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Maternal omega-3 fatty acids and vitamin E improve placental angiogenesis in late-onset but not early-onset preeclampsia

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Abstract
Abnormal placental vasculature is associated with preeclampsia. Preeclampsia is of two types, i.e., early- and late-onset preeclampsia (LOP), both having different etiologies. We have earlier demonstrated low levels of omega-3 fatty acids and vitamin E in women with preeclampsia. The current study examines the effect of maternal omega-3 fatty acids and vitamin E supplementation on angiogenic factors in a rat model of preeclampsia. Pregnant rats were divided into a total of five groups control, early-onset preeclampsia (EOP); LOP; EOP supplemented with omega-3 fatty acid and vitamin E and LOP supplemented with omega-3 fatty acid and vitamin E. Preeclampsia was induced by administering L-nitroarginine methylester (L-NAME) at the dose of 50 mg/kg body weight/day. The vascular endothelial growth factor gene expression and protein levels were lower ($p < 0.01$ for both) in animals from both EOP as well as LOP groups ($p < 0.01$). In the EOP group, the protein levels of VEGF receptor-1 were also lower ($p < 0.01$). Supplementation of omega-3 fatty acids and vitamin E to LOP improved the levels of VEGF and VEGF receptor-1 only in the LOP but not in the EOP group. In the EOP group, the gene expression of hypoxia inducible factor 1 alpha (HIF-1α) in the placenta was higher ($p < 0.05$) and supplementation normalized these levels. Our findings indicate that maternal supplementation of omega-3 fatty acids and vitamin E has differential effect on preeclampsia subtypes.

Keywords Hypoxia inducible factor 1 alpha · Omega-3 fatty acids · Peroxisome proliferator-activated receptor gamma (PPAR-g) · Preeclampsia · Vascular endothelial growth factor receptor · Vascular endothelial growth factor

Introduction

Vascularisation is crucial during pregnancy and facilitates the flow of blood from the maternal side to the growing fetus [1]. During fetal development, there is increased placental vasculogenesis and angiogenesis [2, 3]. Placental vascular growth begins around 21 days of gestation and continues throughout pregnancy [4]. An abnormal development of placental vasculature leads to placental insufficiency, resulting in pregnancy complications like preeclampsia [2].

The characteristic features of preeclampsia are proteinuria and raised blood pressure developing after 20 weeks of gestation. Preeclampsia can be either early-onset preeclampsia (EOP) or late-onset preeclampsia (LOP) both of which have different etiologies. The severe form, early-onset preeclampsia is associated with neonatal morbidity and mortality while LOP is associated with maternal morbidity [5]. Literature on human studies reports the existence of impaired feto-placental blood flow in EOP rather than LOP [6–9]. The angiogenic imbalance and apoptosis is more pronounced in EOP [10, 11].

Pro-angiogenic factor vascular endothelial growth factor (VEGF) influences endothelial cell function. VEGF and its receptors are mediated through the activity of transcription factors like hypoxia inducible factor 1 alpha (HIF-1α) [12, 13]. The association of peroxisome proliferator-activated receptor gamma (PPAR-g) with oxidative stress and angiogenesis is also reported [14]. The regulation of these transcription factors are known to be influenced by nutrients like omega-3 fatty acids [15].

Our earlier studies report reduced erythrocyte omega-3 fatty acids, disturbed placental fatty acid metabolism and altered angiogenesis in women with preeclampsia [16–20].

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These women had lower levels of vitamin E and higher oxidative stress [21]. Vitamin E is reported to increase VEGF expression in monocytes and may help in mediating angiogenesis [22]. Omega-3 fatty acids are also known to influence placental development at various stages of gestation [23]. We, therefore, hypothesize that maternal omega-3 fatty acid and vitamin E supplementation will influence placental angiogenesis in preeclampsia.

The current study reports the effect of maternal omega-3 fatty acids and vitamin E supplementation on placental angiogenic markers and transcription factors at two time points across gestation in an animal model of early and late-onset preeclampsia.

Materials and methods

Study design

The study was initiated after approval from the institutional animal ethics committee (IAEC/CPCSEA/BVDUMC/2670/2017/002/016) and was as per the Committee for the purpose of control and supervision of experimental animal guidelines, Govt of India. In the present study, Wistar albino rats (150–170 g) were obtained from an in-house facility (Bharati Medical College Animal House; Pune). Animals were mated in a male:female ratio of 1:2 and on the following morning the vaginal smears were taken, the presence of sperms was considered as d0 of gestation. On confirmation of pregnancy, dams were assigned to either of the five groups, i.e., 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). In the current study, we have used 8 animals per group. The power of this sample size is calculated based on our earlier study [24] reporting placental vascular endothelial growth factor (VEGF) levels in pregnant Wistar rats. A sample size of 8 per group gave a > 95% of probability of detecting a difference at an alpha of 0.05. In humans, EOP develops before 34 weeks of gestation and LOP develops after 34 weeks of gestation. Gestational day 1–10 in rodents is equivalent to the first trimester of humans and gestational day 10–20 corresponds to the second trimester [25]. Preeclampsia was induced by administering nitric oxide synthase inhibitor N (G)-nitro-L-arginine methyl ester (L-NAME). The dose of L-NAME used was 50 mg/kg/day (Sigma Chemical Co., St. Louis, MO). Late-onset preeclampsia was induced by administering L-NAME from day 14 to 19 of gestation while early-onset preeclampsia was induced by administering L-NAME from day 7 to 19 of gestation. Elevated levels of systolic and diastolic blood pressure in the same model have been reported by us earlier [26].

Diets

The diets used in the present study were prepared in accordance with the AIN-93 guidelines (Table 1). The preeclampsia groups were supplemented with omega-3 fatty acids and vitamin E. The source of omega-3 fatty acids in the present diets was fish oil capsules containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Table 1). The ratio of omega-3 to omega-6 fatty acids in the supplemented diet was 1:1.

Tissue homogenisation and total protein estimation

The placental tissue collected at day 14 and day 20 of gestation dams was homogenized in 1× phosphate buffer saline consisting of protease inhibitors to prepare tissue lysate. Protein assay kit (Pierce BCA; Catalog No: 23225) was used to estimate the total protein content of the placental tissue homogenate. Protein was expressed as mg/ml.

Estimation of angiogenic markers (VEGF, VEGF receptor-1 (VEGFR-1)) and transcription factor (HIF-1α and PPAR-g) protein levels

Protein levels of angiogenic markers like VEGF and VEGFR-1 and transcription factors like HIF-1α and PPAR-g were estimated from the tissue lysates by the ELISA method. VEGF was estimated using the Abcam kit (Catalog No: ab100787), VEGFR-1/Flt-1 using the kit from MyBiosource (Catalog No: MBS704848), HIF-1α and PPAR-g from Cloud Clone Corp. (Catalog No: SEA798Ra and Catalog No: SEA886Ra, respectively).

RNA isolation and cDNA synthesis

Invitrogen Trizol reagent was used for the isolation of total RNA from the placental tissue. The isolated RNA was quantitated using Biophotometer (Eppendorf, Germany). High-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) was used to transcribe total RNA to single-stranded cDNA.

Gene expression of VEGF, VEGFR-1, HIF-1α and PPAR-g

Real-time quantitative PCR (RT-PCR) (Applied Biosystems 7500 Standard system) was used for the gene expression analysis of VEGF, VEGFR-1, HIF-1α and PPAR-g. 100 ng cDNA was used for RT-PCR using the TaqMan Universal PCR Master Mix procured from Applied Biosystems, USA.

Table 1 Diet composition of control and treatment groups

Diet components (g/kg)	Control	EOP	LOP	EOP + O + E	LOP + O + E
Corn starch	398	398	398	398	398
Casein	200	200	200	200	200
Dextrin	132	132	132	132	132
Sucrose	100	100	100	100	100
Soyabean oil	70	70	70	25	25
Fish oil (mega-3)	0	0	0	45	45
Mineral mixture[a]	35	35	35	35	35
Cellulose	50	50	50	50	50
Vitamin mixture[b]	10	10	10	10	10
Vitamin E (IU)	0.3	0.3	0.3	0.6	0.6
Cystine	3	3	3	3	3
Choline chloride	2.5	2.5	2.5	2.5	2.5
Tertiary butyl hydroquinine	0.014	0.014	0.014	0.014	0.014
Total energy (kJ)	15.7	15.7	15.7	15.7	15.7

Dietary groups *C* control, *EOP* early-onset preeclampsia, *LOP* late-onset preeclampsia, *EOP + O + E* early-onset preeclampsia + omega-3 fatty acid + vitamin E supplementation, *LOP + O + E* late-onset preeclampsia + omega-3 fatty acid + vitamin E supplementation

[a]Mineral mixture (g/kg mixture): calcium carbonate, 357; potassium phosphate, 196; potassium citrate, 70.78; sodium chloride, 78; potassium sulfate, 46.6; magnesium oxide, 24; ferric citrate, 6.06; zinc carbonate, 1.65; manganous carbonate, 0.63; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenate, 0.01; ammonium paramolybdate, 0.007; sodium metasilicate, 1.45; chromium potassium sulfate, 0.275; lithium chloride, 0.01; boric acid, 0.08; sodium fluoride, 0.06; nickel carbonate, 0.03; ammonium vanadate, 0.006; sucrose, 221.02

[b]Vitamin mixture (g/kg mixture): nicotinic acid, 3; calcium pantothenate, 1.6; pyridoxine–HCl, 0.7; thiamin-HCl, 0.6; riboflavin, 0.6; D-biotin, 0.02; vitamin B_{12} (in 0.1% Mannitol), 2.5; vitamin E, 30; vitamin A, 0.8; vitamin D-3, 0.25; vitamin K, 0.075; folic acid, 0.2 (control) and sucrose 974.655, will be used to make total weight of the vitamin mixture to 1 kg

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control gene. Relative expression levels of genes were 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 is: GAPDH (Rn99999916_S1), VEGF (Rn01511601_m1), VEGFR-1 (Rn00570815_m1), HIF-1α (Rn01472831_m1) and PPAR-gamma (Rn0044095_m1).

Estimation of fatty acids from placenta

Gas chromatography was used to analyze the placental fatty acids and this procedure has been reported by our group [27]. Fatty acids were expressed as g/100 g fatty acid. Saturated fatty acids include myristic acid (MYR), palmitic acid and stearic acid (STE); total monounsaturated fatty acids include myristoleic (MYRO), palmitoleic (PALO) and oleic acid (OLE); the omega-3 fatty acids include alpha linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) while the omega-6 fatty acids include linoleic acid (LA), gamma linolenic acid (GLA), di-homo-gammalinolenic acid (DGLA), docosapentaenoic acid (DPA) and arachidonic acid (AA).

Statistical analysis

The data are expressed as mean ± SD/mean ± SE (for mRNA levels). 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. A $p < 0.05$ or $p < 0.01$ was considered significant. Two-way ANOVA was used to understand the effect of supplementation and duration on various markers. These data were analyzed using SPSS/PC + package (Version 20, Chicago IL).

Results

Pregnancy outcome

In the current study, L-NAME administration to the pregnant dams resulted in higher systolic and diastolic blood pressure (BP) in both the preeclampsia groups as compared to control ($p < 0.01$ for both). Supplementation of omega-3 fatty acid and vitamin E was able to normalize the BP only in the LOP group but not the EOP group. Litter weight and size were lower ($p < 0.01$) only in the EOP group and supplementation was not beneficial in increasing both litter weight and size.

The absolute and relative placental weights were found to be similar across all the treatment groups both at day 14 and day 20 of gestation.

Placental protein and mRNA levels of VEGF and VEGFR-1 at d14 of gestation

The VEGF protein levels in the EOP group were lower ($p = 0.01$) as compared to control. The mRNA levels of VEGF were also lower ($p = 0.1$) in the EOP group compared to control but were not statistically significant. Supplementation of omega-3 fatty acids and vitamin E was not beneficial in improving the VEGF protein and mRNA levels as compared to the EOP group as it remained lower ($p < 0.05$) than control (Fig. 1a).

Placental VEGFR-1 protein levels were lower in both the EOP and EOP + O + E groups as compared to control ($p = 0.025$ and $p = 0.032$, respectively). However, the mRNA levels of VEGFR-1 were similar across all the groups (Fig. 1a).

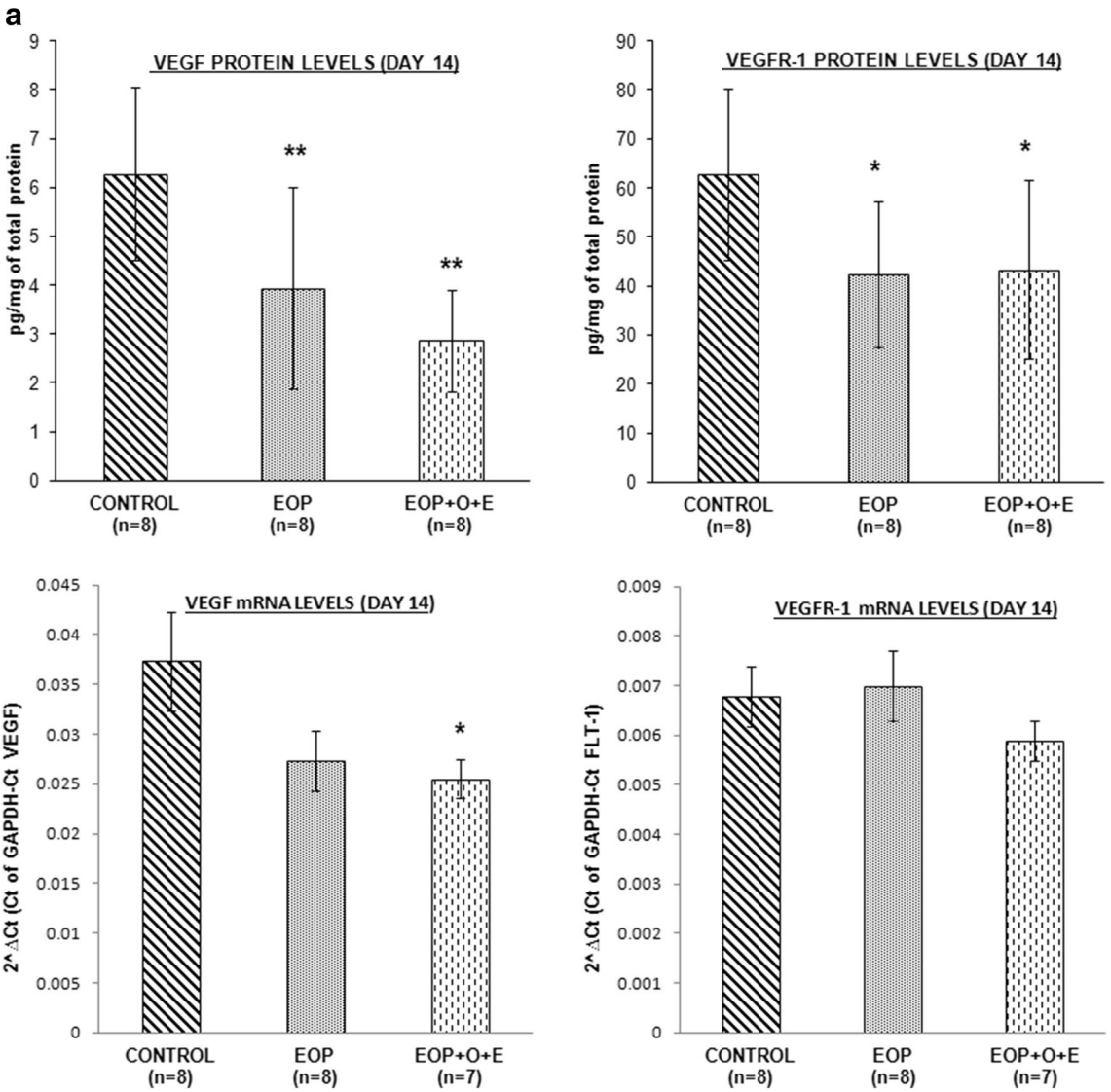

Fig. 1 **a** Angiogenic factors at day 14 of gestation. Values are expressed as mean ± SD (for protein levels) and mean ± SEM (for mRNA levels); $^{**}p < 0.01$, $^{*}p < 0.05$ as compared to control; dietary groups: *C* control, *EOP* early-onset preeclampsia, *EOP + O + E* early-onset preeclampsia supplemented with omega-3 fatty acids and vitamin E. **b** Angiogenic factors at day 20 of gestation. Values are expressed as mean ± SD (for protein levels) and mean ± SEM (for mRNA levels); $^{**}p < 0.01$, $^{*}p < 0.05$ as compared to control, $^{@}p = 0.075$ as compared to EOP. Dietary groups *C* control, *EOP* early-onset preeclampsia, *EOP + O + E* early-onset preeclampsia supplemented with omega-3 fatty acids and vitamin E, *LOP* late-onset preeclampsia, *LOP + O + E* late-onset preeclampsia supplemented with omega-3 fatty acids and vitamin E

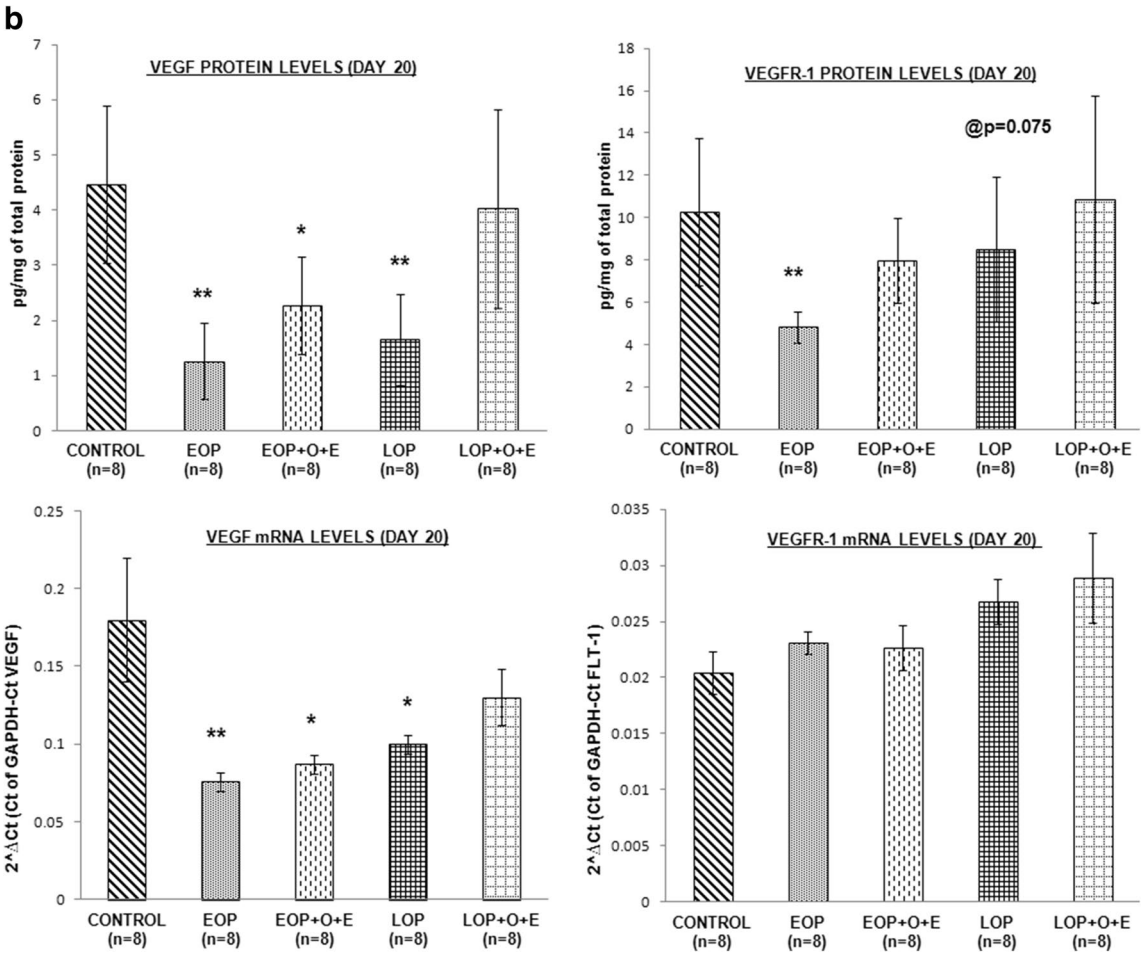

Fig. 1 (continued)

Placental protein and mRNA levels of VEGF and VEGFR-1 at d20 of gestation

VEGF protein levels were lower in both the EOP and LOP groups ($p = 0.000$ for both) as compared to control. The VEGF levels were higher in the LOP + O + E ($p = 0.064$) as compared to the LOP group. However, the VEGF levels in the EOP + O + E group were lower as compared to control ($p < 0.01$) (Fig. 1b). The mRNA levels were lower in both the EOP ($p = 0.012$) and LOP groups ($p = 0.09$). Supplementation was not beneficial in improving the mRNA levels in both the preeclampsia groups (Fig. 1b).

VEGFR-1 protein levels were lower in the EOP ($p = 0.005$) group but not in the LOP group as compared to control. VEGFR-1 protein levels were higher ($p = 0.075$) in the LOP group as compared to EOP group. The gene expression of VEGFR-1 was similar in all the groups (Fig. 1b).

Effect of duration of L-NAME and supplementation of omega-3 fatty acid and vitamin E on VEGF and VEGFR-1 protein and mRNA levels

Two-way ANOVA demonstrates a significant effect of duration ($f = 17.278$, $p < 0.01$) and supplementation ($f = 21.022$, $p < 0.01$) on VEGF protein levels. In case of VEGF mRNA levels along with duration ($f = 29.354$, $p < 0.01$) and supplementation ($f = 5.519$, $p = 0.08$), there was also a combined effect of the duration as well as of supplementation ($f = 3.594$, $p = 0.010$).

Two-way ANOVA demonstrates a significant effect of duration ($f = 145.300$, $p < 0.01$) and supplementation ($f = 5.481$, $p = 0.08$) on VEGFR-1 protein levels in both the EOP and EOP + O + E groups. However, only duration had an effect on VEGFR-1 mRNA levels ($f = 175.128$, $p < 0.01$).

Placental protein and mRNA levels of HIF-1α and PPAR-g at d14 of gestation

HIF-1α protein levels were similar to control in the EOP and EOP + O + E groups. The mRNA levels of HIF-1α were also comparable to control in the EOP and EOP + O + E group (Fig. 2a).

PPAR-g protein levels and mRNA levels were similar in the EOP and EOP + O + E groups as compared to control (Fig. 2a).

Placental protein and mRNA levels of HIF-1α and PPAR-g at d20 of gestation

HIF-1α protein levels were comparable across all treatment groups and control. The HIF-1α mRNA levels were significantly higher in the EOP ($p = 0.015$) group as compared to control and supplementation was able to normalize the HIF-1α mRNA levels. The HIF-1α mRNA levels in the LOP group was lower as compared to EOP group ($p = 0.055$) whereas in the LOP + O + E group they were similar to the control (Fig. 2b).

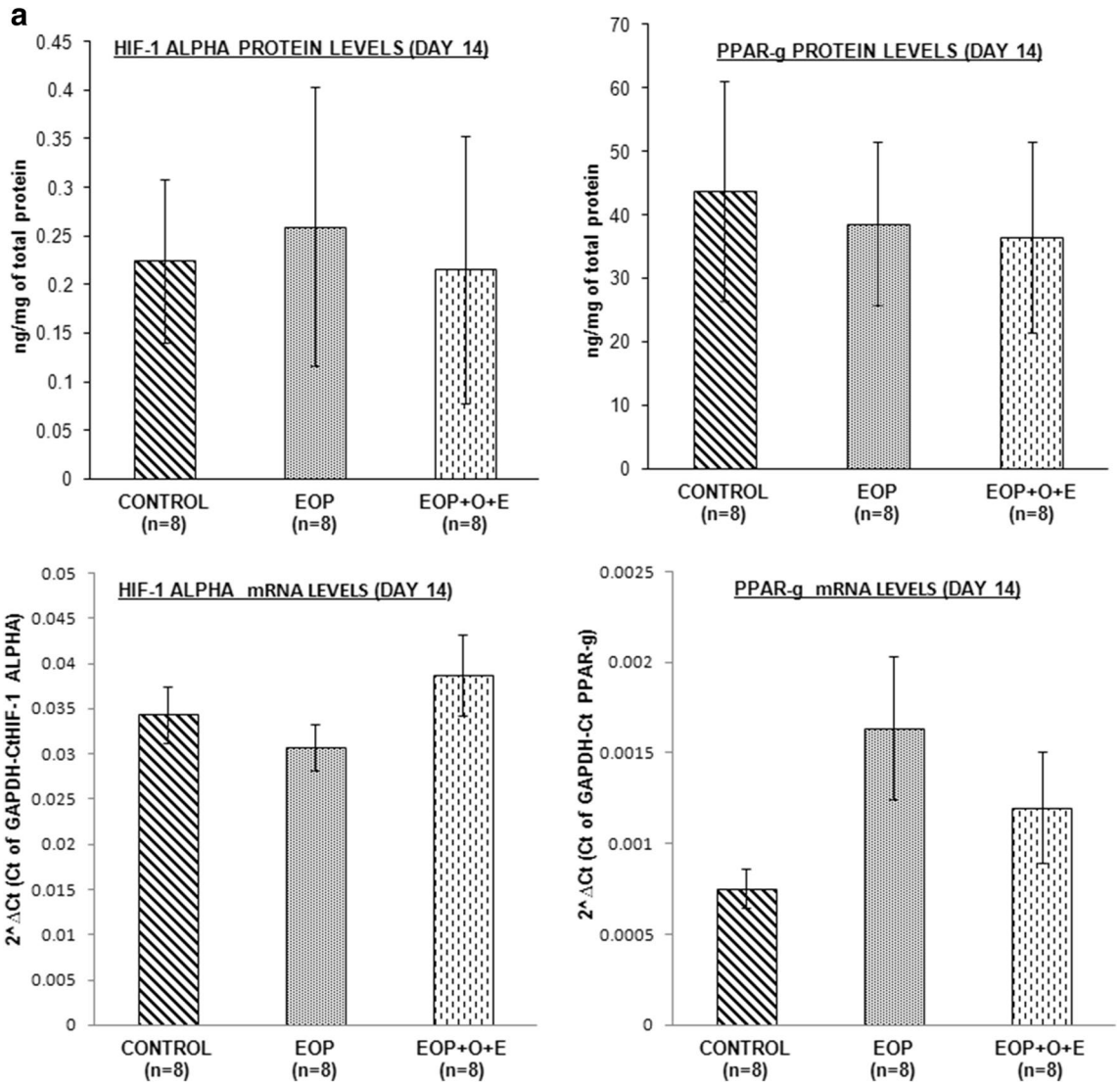

Fig. 2 **a** Transcription factors at day 14 of gestation. Values are expressed as mean ± SD (for protein levels) and mean ± SEM (for mRNA levels). Dietary groups: control (C); early-onset preeclampsia (EOP); early-onset preeclampsia supplemented with omega-3 fatty acids and vitamin E (EOP + O + E). **b** Transcription factors at day 20 of gestation. Values are expressed as mean ± SD (for protein levels) and mean ± SEM (for mRNA levels); $^{**}p < 0.01$ as compared to control, $^{@@}p < 0.01$ as compared to EOP, $^{\#}p < 0.05$ as compared to LOP. Dietary groups: *C* control, *EOP* early-onset preeclampsia, *EOP + O + E* early-onset preeclampsia supplemented with omega-3 fatty acids and vitamin E, *LOP* late-onset preeclampsia, *LOP + O + E* late-onset preeclampsia supplemented with omega-3 fatty acids and vitamin E

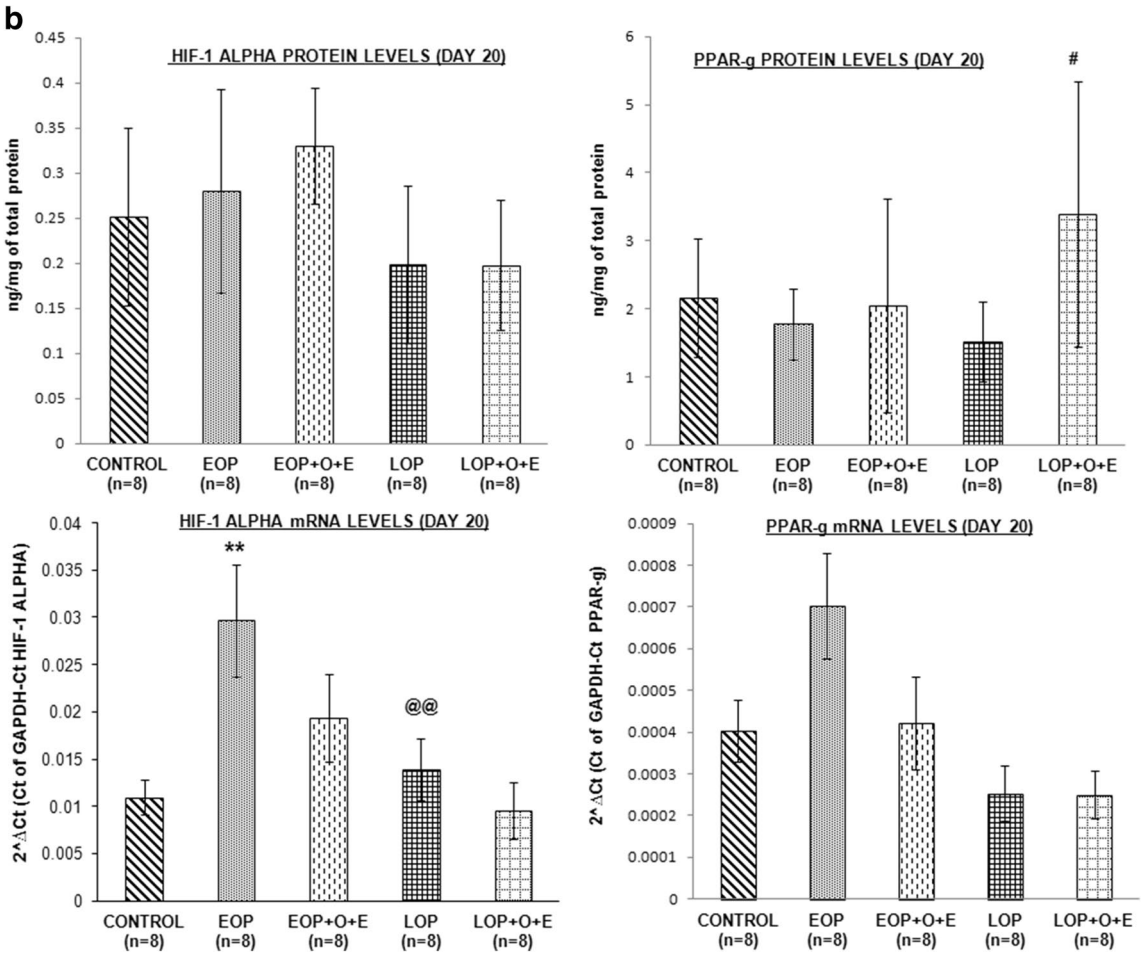

Fig. 2 (continued)

PPAR-g protein levels were similar in the EOP and LOP as compared to control. However, supplementation increased ($p < 0.05$) the PPAR-g (protein levels) only in the LOP + O + E group as compared to the LOP group. The mRNA levels of PPAR-g were similar across all groups (Fig. 2b).

Effect of duration of L-NAME and supplementation of omega-3 fatty acid and vitamin E on HIF-1α and PPAR-g protein and mRNA levels

Two-way ANOVA demonstrated a significant effect of supplementation ($f = 5.037$, $p = 0.01$) on HIF-1 alpha mRNA levels both in the EOP and EOP + O + E groups. Supplementation and duration also had a combined effect on the HIF-1 alpha levels ($f = 9.055$, $p = 0.01$).

Two-way ANOVA only had a significant effect of duration ($f = 134.186$, $p < 0.01$) ($f = 12.428$, $p = 0.001$) on PPAR-g protein and gene expression levels in both the EOP and EOP + O + E groups.

Placental fatty acid levels at day 14 of gestation

Table 2 shows the fatty acid profile in control and treatment groups at day 14 of gestation. The levels of omega-6 fatty acids such as LA, AA and total omega-6 were similar in EOP and control group. In supplementation group, i.e., EOP + O + E the omega-6 fatty acids such as LA, AA and total omega-6 fatty acids were significantly lower ($p < 0.01$) and total omega-3 fatty acids were higher than EOP and control group. Omega-3 fatty acid such as ALA, DPA n-3, EPA, DHA and total omega-3 fatty acid were similar in EOP and control group.

Placental fatty acid levels at day 20 of gestation

Omega-6 fatty acids (LA and AA) were significantly higher ($p < 0.01$) in the EOP group compared to control and LOP group. Supplementation was beneficial in reducing the LA, AA and total omega-6 fatty acid in the EOP + O + E group and LOP + O + E groups. Omega-3 fatty acids such as ALA and total omega-3 fatty acids

Table 2 Placental fatty acid levels at day 14 of gestation

Fatty acids (g/100 g fatty acids)	Control ($n=8$) (mean ± SD)	EOP ($n=8$) (mean ± SD)	EOP + O + E ($n=8$) (mean ± SD)
Linoleic acid	9.33 ± 1.23	9.72 ± 1.11	5.95 ± 1.17**@@
Gamma linolenic acid	0.15 ± 0.05	0.15 ± 0.06	0.24 ± 0.12
Alpha linolenic acid	0.15 ± 0.03	0.19 ± 0.07	0.21 ± 0.07
Dihomogamma linolenic acid	0.39 ± 0.30	0.58 ± 0.56	0.84 ± 0.65
Arachidonic acid	15.81 ± 1.02	15.11 ± 0.78	10.85 ± 1.42**@@
Eicosapentaenoic acid	0.13 ± 0.05	0.17 ± 0.06	1.48 ± 0.80**@@
Omega-3—docosapentaenoic acid	0.29 ± 0.06	0.30 ± 0.04	2.47 ± 0.49**@@
Docosahexaenoic acid	1.69 ± 0.24	1.69 ± 0.32	2.62 ± 0.24**@@
Total omega-3 fatty acids	4.18 ± 0.39	4.15 ± 0.48	6.52 ± 1.09**@@
Total omega-6 fatty acids	25.67 ± 1.80	25.56 ± 1.18	17.88 ± 2.47**@@
SFA	38.18 ± 2.43	39.96 ± 2.36	41.74 ± 1.20**
MUFA	1.43 ± 0.62	1.33 ± 0.57	1.31 ± 0.64

Values are expressed as mean ± SD

Dietary groups *C* control, *EOP* early-onset preeclampsia, *EOP + O + E* early-onset preeclampsia supplemented with omega-3 fatty acids and vitamin E, *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids

$^{**}p < 0.01$ as compared to control, $^{@@}p < 0.05$ as compared to EOP

were lower ($p < 0.01$) in the EOP and LOP groups as compared to control. DHA levels were significantly lower in the LOP group compared to control, whereas, in EOP group it was comparable with control. EPA and DPA n-3 were similar across all groups (Table 3).

Discussion

To the best of our knowledge, this is the first report on the effect of a combined supplementation of omega-3 fatty acids and vitamin E on angiogenic markers and transcription factors in early- and late-onset preeclampsia. The key findings are as follows: (1) placental protein levels and mRNA levels

Table 3 Placental fatty acid levels at day 20 of gestation

Fatty acids (g/100 g fatty acids)	CONTROL ($n=8$) (mean ± SD)	EOP ($n=8$) (mean ± SD)	EOP + O+E ($n=8$) (mean ± SD)	LOP ($n=8$) (mean ± SD)	LOP + O+E ($n=8$) (mean ± SD)
Linoleic acid	11.73 ± 1.04	13.08 ± 1.0**	8.5 ± 0.5**@@	11.3 ± 0.6@@	8.4 ± 0.6**##
Gamma linolenic acid	0.09 ± 0.04	0.15 ± 0.03	0.06 ± 0.006	0.12 ± 0.03	0.05 ± 0.01
Alpha linolenic acid	0.24 ± 0.04	0.09 ± 0.02**	0.04 ± 0.01**@@	0.08 ± 0.06**	0.04 ± 0.01**##
Dihomogamma linolenic acid	0.75 ± 0.10	0.87 ± 0.1	0.97 ± 0.05	0.7 ± 0.04	0.88 ± 0.01
Arachidonic acid	16.42 ± 0.89	17.69 ± 1.16*	13.42 ± 0.59**@@	16.2 ± 0.7@@	12.6 ± 1.4**##
Eicosapentaenoic acid	0.10 ± 0.04	0.19 ± 0.10	2.46 ± 0.47**@@	0.18 ± 0.07	2.22 ± 0.5**##
Omega-3—docosapentaenoic acid	0.36 ± 0.14	0.37 ± 0.1	3.13 ± 0.39**@@	0.22 ± 0.1	2.8 ± 0.4**##
Docosahexaenoic acid	2.79 ± 0.67	2.325 ± 0.36	4.8 ± 0.81**@@	1.84 ± 0.6**	5.2 ± 1.11**##
Total omega-3 fatty acids	3.61 ± 1.72	2.98 ± 0.46*	10.5 ± 1.5*@@	2.2 ± 0.5**	10.4 ± 1.93**##
Total omega-6 fatty acids	32.07 ± 1.49	33.23 ± 1.6	23.3 ± 0.9**@@	31.6 ± 1.02	22.2 ± 1.6**##
SFA	38.78 ± 1.79	39.08 ± 1.5	40.7 ± 0.7	38.7 ± 0.6	40.5 ± 1.3
MUFA	13.05 ± 1.17	11.9 ± 1.2	11.0 ± 0.7	14.0 ± 1.5	12.4 ± 0.8

Values are expressed as mean ± SD

Dietary groups *C* control, *EOP* early-onset preeclampsia, *EOP + O + E* early-onset preeclampsia supplemented with omega-3 fatty acids and vitamin E, *LOP* late-onset preeclampsia, *LOP + O + E* late-onset preeclampsia supplemented with omega-3 fatty acids and vitamin E, *SFA* saturated fatty acids, *MUFA* monounsaturated fatty acids

$^{*}p < 0.05$, $^{**}p < 0.01$ as compared to control, $^{@@}p < 0.01$ as compared to EOP, $^{\#\#}p < 0.05$ as compared to LOP

of VEGF were lower in both EOP and LOP groups, whereas supplementation of omega-3 fatty acids and vitamin E was beneficial only in case of the LOP group; (2) VEGFR-1 protein levels were lower only in the EOP group and supplementation was not beneficial in improving the VEGFR-1 levels; (3) placental HIF-1α mRNA levels were higher in the EOP group. Supplementation was beneficial in normalizing the HIF-1 α mRNA levels. (4) Supplementation increased the protein levels of PPAR-g in the LOP group. (5) Supplementation of omega-3 fatty acids along with vitamin E and duration of the L-NAME exposure as well as their combination had a significant effect on angiogenic markers and transcription factors.

Our earlier study using a rat model of preeclampsia reports lower placental VEGF levels in the late-onset group as compared to control. The study also reports that omega-3 fatty acids supplementation as well as combined supplementation of omega-3 fatty acids and micronutrients (vitamin B_{12} and folate) was beneficial in improving the VEGF levels [24]. It is likely that the improved levels of VEGF in the combined supplementation group could be due to the presence of omega-3 fatty acids as the individual supplementation of vitamin B_{12} and folate did not show a similar effect. However, supplementation of omega-3 fatty acids and the combined supplementation also increased the maternal plasma malondialdehyde levels [28]. To counter balance the oxidative stress, supplementation of antioxidants is important. Further, the earlier study examined the effects of supplementation only in late-onset preeclampsia. It is of importance to understand the effects of supplementation in both the subtypes of preeclampsia. Therefore, the current study was designed to examine the effect of omega-3 fatty acid supplementation along with vitamin E (antioxidant) on the angiogenic markers in both early-onset and late-onset preeclampsia.

In the current study, we observed that placental VEGF protein and mRNA levels were lower in both the preeclampsia subtypes as compared to control. The results of the current study are consistent with earlier reports by others and us which indicate that levels of VEGF are lower in preeclampsia [16, 29–31] indicating a disturbed angiogenesis [32]. Reports on the VEGF mRNA levels in women with preeclampsia are inconclusive with some studies reporting lower mRNA levels [33, 34] and others reporting higher levels [35]. Among the dietary factors, fatty acids and vitamin E are suggested to influence the process of angiogenesis [36]. A cell culture study by Johnsen et al. reports that DHA supplementation stimulated VEGF mRNA expression in HTR8/SVneo cell lines, suggesting the importance of omega-3 fatty acids in regulating placental angiogenesis [37]. Supplementation of vitamin E is reported to neutralize oxidative stress and promote angiogenesis in pregnant ewes [38]. Omega-3 fatty acid and vitamin E supplementation in the current study was beneficial in improving the VEGF protein levels only in the LOP group but not the EOP group.

In this study, the VEGFR-1 protein levels were lower only in the EOP group as compared to control. Binding of VEGF to its membrane bound receptor; VEGFR-1 stimulates the PI3/Akt pathway leading to cell proliferation and migration [39, 40]. The lower VEGFR-1 protein in EOP may be due to increased levels of sVEGFR-1 [41, 42] that is generated by alternative splicing and premature termination of VEGFR-1 pre-mRNA. In this study, supplementation did not improve placental VEGFR-1 levels.

In the present study, the HIF-1α in placental tissue of preeclampsia was comparable in all groups, but the mRNA levels were higher in the EOP group. During hypoxic conditions, HIF-1α increases the expression of VEGF in placental syncytiotrophoblasts and endothelial cells [43, 44]. Several studies have shown increased expression of HIF-1α mRNA in placental tissues of preeclampsia [45, 46]. In the present study, although there was a change in the mRNA levels, there was no difference in the protein levels and it may be because the gene expression could be regulated at the post-transcriptional level resulting in differences in the protein levels and there transcripts [47].

PPARs are nuclear receptor transcription factors which influence placental development [48, 49]. The current study demonstrated lower protein levels of PPAR-g although it was not statistically significant in both EOP as well as in LOP groups. Studies have shown reduced circulating PPAR-g activators in women with preeclampsia as compared to healthy pregnant women [50]. In contrast, there are studies that report no difference between the mRNA or protein levels of PPAR-g in women with preeclampsia and controls [51, 52].

PPAR are known to be activated by ligands such as long-chain polyunsaturated fatty acids. In the current study, in both the preeclampsia subtypes, placental omega-6 fatty acids were higher while omega-3 fatty acid levels were lower as compared to control. The omega-3 fatty acids like EPA and DHA are potent activators of PPARs than the omega-6 fatty acids [53]. However, supplementation of omega-3 fatty acids was beneficial in increasing the protein levels of PPAR-g only in the LOP group. Similarly, a study in rat cardiomyocytes demonstrates that omega-3 fatty acids stimulate PPAR-g gene expression [54].

Studies have suggested that the angiogenic/anti-angiogenic imbalance is milder in LOP rather than in EOP [55, 56]. In the current study, supplementation of omega-3 fatty acids was beneficial in normalizing the angiogenic factors like VEGF and transcription factors like PPAR-g only in the LOP group and not in the EOP group. Possible explanation for this differential effect of supplementation may be due to the following reasons: (1) the optimal fatty acid stores in LOP, (2) higher severity of insult in case of early-onset

preeclampsia group, and (3) milder angiogenic/anti-angiogenic imbalance in the late-onset group. These results also suggest a need for having an optimal fatty acid status at the pre-conceptional stage to reduce the severity of preeclampsia.

Conclusion

The present study indicates that placental angiogenic factors are disturbed in both subtypes of preeclampsia. Maternal omega-3 fatty acid and vitamin E supplementation was beneficial only in the late-onset but not early-onset preeclampsia in normalizing the levels of angiogenic and transcription factors. These findings suggest a differential role of maternal nutrient supplementation on angiogenesis in the two subtypes of preeclampsia. The findings of this study suggest that maternal supplementation with omega-3 fatty acids and vitamin E may be useful in reducing the severity of early-onset preeclampsia. It is likely that optimal omega-3 fatty acid stores during the preconception period and pregnancy may be lead to a better pregnancy outcome. Randomized trials on pregnant women need to be undertaken to evaluate the efficacy and safety of using a combined supplementation of omega-3 fatty acids and vitamin E during early gestation for the prevention of preeclampsia.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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