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Dietary omega-3 and -6 fatty acids affect the expression of prostaglandin E2 synthesis enzymes and receptors in mice uteri during the window of pre-implantation

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ABSTRACT

Considering possible effects of poly-unsaturated fatty acids (PUFA) on embryo implantation more likely through PGs, we investigated effects of dietary omega-3 and -6 PUFA on prostaglandin E2 (PGE2) signaling in mice uterus during pre-implantation period. The mRNA expressions of microsomal- and cytosolic- PGE synthase (mPGES and cPGES) and protein expressions of PGE receptor 2 and 4 (EP2 and EP4) were evaluated in uterus tissues of control as well as omega 3 and omega 6 received mice at days 1–5 of pregnancy. Expression of cPGES gene was not significantly different between groups but the mPGES expression on days 4 and 5 of pregnancy in supplemented groups was higher than controls. Omega-3 significantly decreased EP2 levels on days 3 and 4, while omega-6 caused an increase on days 3–5 of pregnancy. The levels of EP4 were significantly higher in the omega-6 group than other groups on days 4 and 5 of pregnancy. Also the implantation rate was higher in omega -6 compared to omega-3 group ($p = 0.006$). Moreover, there were significant correlations between implantation rate with expression levels of mPGES and EP2. Our results showed negative and positive effects of respectively dietary omega-3 and -6 PUFA on PGE2 signaling and implantation rate.

1. Introduction

The food habits have changed in favor of poly unsaturated fatty acids (PUFA), especially among the population of developed countries, although there is still some ambiguity regarding the consumption ratio between mega-3 (ω3) to omega-6 (ω6) [1]. Successful embryo implantation guarantees the outcome of pregnancy, and so, in order to increase the endometrial receptivity, appropriate changes in the endometrium are required [2–5]. Previous studies have demonstrated the influence of PUFA on embryo implantation and pregnancy outcome [6,7]. PUFA is believed to enforce potential influences on pregnancy via a wide variety of mechanisms (reviewed by Wathes et al. [8]) such as regulation of the prostaglandins (PGs) pathway [9]. PGs, especially the 2-series, play an essential role in embryo implantation [10]. The crucial roles of PGE2 in endometrial vascular permeability, blastocyst spacing, implantation, and decidualization [11], possibly through the PGE receptor 2 and 4 (EP2 and EP4), have been emphasized in the past [12,13]. Moreover, it has been demonstrated that reduced embryo adhesion, following the administration of PG inhibitors, could be restored by the addition of the PGE2 or the EP2 agonists [14].

The PGE2 is derived from PUFA through a certain synthesis pathway that contains various key enzymes such as cPGES, and mPGES [15]. Coyne et al. [9] have reported that dietary PUFA exerts luteotrophic effects in bovine endometrium via an increasing expression of mPGES. Previous studies have shown the inhibitory effect of docosahexaenoic acid (DHA) on PGE2 secretion in human decidual cells [16], bovine [17], and rat uteri [18].

Considering the aforementioned reports, we investigated that if

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the ω3 and ω6 supplementation could affect PGE2 signaling in the uterine tissue of mice during the window of pre-implantation, and consequently, affect the implantation rate.

2. Materials and methods

2.1. Animals and supplementation

Sixty female and 20 male adult albino NMRI mice varying weights of 20.5 ± 3.4 were prepared in the RAZI Institute of Iran. All the mice were kept under controlled conditions (temperature 25 ± 2 °C, 60–70% humidity with 12:12 h light and dark cycles), diet, and water, ad-libitum. The experimental procedures were in accordance with the National Institutes of Health guide for the care and use of Laboratory animals and were approved by the Animal Ethical Committee of the Tabriz University of Medical Sciences (Permit Number: 5/46139). After one week of adaptation, the females were randomly divided into three groups of 20 mice each; normal (fed standard pellets), omega-3 (fed standard pellets + 10% w/w of omega-3), and omega-6 (fed standard pellets + 10% w/w of omega-6). The omega-3 (ω3) supplement was provided as fish oil from Danna Pharma Co. (Tabriz, Iran) and for the omega-6 (ω6), soybean oil was used (Italy). After three weeks of supplementation, three female mice of each group were kept with a male mouse in a separated cage overnight in order to simulate natural mating. Observation of a vaginal plug and spermatozoa in the vaginal smear was considered as Day 1 of pregnancy. The female mice were killed between 09:00–10:00 h of Days 1–5 of pregnancy, and the uterine tissues were collected (four mice for each day in any group). After carefully washing the samples, they were frozen in liquid nitrogen and stored at −70 °C for subsequent measurements. The implantation site counts were evaluated by injecting of 0.1 ml of 1% Chicago blue (Sigma–Aldrich, USA) in the saline via a tail vein according to the described method (Fig. 1) [19].

2.2. Gene expression analysis

Under sterile conditions, the total RNA was extracted (miRCURY™ RNA Isolation Kit, Exiqon, Denmark). The samples were treated with DNase I to avoid genomic DNA contamination and the NG dART RT kit (Eurex, Poland) was applied to synthesize cDNA from the RNA. For the expression analysis following primers were used: cPGES forward 5′-ATGGAGCAGATGATGATTC-3′ and cPGES reverse 5′-GGTTAGAGGAGGCAAGTA-3′; mPGES forward 5′-GTGAGAAGGACTGAGATC-3′ and mPGES reverse 5′-ACTAATGATGACAGAGGAG-3′; and GAPDH forward 5′-GCGACTTCAACAGCAACTC-3′ and GAPDH reverse 5′-GCCGTATTCATTGTCATACCAG-3′. We used the MIC real-time PCR detection system (Bio Molecular Systems, Australia) and the SYBR Green kit (Eurex, Poland) for real-time PCR assay. Triplicate assays with following program were conducted: 10 min of initial denaturation at 95 °C, up to 40 cycles of 10 s in 95 °C for denaturation, 20 s in optimized annealing temperature, and 20 s in 72 °C as extension temperature. The melting curves were evaluated for product verification, and considering that the amplification efficiencies of the target and reference were approximately equal, the ΔΔCT calculation method was used to obtain the relative quantities [20].

2.3. Western blot analysis

For lysing the uterine samples ice cold RIPA Buffer (Sigma–Aldrich, USA), containing protease inhibitors (cOmplete™ Protease Inhibitor Cocktail, Roche, Germany) were used. After centrifugation, the protein concentration was evaluated in the supernatants (Pierce TM BCA protein assay kit, Thermo Fisher Scientific, USA). The samples were electrophoresed at equal concentration of protein (50 mg/lane) in 10% w/v sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Mini-PROTEAN Tetra Cell system (Bio-Rad, USA). After transferring the protein bands form gel to a methanol-preactivated polyvinylidene fluoride (PVDF) membrane (Roche, Germany), the membrane non-specific binding sites were blocked for one hour while gently shaking using dried non-fat milk 3% w/v in TBS plus 0.1% tween-20. Based on the target protein, the membrane was incubated overnight with the anti-prostaglandin E receptor (EP2) antibody diluted at a ratio of 1:700 (ab124419, abcam, USA) or the anti-prostaglandin E receptor (EP4) antibody diluted at a ratio of 1:1000 (ab93486, abcam, USA) at 4 °C. The β-actin was applied as reference using the anti-beta actin antibody (ab103548, abcam, USA). After washing the membrane incubated for one hour at 4 °C with a secondary antibody (anti-rabbit IgG-horseradish peroxidase, A6154, Sigma-Aldrich, USA) diluted at a ratio of 1:5000. The membrane was again washed and the bands were visualized using the Clarity™ Western ECL Substrate (Bio-Rad, USA). In order to identify the protein bands' molecular weight markers, (Thermo Scientific™, USA) was used. The densities of EP2, EP4, and β-actin were determined using the Image J software package and the relative density of each target protein was calculated to β-Actin.

2.4. Statistical analysis

The normal distribution of data was confirmed by the Kolmogorov–Smirnov test. The One-Way ANOVA Test following Tukey's Post Hoc Test was conducted to compare the data among groups as well as among different pregnancy days in each group. The Pearson Correlation Test was used to investigate the possible

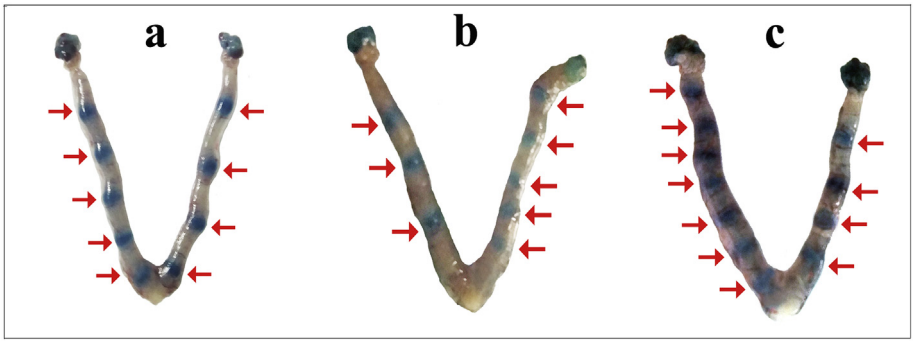


Fig. 1. Embryo implantation sites on the uterine horns of (a) control (b) omega-3 and (c) omega-6 groups at the fifth day of pregnancy; the red arrows indicate implantation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Relative mRNA expression of cPGES and mPGES genes in mouse uterus during embryo pre-implantation period.

	Day 1	Day 2	Day 3	Day 4	Day 5
Control group					
cPGES/GAPDH	1.94 ± 0.19	4.87 ± 0.85^{a}	3.95 ± 0.57^{a}	3.14 ± 0.35^{b}	$1.57 \pm 0.85^{b,c,d}$
mPGES/GAPDH	5.06 ± 1.16	4.36 ± 0.89	3.72 ± 1.03	3.01 ± 0.49^{a}	$1.76 \pm 0.55^{a,b,c}$
Omega-3 group					
cPGES/GAPDH	1.82 ± 0.42	5.06 ± 1.49^{a}	4.27 ± 0.69^{a}	3.06 ± 0.05^{b}	$1.38 \pm 0.24^{b,c,d}$
mPGES/GAPDH	4.88 ± 1.37	3.84 ± 0.36	$2.33 \pm 0.87^{a,\$}$	$2.23 \pm 0.79^{a,b,\$}$	$1.49 \pm 0.66^{a,b}$
Omega-6 group					
cPGES/GAPDH	2.18 ± 0.25	5.06 ± 0.49^{a}	4.33 ± 0.43^{a}	3.26 ± 0.95^{b}	$1.98 \pm 0.48^{b,c,d}$
mPGES/GAPDH	4.64 ± 0.36	4.22 ± 0.35	$2.29 \pm 0.04^{a,b,\$}$	$1.24 \pm 0.20^{a,b,c,\$,\dagger}$	$0.74 \pm 0.13^{a,b,c,\$}$

Data are presented as mean of $\Delta C_T \pm SD$; $\Delta C_T = C_{T\,(target\ gene)} - C_{T\,(Ref.\ gene)}$; **cPGES**, cytosolic prostaglandin E_2 synthase; **mPGES**, microsomal prostaglandin E_2 synthase; **GAPDH**, Glyceraldehyde-3-phosphate dehydrogenase.
Significant difference ($p < 0.05$) in comparison with [a] Day 1, [b] Day 2, [c] Day 3 and [d] Day 4.
Significant difference in comparison with [$]control group or [†]supplemented group at the same day of pregnancy.

association between the evaluated parameters on Day 5 of the pregnancy and the pregnancy rate. All statistical analyses were conducted using the SPSS software (Version 16). The differences between the means were considered significant when p was 0.05 or less.

3. Results

In the control group, the expression of cPGES was at a high level on the first day of pregnancy, and then, it experienced a sharp reduction on Day 2. The expression again started to increase with a mild slope between Day 2 and 4, and greatly increased on the fifth day (Table 1). Similar patterns of mRNA expression for the cPGES gene was found in both, the omega-3 and the -6 supplemented groups. As presented in Table 1, the mPGES expression levels in the uteri of normal mice were nearly at low and baseline levels between Days 1 and 3 of pregnancy, and gently rose on Day 4, and then, shot up on the implantation day. Unlike the control group, the increase in the mPGES expression began from Day 3 instead of Day 4 of pregnancy (Table 1). Our results indicated that the mPGES expression levels on Days 4 and 5 of pregnancy in both the omega-3 and -6 groups were higher in comparison with the control group ($p < 0.05$).

The results of protein expression of EP2 and EP4 in the uterine tissue are illustrated in Fig. 2. The EP2 protein expression pattern in the control (Fig. 2a), the omega-6 (Fig. 2c), and, the omega-3 (Fig. 2b) groups were about similar as the expression levels were low on the first and the second day of pregnancy, but started to enhance from Day 3, and reached a high and approximately constant level on Days 4 and 5. However, the EP2 protein amounts on Days 3 and 4 of pregnancy were significantly lower in the omega-3 group compared to the control group (Fig. 3a). Unlike the omega-3 group, the EP2 expression levels in the uterine tissues of the mice that had been supplemented with omega-6 fatty acids were significantly higher than the control group mice on Days 3, 4, and 5 of pregnancy (Fig. 3a).

Western blot analysis of EP4 protein levels showed that the receptor expressions were at very low levels on the first and the second days of pregnancy in uteri of all groups (Fig. 2a, b, and c). The EP4 protein levels were high on Days 3 and 4 of pregnancy in all three groups of mice (Fig. 2a and b). A comparison of the EP4 protein levels among the various groups during the window of pre-implantation revealed that the protein levels were significantly higher in the omega-6 group in comparison to both, the control and the omega-3 groups, on the fourth and fifth days of pregnancy ($p < 0.05$, Fig. 3b).

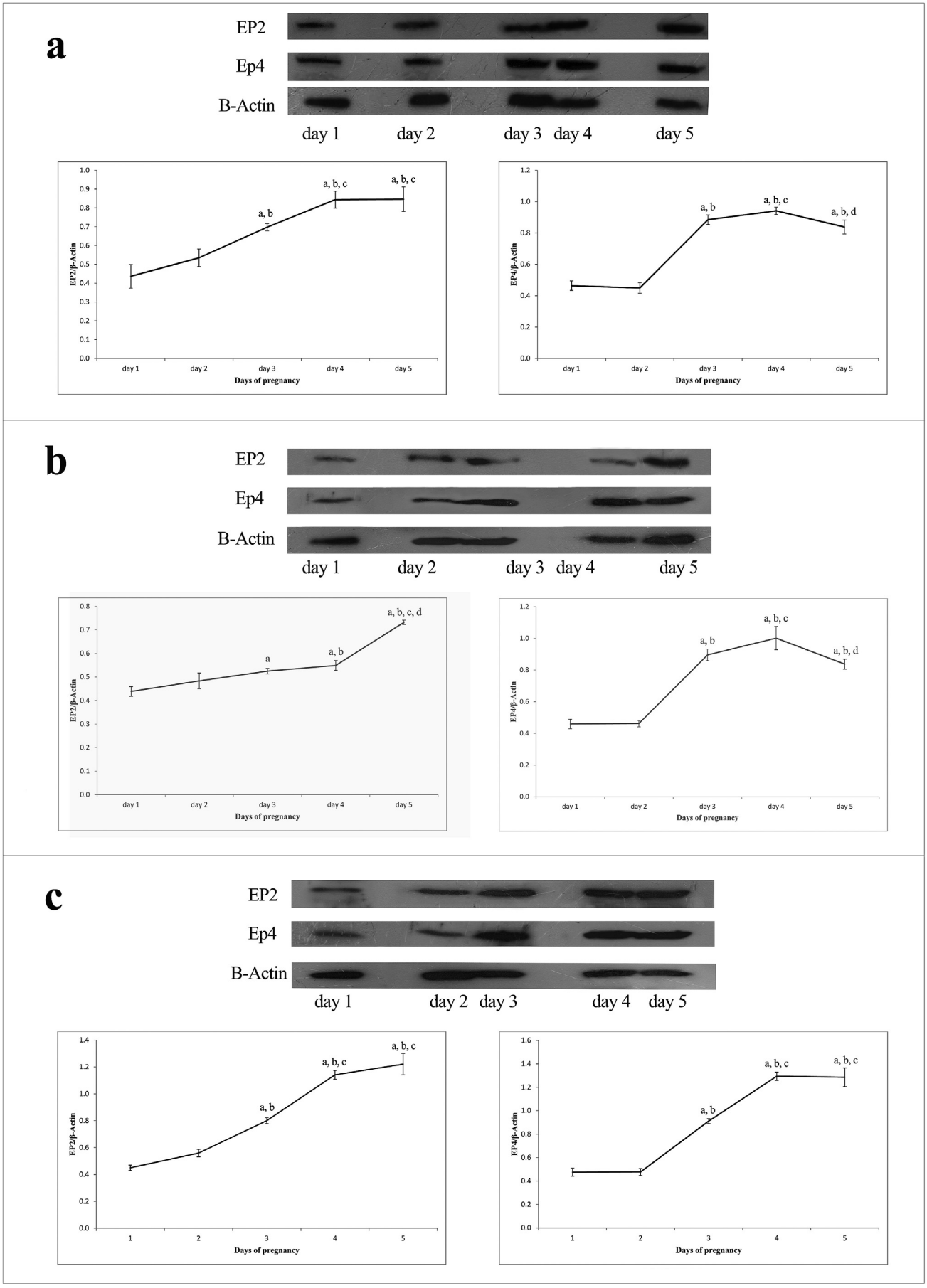
There were statistically significant correlations between the expressions of EP2 with EP4 ($r = 0.878$, $p < 0.001$), EP2 with mPGES ($r = 0.853$, $p < 0.001$), and EP4 with mPGES ($r = 0.639$, $p < 0.002$). The cPGES expression levels did not have statistical correlations with other evaluated factors ($p > 0.05$).

The embryo implantation rate in the control, the omega-3, and the -6 groups were 9.5 ± 1.29, 8 ± 0.82, and 11 ± 0.82, respectively ($p = 0.006$). Moreover, significant correlations were obtained between the implantation rate and the expression levels of mPGES and EP2 in control group, EP2 in the omega-3 groups and EP4 in omega-6 group (Table 2).

4. Discussion

Similar to a report by Ni et al. [21], we found reduced expression levels of cPGES on Days 2–4 of pregnancy, which then showed a strong expression on the embryo implantation day (Day 5). High levels of cPGES mRNA on the fifth day of pregnancy could be explained by the results of previous studies that indicated high expression of cPGES at implantation sites and with more intensity in decidualized cells [21] which suggests the role of cPGES in embryo implantation and pregnancy initiation. However, we did not find any statistical correlation between the implantation rate and cPGES expression. Our results showed that the high mRNA expression of cPGES in the uteri of mice on the first day of pregnancy was possibly due to mating and the activation of consequent inflammatory signaling [22]. Furthermore, we found that dietary omega-3 or -6 fatty acids could not affect the uterus expression of cPGES during the window of implantation. Since the exact regulating mechanism of the cPGES gene expression remains to be determined [22], the explanation of our results is complicated. However, in supporting our results, it has been reported that this gene is expressed constitutively and is not influenced by external stimuli [22] such as arachidonic acid (n-6 fatty acid) [23].

The mRNA expression pattern of mPGES during the pre-implantation period in all three groups was roughly similar and was in accordance with previously reported observations [12]. High expression of mPGES on implantation day and the day before that could pave the way for embryo implantation as we found a positive correlation between the expression levels of mPGES on Day 5 and the embryo implantation rate. Besides this, the results of the present study showed that dietary supplementation with both omega-3 and -6 significantly increased the mPGES expression levels on Days 4 and 5 of pregnancy. Other studies have also shown that PUFA could influence the activation and expressions of enzymes that are involved in the PGs pathway. In this context, the elevated expressions of PLA1, PGFS, PTGS1, and PTGS2 were demonstrated following omega-6 fatty acids supplementation [24,25]. In support of our results, Coyne et al. [9] indicated an increase in mRNA expression of mPGES1 in bovine endometrium following a dietary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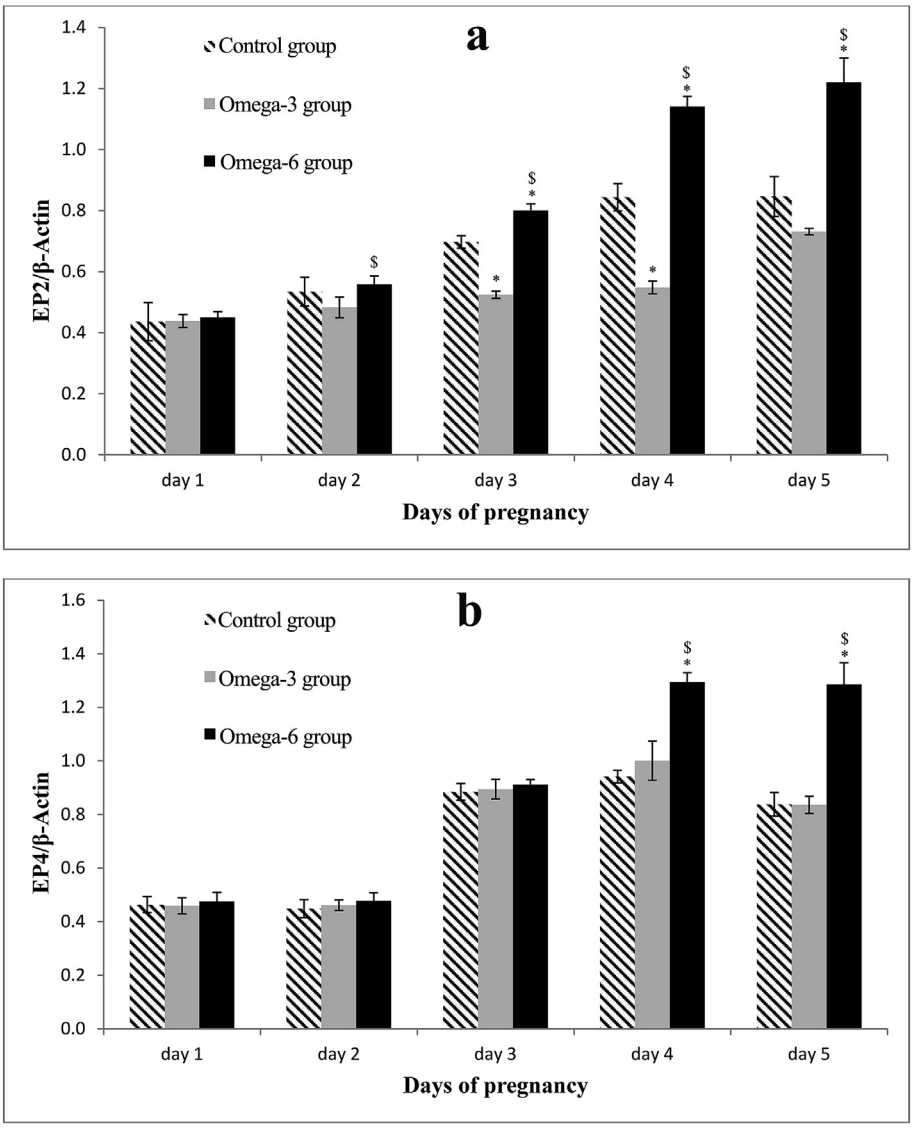

Fig. 3. Comparison of protein expression of (**a**) prostaglandin E receptor 2 (EP2) and (**b**) prostaglandin E receptor 4 (EP4) in uterine tissue among control, omega-3 and omega-6 groups. Significant difference ($p < 0.05$) in comparison with *control group and $omega-3 group.

Table 2
Correlation of implantation rate with expression of cPGES, mPGES, EP2 and EP4 in mouse uterine tissue at day 5 of pregnancy (implantation day).

	cPGES		mPGES		EP2		EP4	
	r	p	r	p	r	p	r	p
Control group								
Implantation rate (n)	0.926	0.074	0.993	**0.007**	0.985	**0.015**	−0.101	0.899
Omega-3 group								
Implantation rate (n)	0.833	0.167	0.092	0.908	0.998	**0.002**	−0.788	0.212
Omega-6 group								
Implantation rate (n)	0.437	0.563	0.880	0.120	0.940	0.060	0.971	**0.029**

cPGES, cytosolic prostaglandin E_2 synthase; **mPGES**, microsomal prostaglandin E_2 synthase; **EP2**, prostaglandin E receptor 2; **EP4**, prostaglandin E receptor 4. Bold-face numbers show statistically significant values ($p < 0.05$).

n-3 PUFA supplementation. In addition, it has been documented that supplementation with both EPA (n3 fatty acids) and AA (n6 fatty acid) increases the mPGES1 expression [25]. It has been concluded that an increase in mPGES expression after omega-3 and -6 supplementations could augment PGE2 levels, and consequently, exert potential luteotrophic effects on the uterus in favor of embryo

Fig. 2. Immunoblotting and quantitation of prostaglandin E receptor 2 and 4 (EP2 and 4) in uterine tissue of (**a**) control, (**b**) omega-3 and (**c**) omega-6 groups. Significant difference ($p < 0.05$) in comparison with [a] Day 1, [b] Day 2, [c] Day 3 and [d] Day 4.

implantation [9]. Although we did not evaluate PGE2 levels in the uterus samples, considering a wide variety of evidence about the increasing [26] and the decreasing [27,28] of series 2 PGs, especially PGE2 following the supplementation with omega-6 and omega-3 fatty acids, respectively, it can be inferred that the observed elevated uterine expression of mPGES following the omega-3 fatty acids supplementation could be in favor of the series 3 PGs production rather than the series 2 PGs. As mentioned previously, the increased levels of EPA (omega-3 fatty acid) can result in an elevated production of series 3 PGs [29], perhaps due to the replacement of AA with EPA, which leads to a lack of enough AA for the production of series 2 PGs. In the present study, the expression levels of mPGES were higher in the omega-6 group than omega-3 group on Day 4 of pregnancy. Such a difference could be due to the positive feedback of PGE¬2 on mPGES expression [30], since on the basis of the aforementioned causes, the production of PGE2¬ in the omega-6 group could be possibly higher than omega-3 received mice. However, such a difference was not found on Day 5 of pregnancy, probably due to the presence of the embryo that, in turn, can increase mPGES expression—similar effects have been seen in the Corpora Lutea of Pig [31].

The EP2 protein expression pattern in all three groups was in accordance with the previous findings [32], as the expression levels were low on early days of pregnancy, reaching a high and levelling on Days 4 and 5. The elevated protein levels of EP2 on the fourth and fifth days of pregnancy imply the possible roles of this receptor in embryo implantation as well as endometrium decidualization as we found a positive correlation between EP2 levels on Day 5 of pregnancy and the implantation rate. In line with this finding, an enhancement in embryo adhesion following endometrial EP2 activator administration has been reported [14]. The present study showed that the dietary supplementation of omega-6 fatty acids unlike omega-3 caused an increase in the protein levels of EP2. Increasing the effect of omega-6 fatty acids on PGE2 receptors has been confirmed previously as it has been demonstrated that corn oil diet (rich omega-6 fatty acids) could raise PGE2 receptor density in the macrophages [33]. One of the possible explanations for reduction in EP2 levels following the omega-3 fatty acid supplementation is that the omega-3 fatty acids could activate PPARγ [34] and PPARγ, in turn, suppresses EP2 mRNA and protein expression [35].

Our results proved that omega-3 supplementation could not affect the uterus expression of EP4, which was in consistent with previous findings which found no significant effect of flaxseed and fish oil supplementation on expression of EP4 [36] [37]. We found that omega-6 rich diet significantly increased protein levels of EP4 on Days 4 and 5 of pregnancy in comparison to the corresponding day of control and omega-3 groups.

The embryo implantation rate in the mice that had received omega-6 was significantly higher than the mice with omega-3 supplementation ($p = 0.006$). In support of this finding, it has been derived that the number of ovulated oocytes and presumed zygotes was lower in mice fed on a diet rich in omega-3 fatty acids and the percentage of oocytes trapped in luteinized follicles was higher in the omega-6 supplemented group [28]. Therefore, a lower implantation rate in the omega-3 group in comparison to the mice supplemented with omega-6 could be partly due to the decreased number of oocytes and zygotes. Another explanation for this difference could be due to the high expression of PGE2 producing enzyme as well as PGE2 receptors on the day of implantation in the omega-6 group, which would be in favor of uterine receptivity and embryo implantation [38].

In conclusion, our results showed the positive effects of omega-3 and omega-6 supplementation on mRNA expression of mPGES. In addition, we observed the increasing and decreasing effects of omega-6 and omega-3 fatty acids, respectively, on the uterus expression of EP2 during embryo implantation. Moreover, the EP4 levels elevated on Days 4 and 5 of pregnancy in the omega-6 supplemented group, but not the omega-3 group.

Conflicts of interest

There are no conflicts of interest.

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Transparency document

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