Health, Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth (Deemed to be University), Pune, Pune-Satara Road, Pune, 411043, India.
E-mail address: srjoshi62@gmail.com (S. Joshi).

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growth [15]. We have recently hypothesized that oxidative stress, altered angiogenesis and apoptosis are inter-related events in the pathophysiology of preeclampsia [16].

Nutrients like omega-3 fatty acids are suggested to influence apoptotic markers. Women supplemented with docosahexanoic acid (DHA) during pregnancy are reported to have lower placental caspase-3 levels as compared to women who were not supplemented [17]. Similarly, pregnant rats, fed a DHA rich diet, when subjected to hypoxic conditions demonstrated lower levels of neuronal apoptosis [18]. Our earlier studies report reduced erythrocyte omega-3 fatty acids, disturbed placental fatty acid metabolism and lower vitamin E levels in women with preeclampsia [12,19,20]. Omega-3 fatty acids are highly susceptible to peroxidation due to the presence of high number of double bonds in their structure and hence supplementing vitamin E (an antioxidant) along with docosahexaenoic acid (DHA) will help reduce lipid peroxidation.

Evidence suggests that dysregulation of apoptosis can lead to embryonal death and is involved in the pathophysiology of various inflammatory diseases [21]. We have recently reported increased embryotoxicity in a rat model of preeclampsia and supplementation of omega-3 fatty acid and vitamin E (antioxidant) was beneficial in case of late onset preeclampsia [22]. It is likely that the increased embryotoxicity observed in preeclampsia is associated with increased placental apoptosis. Also it remains to be established whether supplementation of omega-3 fatty acids and vitamin E are able to reduce placental apoptosis. Further, the amount of apoptosis in placental villi is known to change across gestation; it is the lowest initially in pregnancy and subsequently increases as pregnancy progresses [23].

Trophoblast apoptosis includes the extrinsic and intrinsic pathways culminating in the activation of caspases. Reactive oxygen species has been shown to upregulate the production of BAX proteins in the cytosol [24]. Expression of Bcl-2 is also known to be influenced by various angiogenic markers like vascular endothelial growth factor (VEGF) [25]. It is well established that the central executioners of apoptosis are the caspases which include the initiator caspases (caspase-8) and executioner caspases (caspase-3). Therefore, the current study reports the effect of maternal omega-3 fatty acids and vitamin E supplementation on the above placental apoptotic markers in early as well as late onset preeclampsia using a rat model at two time points across gestation.

2. Materials and method

2.1. Study design

The present study was initiated after obtaining the approval from the Institutional Animal Ethics Committee (IAEC/CPCSEA/BVDUMC/2670/2017/002/016). In the present study 72 female and 35 male Wistar albino rats (150-170 gms) were obtained from Bharati Medical College Animal House; Pune. The details of the study design are reported by us earlier [22]. All animals were maintained in a room with a 12:12 h light/dark cycle, controlled humidity and temperature. Water and food available was given *ad libitum*.

Animals were mated in a male: female ratio of 1:2; on the following morning the vaginal smears were taken and observed under a microscope using 10× magnification to confirm mating. The presence of sperms was considered as day 0 of gestation. Pregnant dams (n = 64) were randomly divided into control and 4 treatment groups. The five groups (n = 8 per group) were as follows: Control (C); Early onset preeclampsia (EOP); Late onset preeclampsia (LOP); Early onset preeclampsia + omega-3 fatty acid supplementation + Vitamin E (EOP + O + E) and Late onset preeclampsia + omega-3 fatty acid supplementation + Vitamin E (LOP + O + E). Supplementation of omega-3 fatty acids and vitamin E to the supplemented groups was started after confirmation of pregnancy i.e day 0 of gestation. Diets were prepared as per AIN-93 guidelines. The ratio of omega-3 to omega-6 fatty acids was 1:1. The source of omega-3 fatty acids was fish oil capsules (Mega-3, Dr. Reddy's Laboratories, India).

To induce preeclampsia, nitric oxide synthase inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME) (Sigma Chemical Co., St. Louis, MO, USA) was administered through oral gavage at a dosage of 50 mg/kg/d. To induce EOP, the animals from the EOP and EOP + O + E groups received L-NAME once a day for 13 consecutive days starting from day 7 to day 19 of gestation. To induce LOP, the animals from the LOP and LOP + O + E group received L-NAME once a day for 6 consecutive days starting from day 14 till day 19 of gestation.

Animals from the Control; EOP and EOP + O + E groups were sacrificed at two time points i.e d14 and d19 of gestation. The pregnant dams sacrificed at d14 of gestation received these diets for 14 days while those dissected on d19 of gestation received the diets for a period of 19 days. The animals from the LOP and LOP + O + E groups were dissected at d19 of gestation so they received diets for a period of 19 days. Anesthetic ether (Diethyl ether) was used to euthanize the dams and blood and placenta was collected.

Preeclampsia was confirmed by an increase in blood pressure and alterations in intrauterine development in both; EOP and LOP groups and has been reported by us earlier [22]. Briefly, L-NAME administration to the dams resulted in higher systolic and diastolic blood pressures ($p < 0.01$) in both EOP and LOP groups. Similarly, these groups had higher percentage of fetal resorptions and embryotoxicity (deformities and hematomas).

2.2. Analysis of dam plasma oxidative stress marker malondialdehyde (MDA)

Dam plasma levels of malondialdehyde (MDA) were estimated through spectrophotometric assay for MDA by using a kit from Percipio Biosciences OxisResearch (BIOXYTECH MDA-586 USA, Catalog No: 21044).

2.3. Preparation of placental tissue lysates and estimation of total protein

The total placental tissue was homogenized in 1X phosphate buffer saline consisting of protease inhibitors to prepare the tissue lysate. The total protein content was estimated using the Pierce BCA protein assay kit (Catalog No: 23225).

2.4. Analysis of placental protein levels of Caspase-3, Caspase-8, BAX and Bcl-2

Protein levels of caspase-3, caspase-8, BAX and Bcl-2 were analyzed from the tissue lysates by the ELISA method. Caspase-3, caspase-8, BAX were estimated using the Cusabio kits (Catalog No: CSB-E08857r, Catalog No: CSB-E14912r and Catalog No: CSB-EL002573RA respectively), Bcl-2 was estimated using the My Biosource kit (Catalog No: MBS704498).

2.5. RNA isolation and cDNA synthesis

The total RNA was isolated from the placental tissue using the Trizol reagent (Ambion by Life Technologies Ref- 15596018) and was quantified using the Biophotometer (Eppendorf, Germany). Reverse transcription of total RNA to single stranded cDNA was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems by Thermo Fischer Scientific, Foster City, CA, USA Ref- 4366596).

2.6. Analysis of mRNA levels of Caspase-3, Caspase-8, BAX and Bcl-2

The mRNA levels of caspase-3, caspase-8, BAX and Bcl-2 were evaluated using the Real-time quantitative PCR (RT-PCR) (Applied Biosystems 7500 Standard system). 100 ng cDNA was used for RT-PCR using the TaqMan Universal PCR Master Mix procured from Applied Biosystems, USA. To normalize the variation in the quality of RNA and the amount of input cDNA from the sample, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control gene. Relative expression levels of genes was calculated and expressed as

$2^{\Delta Ct}$. In $2^{\Delta Ct}$, ΔCt is the difference between the Ct of GAPDH and Ct of the target gene. The TaqMan assays ID (Applied Biosystems, USA) for the above mentioned genes are: GAPDH (Rn99999916_s1), caspase-3 (Rn00563902_m1), caspase-8 (Rn01440170_m1), BAX (Rn01480161_g1) and Bcl-2 (Rn99999125_m1).

2.7. Statistical analysis

Protein levels are expressed as mean ± SD and mRNA levels are expressed as mean ± SE. One-way ANOVA was used to compare means and post-hoc Tukey was used to test the differences among the means for various treatment groups at conventional levels of significance ($p < 0.05$). Two-way ANOVA was used to compare means of EOP and EOP + S at two different time points to understand the effect of supplementation and duration on various apoptotic markers. The data was analyzed using SPSS/PC + package (Version 20, Chicago IL).

3. Results

3.1. Dam plasma MDA levels at d14 and d20of gestation

Dam plasma MDA levels at d14 were higher, but not statistically significant in the EOP group. At d20 of gestation maternal plasma MDA levels were higher in the EOP group ($p < 0.01$) as compared to control and LOP groups, and supplementation to this group (EOP + O + E) resulted in reduced MDA levels as compared to EOP ($p < 0.05$), whereas in LOP and LOP + O + E groups MDA levels were similar to the control group (Table 1).

3.1.1. Effect of duration of L-NAME and supplementation of Omega-3 fatty acid and vitamin E on MDA levels

Two-way ANOVA demonstrates a significant effect only of supplementation ($f = 5.882$, $p = 0.006$) on the maternal plasma MDA levels.

3.2. Placental protein and mRNA levels of BAX at d14 and d20 of gestation

BAX protein and mRNA levels were similar across all the groups at d14 of gestation. At d20 of gestation, BAX protein levels were significantly higher in the EOP group ($p < 0.05$) compared to control. In LOP and LOP + O + E groups, BAX protein levels were similar to control. BAX mRNA levels were similar across all the groups (Fig. 1).

3.2.1. Effect of duration of L-NAME and supplementation of Omega-3 fatty acid and vitamin E on BAX levels

Two-way ANOVA demonstrates a significant effect of duration of L-NAME on protein ($f = 61.495$, $p < 0.01$) as well as for mRNA levels ($f = 7.129$, $p = 0.011$) of BAX.

Table 1
Dam Plasma Malondialdehyde (MDA) levels and Placental Apoptotic Index at d14 and d20 of gestation.

DURATION	GROUPS	MDA	APOPTOTIC INDEX
Day 14 of gestation	**CONTROL**	5.34 ± 2.95	169.42 ± 116.20
	EOP	7.57 ± 3.08	278.45 ± 148.69**
	EOP + O + E	6.98 ± 2.55	278.99 ± 120.28**
Day 20 of gestation	**CONTROL**	6.12 ± 0.49	33.58 ± 21.43
	EOP	8.94 ± 0.599**	200.48 ± 97.78**
	EOP + O + E	6.74 ± 0.57@@	180.19 ± 73.26**
	LOP	6.92 ± 0.72@@	136.78 ± 49.91**
	LOP + O + E	6.14 ± 0.591	95.30 ± 59.25

Values are expressed as Mean ± SD. p: Level of Significance; **$p < 0.01$ as compared to control, @@$p < 0.01$ as compared to early onset preeclampsia. Dietary Groups: Control (C); Early Onset Preeclampsia (EOP); Early Onset Preeclampsia Supplemented with Omega-3 Fatty Acids and Vitamin E (EOP + O + E); Late Onset Preeclampsia (LOP); Late Onset Preeclampsia Supplemented with Omega-3 Fatty Acids and Vitamin E (LOP + O + E); MDA: malondialdehyde; Apoptotic Index: Bcl-2 associated X-protein (BAX)/B cell lymphoma 2 (Bcl-2).

3.3. Placental protein and mRNA levels of Caspase-8 at d14 and d20 of gestation

At d14 of gestation, protein and mRNA levels of caspase-8 were similar across all the groups. Whereas, at d20 of gestation, protein levels of caspase-8 were significantly higher only in the EOP group ($p < 0.01$) compared to control, and supplementation reduced the higher levels in EOP + O + E ($p = 0.051$) when compared with EOP. There was no difference in the caspase-8 protein levels between the control, LOP and LOP + O + E groups.

mRNA levels were higher in the EOP group compared to control and LOP groups ($p < 0.01$ for both). Supplementation reduced the caspase-8 mRNA levels in the EOP + O + E group compared to EOP ($p < 0.05$). mRNA levels were also higher in LOP group ($p < 0.01$) compared to control and supplementation was beneficial in reducing the caspase-8 levels in LOP + O + E group (Fig. 2).

3.3.1. Effect of duration of L-NAME and supplementation of Omega-3 fatty acid and vitamin E on Caspase-8 levels

Two-way ANOVA demonstrates a significant effect of duration of LNAME on caspase-8 protein levels ($f = 82.481$, $p < 0.01$). For mRNA levels of caspase-8 significant effect was seen for supplementation ($f = 8.553$, $p = 0.001$) as well as a combined effect of supplementation and duration of L-NAME ($f = 5.741$, $p = 0.006$).

3.4. Placental protein and mRNA levels of Caspase-3 at d14 and d20 of gestation

Caspase-3 protein levels were higher only in the EOP group ($p < 0.01$) compared to the control group and supplementation reduced the caspase-3 levels at d14 of gestation. mRNA levels of caspase-3 were similar across all the groups. At d20, protein levels of caspase-3 were also significantly higher in the EOP group compared to control and LOP group ($p < 0.01$ for both) and supplementation to this group was beneficial in reducing caspase-3 levels and were statistically significant when compared with EOP ($p < 0.01$). mRNA levels of caspase-3 at d20 of gestation were similar across all the groups (Fig. 3).

3.4.1. Effect of duration of L-NAME and supplementation of Omega-3 fatty acid and vitamin E on Caspase-3 levels

Two-way ANOVA demonstrates a significant effect of supplementation ($f = 14.557$, $p < 0.01$) on protein levels of Caspase 3 whereas duration of L-NAME had a significant effect ($f = 6.457$, $p = 0.015$) on mRNA levels.

3.5. Placental protein and mRNA levels of Bcl-2at d14 and d20 of gestation

At d14 of gestation, protein and *m*-RNA levels of Bcl-2 were comparable in EOP and control whereas, supplementation reduced the protein and *m*-RNA levels ($p < 0.05$) of Bcl-2 in EOP + O + E group. At d20 of gestation placental protein and mRNA levels were lower in all the groups ($p < 0.01$ for all) compared to control (Fig. 4).

3.5.1. Effect of duration of L-NAME and supplementation of Omega-3 fatty acid and vitamin E on Bcl-2 levels

Two-way ANOVA demonstrates a significant effect of supplementation ($f = 16.381$, $p < 0.01$) as well as combined effect of duration and supplementation on Bcl-2 protein levels ($f = 4.108$, $p = 0.023$). For *m*-RNA duration of L-NAME ($f = 72.980$, $p < 0.01$), supplementation ($f = 21.185$, $p < 0.01$) as well as their combination ($f = 20.490$, $p < 0.01$) had a significant effect.

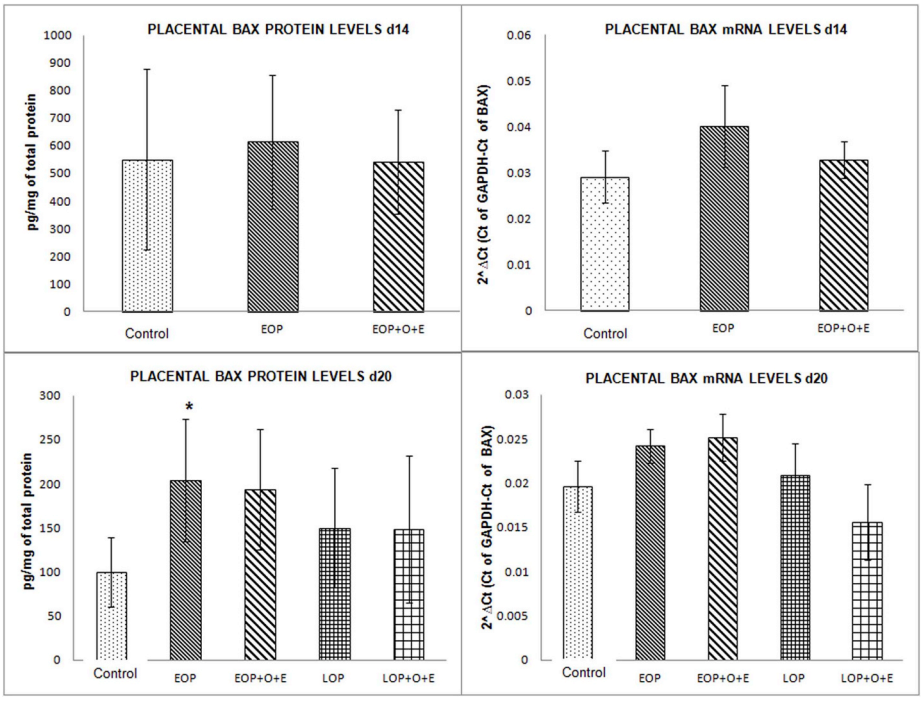

Fig. 1. Placental protein and mRNA levels of BAX at d14 and d20 of gestation.
Values are expressed as Mean ± SD (for protein) and Mean ± SE (for mRNA). p: Level of Significance; *p < 0.05 as compared to control. Dietary Groups: Control; Early Onset Preeclampsia (EOP); Early Onset Preeclampsia Supplemented with Omega-3 Fatty Acids and Vitamin E (EOP + O + E); Late Onset Preeclampsia (LOP); Late Onset Preeclampsia Supplemented with Omega-3 Fatty Acids and Vitamin E (LOP + O + E); BAX: Bcl-2 associated X-protein.

3.6. Apoptotic index (ratio of BAX/Bcl-2) at d14 and d20 of gestation

At d14 of gestation, apoptotic index was significantly higher in EOP as well as EOP + O + E group compared to control ($p < 0.01$ for both). At d20 of gestation, apoptotic index was significantly higher in both EOP and LOP group compared to control ($p < 0.01$ for both). Supplementation was beneficial in reducing the apoptotic index in LOP group only (Table 1).

3.6.1. Effect of duration of L-NAME and supplementation of Omega-3 fatty acid and vitamin E on apoptotic index

For Apoptotic Index two-way ANOVA demonstrates a significant effect of duration of L-NAME ($f = 12.799$, $p = 0.01$), supplementation ($f = 5.813$, $p = 0.01$) but there was no combined effect of supplementation and duration of L-NAME.

4. Discussion

To the best of our knowledge, this is the first study to have examined the effect of a combined supplementation of omega-3 fatty acids and vitamin E on apoptotic markers in subtypes of preeclampsia. The key findings are as follows: 1) Protein levels of BAX were higher at d20 of gestation in the EOP but not in the LOP group as compared to control. 2) Placental protein levels of caspase-8 were also higher in the

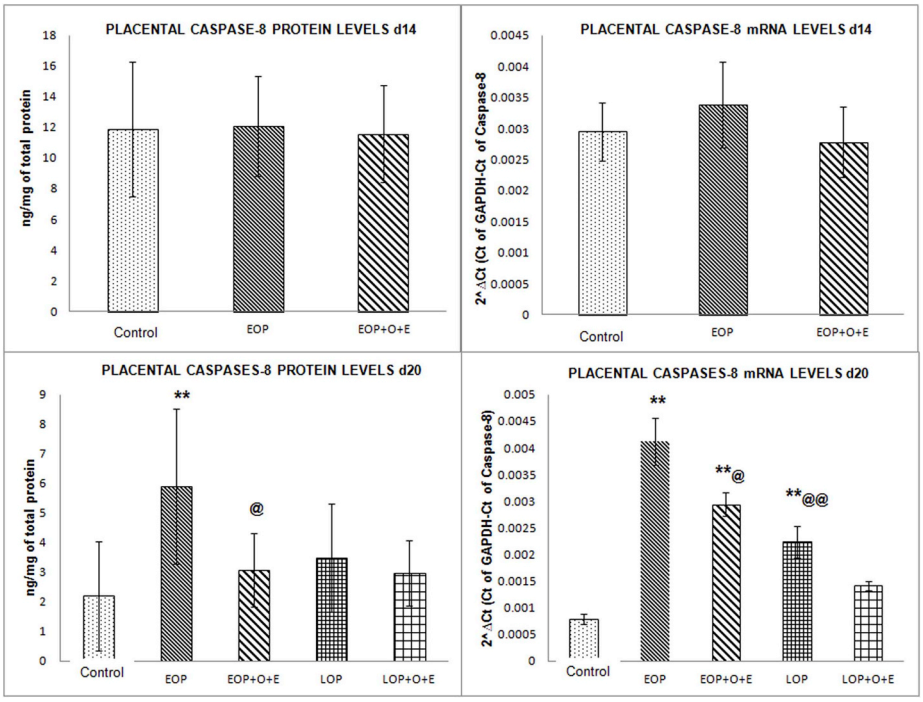

Fig. 2. Placental protein and mRNA levels of Caspase-8 at d14 and d20 of gestation.
Values are expressed as Mean ± SD (for protein) and Mean ± SE (for mRNA). p: Level of Significance; **p < 0.01 as compared to control, $^{@@}p < 0.01$, $^{@}p < 0.05$ as compared to early onset preeclampsia. Dietary Groups: Control (C); Early Onset Preeclampsia (EOP); Early Onset Preeclampsia Supplemented with Omega-3 Fatty Acids and Vitamin E (EOP + O + E); Late Onset Preeclampsia (LOP); Late Onset Preeclampsia Supplemented with Omega-3 Fatty Acids and Vitamin E (LOP + O + E).

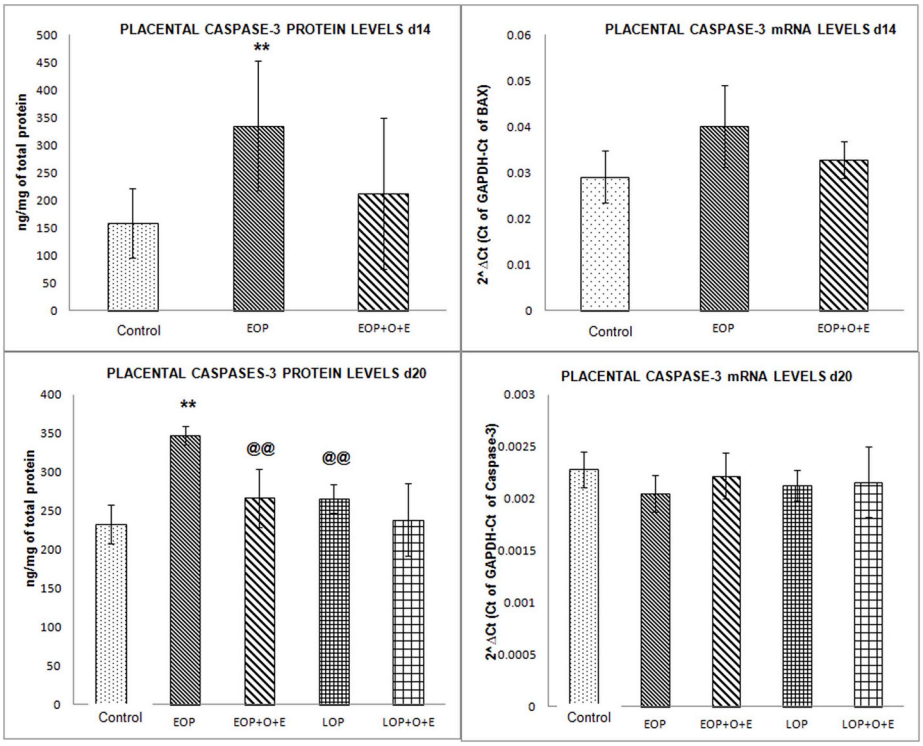

Fig. 3. Placental protein and mRNA levels of Caspase-3 at d14 and d20 of gestation.
Values are expressed as Mean ± SD (for protein) and Mean ± SE (for mRNA). p: Level of Significance; **p < 0.01 as compared to control, @@p < 0.01 as compared to early onset pre-eclampsia. Dietary Groups: Control (C); Early Onset Preeclampsia (EOP); Early Onset Preeclampsia Supplemented with Omega-3 Fatty Acids and Vitamin E (EOP + O + E); Late Onset Preeclampsia (LOP); Late Onset Preeclampsia Supplemented with Omega-3 Fatty Acids and Vitamin E (LOP + O + E).

EOP group as compared to LOP and control at d20 of gestation and 3) Caspase-3 protein levels were higher only in EOP group as compared to control at both time points i.e. d14 and d20 of gestation 4) Bcl-2 protein levels were lower in both the subtypes of preeclampsia as compared to control at d20 5) Supplementation did not improve the levels of BAX and Bcl-2, although it reduced the levels of caspase-8 and caspase-3 5) Apoptotic index was higher in both EOP and LOP groups as compared to control. However, supplementation was beneficial in reducing the apoptotic index only in the LOP group.

Our results showed that increased duration of L-NAME administration resulted in higher levels of BAX, caspase-8, caspase-3 and apoptotic index whereas it lower levels of Bcl-2. Longer duration of omega-3 fatty acid and vitamin E supplementation did not influence the levels of BAX and Bcl-2, but was beneficial in reducing protein levels of caspase-8, caspase-3 and the apoptotic index.

Reports from literature also refer to the L-NAME induced rat model

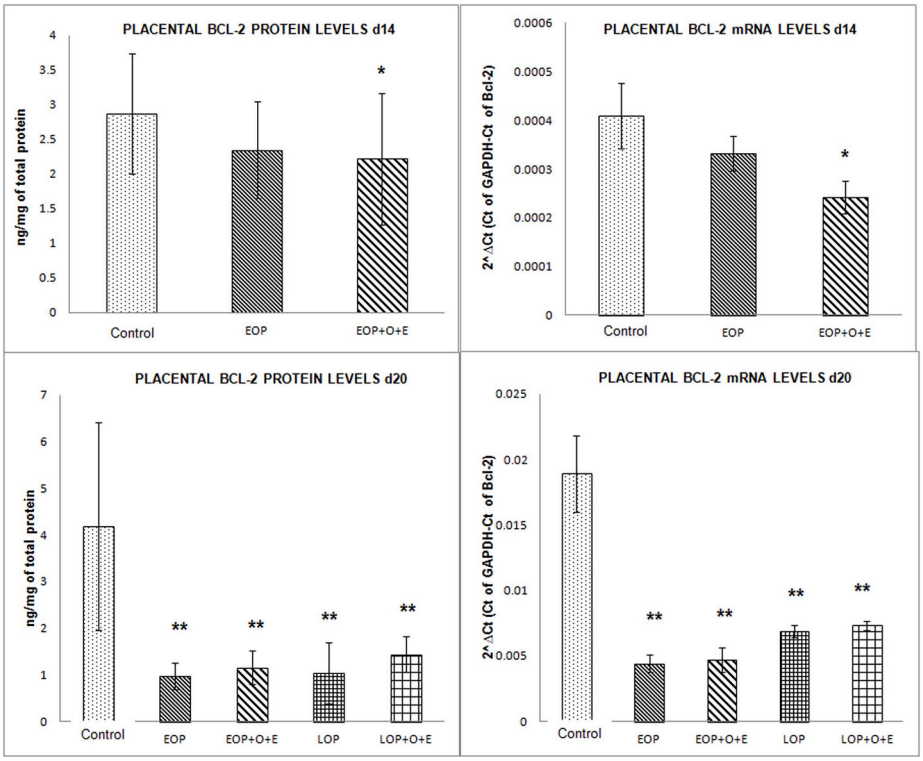

Fig. 4. Placental protein and mRNA levels of Bcl-2 at d14 and d20 of gestation.
Values are expressed as Mean ± SD (for protein) and Mean ± SE (for mRNA). p: Level of Significance; **p < 0.01, *p < 0.05 as compared to control. Dietary Groups: Control (C); Early Onset Preeclampsia (EOP); Early Onset Preeclampsia Supplemented with Omega-3 Fatty Acids and Vitamin E (EOP + O + E); Late Onset Preeclampsia (LOP); Late Onset Preeclampsia Supplemented with Omega-3 Fatty Acids and Vitamin E (LOP + O + E); Bcl-2: B cell lymphoma 2.

as a preeclampsia model [26,27]. In a series of our earlier studies we have reported higher oxidative stress, altered placental fatty acid metabolism and impaired angiogenesis in women with preeclampsia [11,19,20and28]. Similar results have been replicated in the current study as well as in our study using an L-NAME induced rat model [22,29]. Human preeclampsia is characterized by increased blood pressure which results in intrauterine growth restriction and in some severe cases also leads to miscarriages. Similar findings were also observed in the current animal model where the preeclampsia groups had a higher percentage of fetal resorptions and deformities as compared to the control group and has been reported by us earlier [22].

In humans, preeclampsia which develops before 34 weeks of gestation is referred to as EOP and is a deep placental disorder, LOP develops at or after 34 weeks of gestation. The rationale for the selection of d14 and d20 of gestation has been explained in detail by us earlier [22]. In the current study the terms EOP and LOP are based upon exposure to L-NAME and may not accurately mimic human conditions. Nevertheless, this is the first study to describe the effects of maternal nutrient supplementation on the two phenotypes of preeclampsia.

Oxidative stress is result of an imbalance between pro-oxidants and anti-oxidants. This disruption of redox control results in increased production of reactive oxygen species (ROS) like superoxide, hydrogen peroxide, and the hydroxyl radical which damage biomolecules such as proteins, RNA, DNA and lipids. Damage to the proteins results in protein carbonylation, leading to the loss of protein function and aggregation while RNA oxidation i.e. 8-hydroxyguanosine leads to defective protein synthesis and DNA damage at the guanine residues, results in mutations. Degradation of lipids leads to the formation of lipid peroxides [13]. Lipid peroxidation is a chain reaction initiated by free radicals, which can propagate itself resulting in the formation of several lipid peroxides [30]. MDA is one of the by-products of the lipid peroxidation process and is the most frequently used biomarker of oxidative stress [31].

In the current study, dam MDA levels were higher only in the EOP group compared to control. The results of the current study are supported by earlier studies in humans, where circulating levels of MDA are higher in preeclampsia compared to normotensive pregnancies [20,32–34]. Supplementation of omega-3 fatty acids and vitamin E to the EOP group was beneficial in reducing the plasma MDA levels. These findings are supported by a review which indicates that omega-3 fatty acid supplementation ameliorates oxidative stress [35]. The current study did not examine the other markers of oxidative stress and is a limitation of the study.

In this study, apoptosis was majorly observed to be higher at d20 of gestation and not at d14 which may possibly be due to the fact that the amount of apoptosis in placental villi is the lowest in the first trimester and increases at the end of gestation [36]. The placental BAX protein levels were similar at d14 and higher only in the EOP group at d20 of gestation; although there was no change in the mRNA levels at both time points. These results are supported by various studies in humans, animal and *in vitro* studies in HTR-8/SVneo cell lines where, BAX protein levels were elevated in the preeclampsia group compared to control and mRNA levels were similar among the groups [37–39]. In contrast, in the LOP group the BAX levels were comparable to the control group. No change in BAX levels have also been reported in placental tissues in women with preeclampsia [40,41]. One possible explanation for our findings may be attributed to the higher MDA levels in the EOP group and is supported by an earlier report which suggests that higher MDA levels upregulate proapoptotic markers in the placenta [42]. In this study the levels of BAX did not reduce as a result of supplementation (EOP + O + E group) as compared to the EOP group, although the levels of MDA reduced and needs to be explored further. It is known that oxidative stress upregulates the production of BAX and can lead to the activation of caspases [43].

Caspases are the intracellular machinery mediating apoptosis. Caspase-8 is an initiator caspase involved in the activation of the executioner caspase-3 [44]. The placental protein and mRNA levels of caspase-8 in the current study were similar at d14. At d20 of gestation the protein levels were higher only in the EOP group although mRNA levels were higher in both the groups. These findings are similar to earlier studies in humans, and animals which indicate higher caspase-8 levels in the placenta in preeclampsia as compared to control [45,46]. Supplementation to the EOP group was beneficial in reducing both the protein and mRNA levels of caspase 8 at d20 of gestation.

The protein levels of placental caspase-3 were higher only in the EOP group at both time points i.e. d14 as well as d20 of gestation. There are various studies in the human preeclampsia placenta which indicate higher caspase-3 levels [47–49]. In contrast, similar caspase-3 protein levels in control and L-NAME induced preeclampsia group are also reported [39]. In our study in the LOP group, caspase-3 protein levels were similar to control suggesting that the duration of L-NAME induction influences the levels of caspase 3. In the current study, supplementation of omega omega-3 fatty acids along with vitamin E to the EOP group was beneficial in reducing the protein levels of caspase-3.

We observed lower protein as well as gene expression of Bcl-2 in both the subtypes of preeclampsia compared to control at d20 of gestation. This is similar to the previous studies [50,51]. Supplementation of omega-3 fatty acids and vitamin E was not beneficial in increasing the Bcl-2 levels, and remained lower as compared to the control group. Future studies need to be carried out to examine the role of Bcl-2 family proteins in preeclampsia.

The ratio of BAX to Bcl-2 is often considered a useful index of cell death or survival [52,53]. Apoptosis rate is thought to increase in preeclampsia as a result of hypoxic injury and oxidative stress which triggers the intrinsic pathway of apoptosis [2]. In the present study apoptotic index was higher both in the EOP and LOP groups. Supplementation was able to reduce the apoptotic index only in the LOP group (Table .1).

In this study, the effect of supplementation on apoptotic markers may be as follows; Nitric oxide (NO) is responsible for vasodilation which is essential for blood pressure regulation. L-NAME acts as a competitive inhibitor for the enzyme eNOS and reduces the NO levels leading to vasoconstriction, impaired angiogenesis, higher oxidative stress and apoptosis in the placenta. It is also reported that NO prevent apoptosis by interfering with the activation of the caspase cascade [54]. Omega-3 fatty acids are known to increase the transcription and bioavailability of the eNOS enzyme [55] which results in high NO levels. VEGF has been reported to act as an agonist for eNOS activation [56]. We have recently demonstrated increased VEGF levels in the preeclampsia groups as a consequence of omega-3 fatty acid and vitamin E supplementation [57]. This suggests a potential role of omega-3 fatty acids in lowering the blood pressure, regulating angiogenesis and apoptosis.

In the present study, although there was a change in the protein levels, there was no difference in the mRNA levels. This may be either due to a) The procaspases which are known to start proteolytic cascade and activate downstream executioner procaspases resulting in higher protein levels of activated caspase-3 b) posttranscriptional regulation of gene expression c) difference in synthesis time and stability for RNA and proteins.

During normal pregnancy the balance between trophoblast apoptosis and proliferation results in normal trophoblast invasion [58]. Both proapoptotic and antiapoptotic molecules regulate apoptosis. Controlling the activity of caspases is essential for the appropriate regulation of cell death or survival. Inhibitors of apoptosis proteins (IAPs) are a family of proteins that control cell death in normal pregnancy [59]. Apoptosis of trophoblast cells is a natural event for normal placental development, however it is exaggerated in pathological conditions such as preeclampsia, due to an imbalance in the antiapoptotic and proapoptotic markers. Growth factors are known to induce IAP's in normal placental development. We have earlier demonstrated lower maternal placental growth factor levels in preeclampsia [28]. Lower levels of

IAP's such as XIAP, cIAP-1 and 2, TRPM-2, and survivin RNA have been reported in the preeclampsia placenta [60].

The possible explanation for our results may be as follows; in the control group there is a balance between proapoptotic and anti-apoptotic makers which leads to trophoblast survival and proliferation which is important for normal placentation. However, in the EOP group the level of MDA (a marker of oxidative stress) is high which activates the apoptotic pathways resulting in increased levels of apoptotic markers and lower levels of antiapoptotic markers which lead to abnormal placentation and embryotoxicity. Supplementation of omega-3 fatty acids and vitamin E to this group lowers the MDA levels and normalizes some of the proapoptotic markers like caspase-8 and caspase-3. However, supplementation could not normalize the Bcl-2 family proteins; resulting in a high apoptotic index as compared to control. LOP is a less severe form of the disorder, where the MDA levels and proapoptotic markers were comparable to control, but the antiapoptotic marker Bcl-2 was low suggesting increased apoptosis. Supplementation to the LOP group lowered the apoptotic index. To the best of our knowledge, there are no studies which have examined the effect of maternal omega-3 fatty acid and vitamin E supplementation on the two sub types of preeclampsia.

To summarize, in the current study, proapoptotic markers like BAX, caspase-8 and caspase-3 were higher only in the EOP but not in the LOP group and may be attributed to the severity of the disorder due to the increased exposure to L-NAME in the EOP group. We have earlier reported increased embryotoxicity and reduced conceptuses size in the EOP group and this study suggests that increased apoptosis may have contributed to the higher embryotoxicity. Our findings indicate that maternal supplementation with omega-3 fatty acids and vitamin E will help in reducing some of the proapoptotic markers and thereby reduce the severity of preeclampsia. Adequate pre-conceptional levels of omega-3 fatty acids and vitamin E may help in ameliorating the risk of preeclampsia. Future animal and human studies need to be undertaken to examine the same. It is also essential to examine the effects of varying doses of omega-3 fatty acids and vitamin E supplementation on the placental apoptotic markers.

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Declaration of competing interest

The author(s) declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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