Eicosapentaenoic Acid Alleviates Inflammatory Response and Insulin Resistance in Pregnant Mice With Gestational Diabetes Mellitus

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Summary

This study investigated the effect of eicosapentaenoic acid (EPA) on insulin resistance in pregnant mice with gestational diabetes mellitus (GDM) and underlying mechanism. C57BL/6 mice fed with a high-fat diet for 4 weeks and the newly gestated were selected and injected with streptozotocin for GDM modeling. We demonstrated that the fasting insulin levels (FINS) and insulin sensitivity index (ISI) in serum and blood glucose level were significantly higher in GDM group than in normal control (NC) group. The low or high dose of EPA intervention reduced these levels, and the effect of high dose intervention was more significant. The area under the curve in GDM group was higher than that of NC group, and then gradually decreased after low or high dose of EPA treatment. The serum levels of TC, TG and LDL were increased in GDM group, while decreased in EPA group. GDM induced down-regulation of HDL level, and the low or high dose of EPA gradually increased this level. The levels of p-AKT2^{Ser}, p-IRS-1^{Tyr}, GLUT4, and ratios of pIRS-1^{Tyr}/IRS-1 and pAKT2^{Ser}/AKT2 in gastrocnemius muscle were reduced in GDM group, while low or high dose of EPA progressively increased these alterations. GDM enhanced TLR4, NF-κB p65, IL-1β, IL-6 and TNF-α levels in placental tissues, and these expressions were declined at different dose of EPA, and the decrease was greater at high dose. We concluded that EPA receded the release of inflammatory factors in the placental tissues by inhibiting the activation of TLR4 signaling, thereby alleviating the IR.

Key words

Gestational diabetes mellitus • Eicosapentaenoic acid • Insulin resistance • Inflammatory response • TLR4

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Introduction

Gestational diabetes mellitus (GDM) is defined as normal or potentially abnormal pre-pregnancy glucose metabolism, with reduced glucose tolerance primarily observed during pregnancy. In China, the current screening is commonly performed using a 75 g oral glucose tolerance test (OGTT) at 24-28 weeks of gestation [1]. In recent years, the incidence of GDM is on the rise, and patients with GDM are more likely to have recent complications such as pregnancy-induced hypertension, hyperamniotic fluid, premature rupture of membranes and preterm delivery, giant babies, neonatal hypoglycemia, and fetal distress [2,3]. GDM is a multifactorial disorder, and current research suggests that its development may be related to genetic factors, inflammatory factors, leptin and adipokine secretion [4,5]. The current clinical treatment of gestational diabetes includes diet, exercise, medication and novel treatment, such as probiotics. There is no clear evidence that any one treatment has the absolute superiority.

Eicosapentaenoic acid (EPA) is a polyunsaturated fatty acid extracted from deep-sea fish and belongs to the omega-3 PUFA group of polyunsaturated fatty acids [6-8]. Polyunsaturated fatty acids are lipids beneficial to human health and play an important role in suppressing TLR4-mediated intrinsic immune and

inflammatory responses [9]. Insulin resistance (IR) is central to the pathogenesis of gestational diabetes, and the chronic low-level persistent inflammation occurring on the placental surface may contribute to the formation and maintenance of IR. The inflammatory response on the placental surface can be activated when metabolites of pathogens such as bacteria and viruses (e.g. LPS) transferred to the maternal placenta, or when a poor diet induces abnormal metabolic states such as the saturated fatty acid secondary metabolites in the blood [10,11]. Since these surface cells express a large number of Toll-like receptor 4 (TLR4) receptors, when the ligands produced by various external or internal environmental disturbances bind to TLR4 receptors, the downstream target molecules can be activated, thus causing sustained inflammatory responses [12]. Therefore, EPA may improve IR and anti-hyperglycemic and anti-hyperlipidemic effects by inhibiting TLR4-mediated inflammatory responses on the placental surface.

In view of the above basis, the aim of this paper is to investigate the effect and mechanism of EPA regulating inflammatory factor levels on IR in pregnant mice with GDM. It is demonstrated that EPA may reduce the expression level of inflammatory factors on the placental surface by inhibiting the activation of TLR4, thus ameliorating IR.

Methods

Laboratory animals

The 4-5-week C57BL/6 mice were provided by Beijing Huafu Kang Biotechnology Co., Ltd. Mice were housed in 25±2 °C temperature, 45±5 % humidity, and 12/12 h light for a week with a normal diet. Then, the mice were randomly divided into 2 groups given either a normal diet or a high-fat diet (Table 1 for the specific formula) for 4 weeks. 12 mice weighing more than 18 g were selected in the NC group, and those weighing more than 22 g were selected in the high-fat diet group. The two groups were caged together with a 2:1 ratio of male to female, and a pregnancy examination was performed at 07:00 in the next day. The day the vaginal plug appeared was classified as gestation day 0 (GD 0). The animal study was approved by Animal Ethics Committee of the North China University of Science and Technology Affiliated Hospital and the protocols were in accordance with the Guide for the Care and Use of Laboratory Animals (1985).

Table 1. Regular maintenance versus high fat feed formulations (n, %).

Nutrients	**General maintenance feed**		**High fat feed**	
	Weight (g)	Energy supply ratio (%)	Weight (g)	Energy supply ratio (%)
Protein	12.5	13.5	23.6	21.0
Carbohydrates	72.3	77.0	40.5	33.9
Fat	4.1	9.5	23.5	45.1
Saturated fatty acids	1.6	4.2	7.6	14.2
Polyunsaturated fatty acids	1.9	4.6	8.5	16.0
n-3 PUFA	0	0.0	0.5	1.1
n-6 PUFA	0.4	0.8	7.4	14.0

Experimental animal modeling, grouping and EPA intervention

The GDM model was established followed previous descriptions [13,14]. Mice fed with a high-fat diet, weighing >22 g and pregnant were selected for GDM modeling. The mice were fasted from 18:00 on GD4 (normal water intake) to 06:00 on GD5, and injected intraperitoneally with streptozotocin (STZ) solution (45 mg/kg-bw, Jiuding, Biotechnology Co., Ltd., Beijing) on GD5, 6 and 7, with 24 h of interval time.

After each injection, the blood samples were collected in the early morning of the next day by cutting the tip of the tail, and the random blood glucose was detected using a glucometer (Sanuo Pharmaceutical Technology Co., Ltd., Shanghai). GDM model was considered successful if one or more times of random blood glucose ≥11.1 mmol/l occurred within 72 h after injection. The successful modeling GDM mice were

randomly divided into GDM group, low-dose EPA intervention group (GDM+LI) and high-dose EPA intervention group (GDM+HI). Each group contains 8-12 mice. In addition, 10 normal pregnant mice at the same pregnancy time without DM were selected as a normal control (NC) group.

During the gestation period GD8-18, the NC (pregnant mice without DM) and GDM groups were given equal volume of 5 ‰ sodium carboxycellulose solution by gavage (100 μl), the GDM+LI and GDM+HI groups were treated with 100 mg/kg-bw (100 μl) and 300 mg/kg-bw (100 μl) of EPA (Jinbite Biotechnology Co., Ltd., Beijing) by gavage, respectively. EPA was diluted in 5 ‰ sodium carboxycellulose solution and the gavage was performed once a day. For gavage, the throat and esophagus of mice should be kept in line as much as possible. The gavage needle was pressed from the mouth of mice to the root of the tongue and inserted slowly along the swallowing movement of mice.

Enzyme-linked immunosorbent assay (ELISA)

Orbital blood collection was performed to isolate the serum, and the gastrocnemius muscle and placenta were retained after sacrifice. The corresponding mouse ELISA kits were used to measure the serum insulin content, total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) and high density lipoprotein (HDL) according to the instructions. All the mouse ELISA kits were purchased from Xiamen Huijia Biotechnology Co., Ltd. After adding the sample, washing the plate, and filling with the working solution of the chromogenic substrate, 50 μl of the termination solution is mixed to stop reaction. The absorbance value at 450 nm in each well was recorded using an enzyme reader. The formula for insulin sensitivity index (ISI) is as follows: ISI = ln [1/(FINS-FPG)].

Oral glucose tolerance test (OGTT)

The 50 % high-glucose solution was prepared using normal saline. Mice were fasted for 12 h with no food and only water before performing the OGTT. After stable holding, 0.1 ml of 50 % high-glucose solution was immediately injected into the stomach using a gavage needle. The blood glucose level was measured at 0 min, 30 min, 60 min, 90 min, and 120 min. The area under the curve (AUC) was calculated as follows: AUC=1/2×(A+B)×0.5+1/2×(B+C)×1.5. The A, B and C represented the blood glucose levels at 0 min, 60 min, and 120 min.

Western blot

Total protein from mouse gastrocnemius muscle tissue was extracted, and a BCA protein kit (Solarbio, Beijing, China) was used to quantify the protein concentration. The SDS-PAGE were performed using a bromophenol indicator. A 5 % gel concentrate and a 10 % separator gel were selected and 20 μl of protein samples were taken for loading. After electrophoresis, the proteins were transferred to PVDF membranes by electro-transfer at a constant current of 200 mA for 90 min. After being blocked in 5 % skim milk for 2 h, the PVDF membranes were incubated with primary antibodies against anti-IL-1β, anti-IL-6, anti-TNF-α, anti-NF-κB p65, anti-GLUT4, anti-pIRS1^{Tyr}, anti-IRS1, anti-pAKT2^{Ser}, anti-AKT2 and anti-β-actin (ABclonal, USA; 1:1000) overnight at 4 °C. The membranes were washed 3 times using TBST, and then incubated with secondary antibodies for 2 h at room temperature. After washing, the membranes were imaged chromogenically using ECL chemiluminescent solution.

Immunohistochemical staining

The sections were dehydrated in a gradient of ethanol and the sections were rinsed three times using PBS solution. Then, 30 μl of trypsin was added dropwise on each section, following by digestion at 37 °C for 30 min. After adding the peroxidase blocker (20 μl) and incubation for 20 min, the primary antibody anti-TLR4 (ABclonal, USA; 1:400), secondary antibody, and DAB was added in order. Next, the sections were dehydrated, transparent, sealed, and the specimens were stained with anti-TLR4. The mean optical density of yellow luminescence was analyzed using the ImageJ software. The above results were averaged in three tests by different professionals.

Statistical methods

All the data were expressed as mean ± standard deviation (mean ± SD). For data comparison, the normal distribution analysis and consistency of variance were tested, following by one-way ANOVA with Tukey's *post hoc* test. Differences were statistically significant when $P<0.05$.

Results

Impact of EPA intervention on IR

A total of 36 mice were employed for GDM modeling, 32 were successfully modeled and 26 showed symptoms of polyphagia and polyuria. The

number of mice in each group was adjusted to 8 by taking the smallest number of surviving mice in initial group. As shown in Table 2, the blood glucose level of pregnant mice was significantly higher in the GDM group compared to the NC group. In comparison with GDM group, the blood glucose level was reduced in GDM+LI group and GDM+HI group, and the GDM+HI group reduced more significant (P<0.01). The result in Figure 1A showed that the body weight of mice was gradually increased from GD9-11 to GD18, suggesting the growth of embryo. In Figure 1B-C, the insulin levels in pregnant mice were significantly higher in the GDM group compared to the NC group, while these levels were decreased in GDM+LI group and GDM+HI group, ISI was significantly higher in the GDM group compared to the NC group, while this index was declined in the GDM+LI group and the GDM+HI group, revealing more significant decrease in GDM+H group (P<0.01). These results suggested that IR was induced in GDM and the EPA intervention reduced IR level.

Table 2. Comparison of fasting blood glucose in each group (x ± s).

Groups	**n**	**FPG/mmol/l**
NC	8	8.2 ± 0.6
GDM	8	$16.5 \pm 0.6^{***}$
GDM+LI	8	$13.72 \pm 0.6^{\#\#}$
GDM+HI	8	$10.62 \pm 0.6^{@@}$
F		89.7
P		0.000

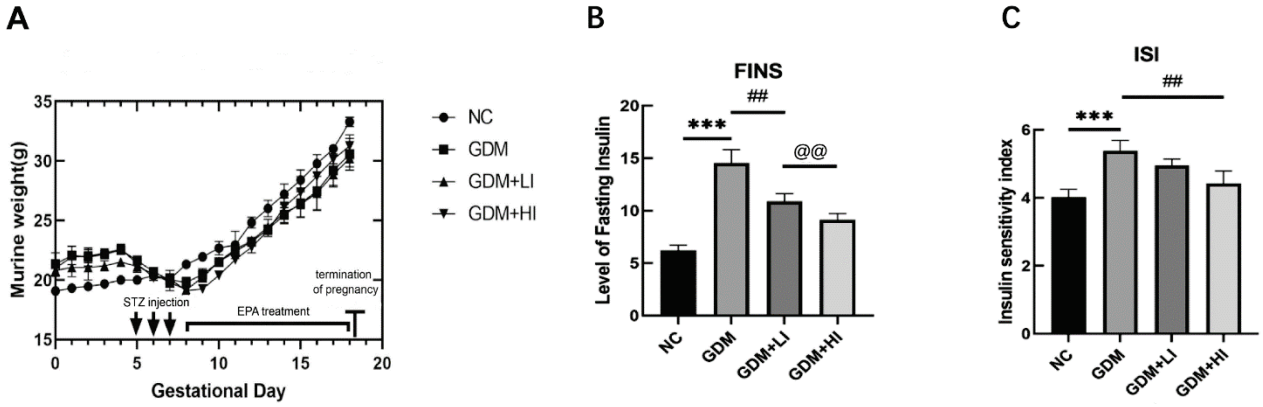

Fig. 1. Comparison of FINS and ISI in serum between groups. (**A**) Comparison of body weight; (**B**) Comparison of fasting insulin (FINS) levels; (**C**) Comparison of insulin sensitivity index (ISI). One-way ANOVA followed by Tukey's *post hoc* test was used for the analysis. Data are presented as mean ± SD. *** P<0.001 vs. NC group; ## P<0.01 vs. GDM group; @@ P<0.01 vs. GDM+LI group.

Impact of EPA on OGTT and serum lipid levels

The impaired glucose tolerance is a characteristic of gestational diabetes, and OGTT can partly reflect the change of glucose tolerance. Mice were given 0.1 ml of 50 % glucose solution after fasting for 12 h. The glucose levels at indicated time points were detected and the AUC area under OGTT curve of each pregnant mouse was calculated. It was observed that the AUC in GDM group was significantly higher than that of NC group (P<0.01), and the AUC in GDM+LI group was lower than that of GDM group but higher than that of GDM+HI group (P<0.05, Fig. 2A-B). The serum lipid levels in GDM model mice were measured. Compared with NC group, the levels of TC, TG and LDL of pregnant mice was significantly increased in GDM group (P<0.01), while significantly decreased after treatment of low (GDM+LI) or high (GDM+HI) dose of EPA (P<0.05). However, there is no significant difference of these levels between low or high dose of EPA treatment (Fig. 2C-E). Differently, the HDL level was markedly reduced in pregnant mice of GDM group and then increased after low or high dose of EPA treatment

(P<0.05). The HDL level was higher in high dose of EPA group than that of low dose group (P<0.05, Fig. 2F). The above results suggested that EPA improved the glucose tolerance and reduced serum lipid levels of GDM mice, and there may be a dose-effect trend between high and low doses.

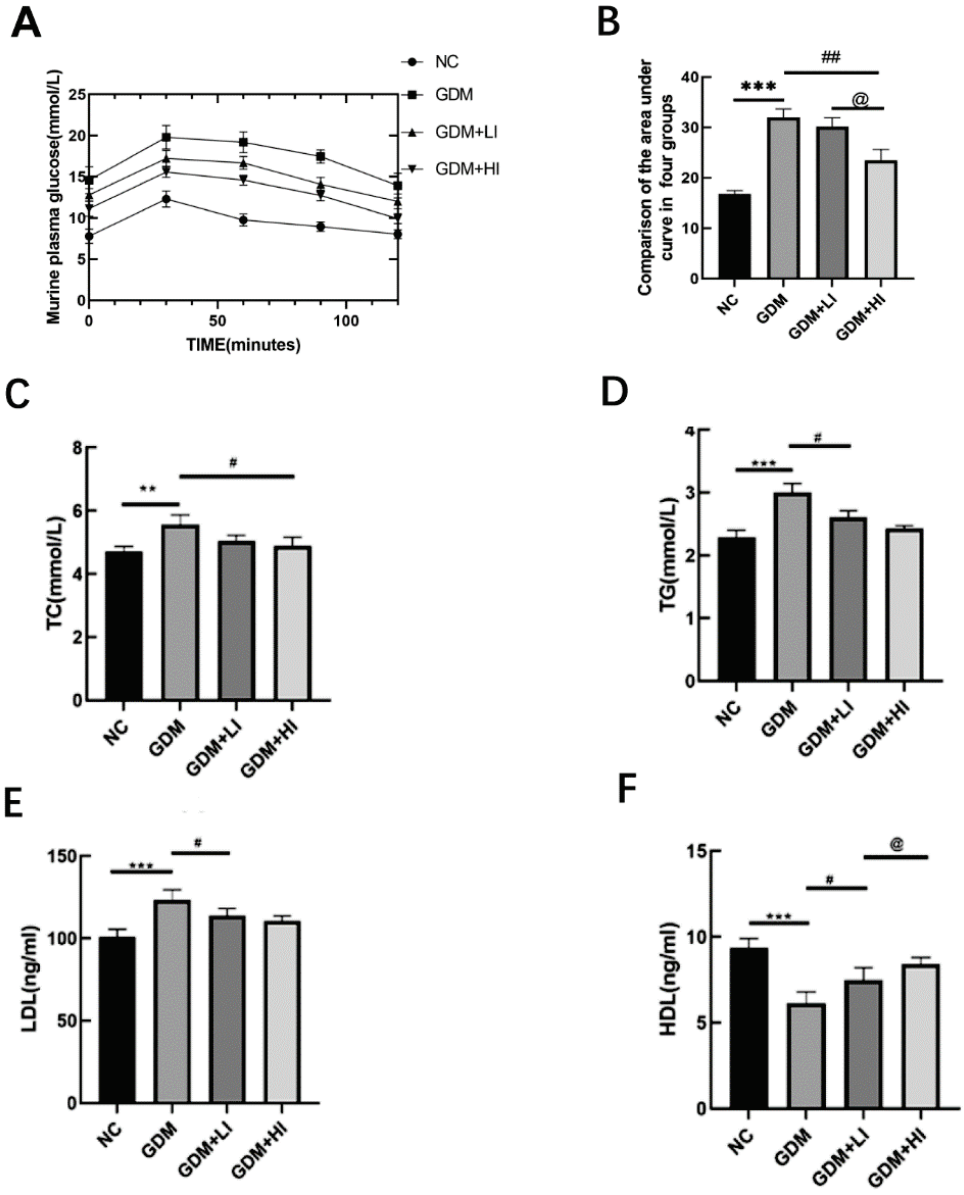

Fig. 2. Impact of EPA on OGTT and serum lipid levels. (**A**) Comparison of OGTT oral glucose tolerance at indicated time points; (**B**) Comparison of area under the curve (AUC) among groups. (**C-F**) Serum levels of TG, TC, LDL and HDL were measured by ELISA. One-way ANOVA followed by Tukey's *post hoc* test was used for the analysis. Data are presented as mean ± SD. ** P<0.01, *** P<0.001 vs. NC group; # P<0.05, ## P<0.01 vs. GDM group; @ P<0.05 vs. GDM+LI group.

Effect of EPA on expressions of insulin signaling proteins in gastrocnemius muscles

The PI3K-AKT pathway is an important pathway for insulin resistance, in which IRS-1, AKT2, GLUT-4 and pIRS-1Tyr, pAKT2Ser are the key molecules [15]. We examined the expression levels of these key factors in pregnant mice by western blot to explore the potential mechanism of EPA intervention affecting IR. As revealed Figure 3A-D, the expression levels of pIRS-1Tyr and pAKT2Ser, as well as the ratios of pIRS-1Tyr/total IRS-1 and pAKT2Ser/total AKT2, were significantly reduced in the GDM group compared to the NC group, while these levels were increased in GDM+LI group and GDM+HI group, and the high-dose EPA displayed more incremental effect (P<0.05). The differences of total IRS-1 and AKT2 levels were not

statistical significant between groups (P>0.05). In addition, the Figure 3E-F showed that the expression of GLUT4 in pregnant mice was lower in the GDM group compared to the NC group (P<0.001), while this expression was elevated in the GDM+LI group when compared to the GDM group (P<0.001). Furthermore, compared with the GDM+LI group, the expression of GLUT4 in pregnant mice was higher in the GDM+LI group (P<0.001). The above data suggest that EPA increases the expression levels of insulin signaling proteins in gastrocnemius muscles, which may be a reason for the improvement of IR.

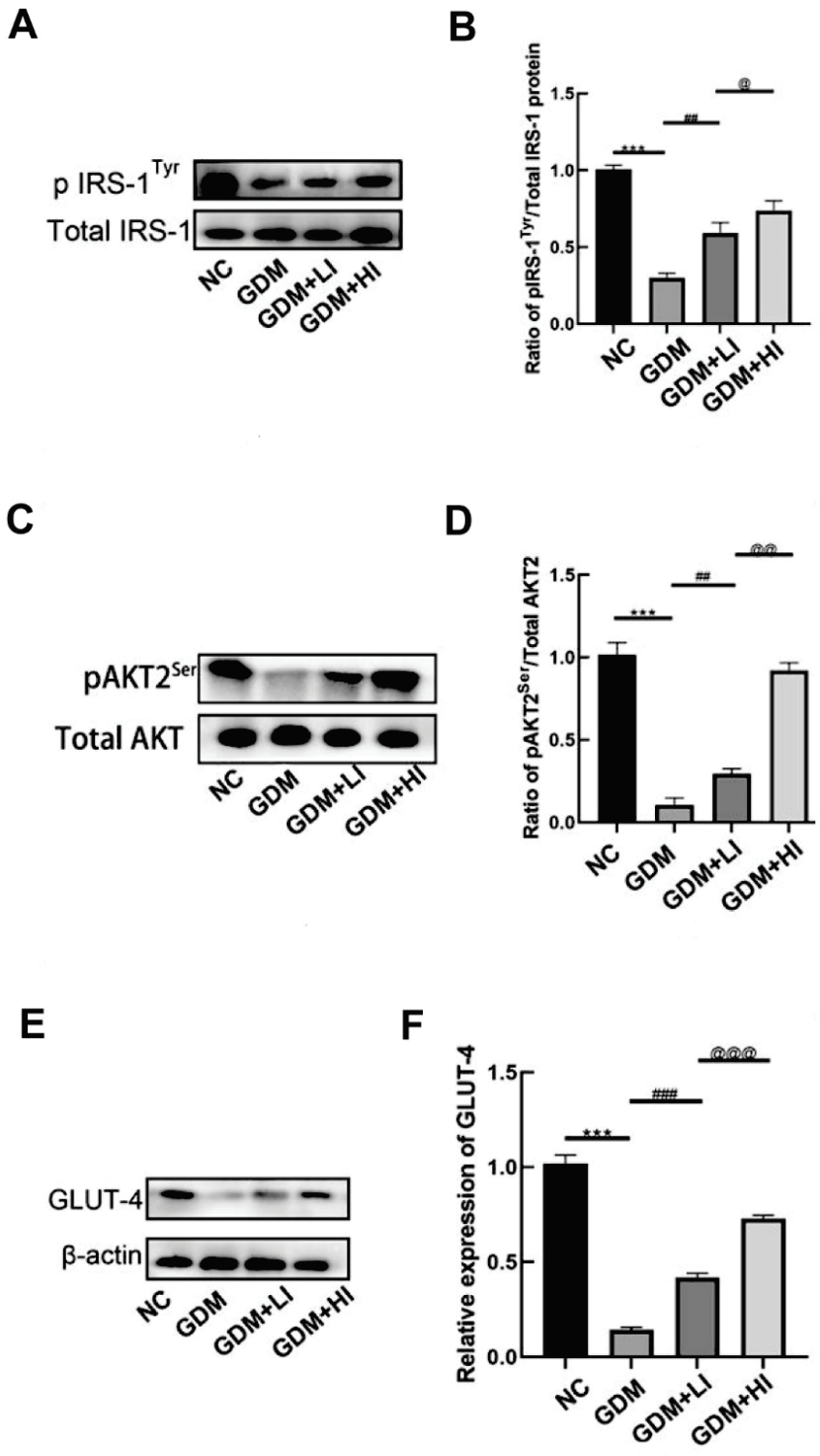

Fig. 3. Comparison of the insulin signaling proteins in gastrocnemius muscles between groups. (**A-D**) Western blot detections of pIRS-1^{Tyr}, IRS-1, pAKT2^{Ser}, AKT2, and ratios of pIRS-1^{Tyr}/IRS-1 and pAKT2^{Ser}/AKT2 in each group; (**E-F**) Western blot detection of GLUT-4. One-way ANOVA followed by Tukey's *post hoc* test was used for the analysis. Data are presented as mean ± SD. *** P<0.001 vs. NC group; ## P<0.01, ### P<0.001 vs. GDM group; @ P<0.05, @@ P<0.01, @@@ P<0.001 vs. GDM+LI group.

Effect of EPA on the expression levels of inflammatory factors in placenta tissues

Inflammatory response is a trigger for increased IR [16]. The expression of placental inflammatory factors IL-1β, IL-6 and TNF-α were detected to observe the inflammatory responses in the GDM group with EPA intervention. The results in Figure 4A-F confirmed that placental IL-1β, IL-6 and TNF-α levels were significantly increased in the GDM group compared to the NC group (P<0.01). The placental IL-1β, IL-6 and TNF-α levels were down-regulated in GDM+LI group when compared to the GDM group, and reduced more in the GDM+HI group (P<0.01). These data suggest that the inflammatory factors play a role in the development of GDM and EPA intervention reduces the inflammatory response during GDM.

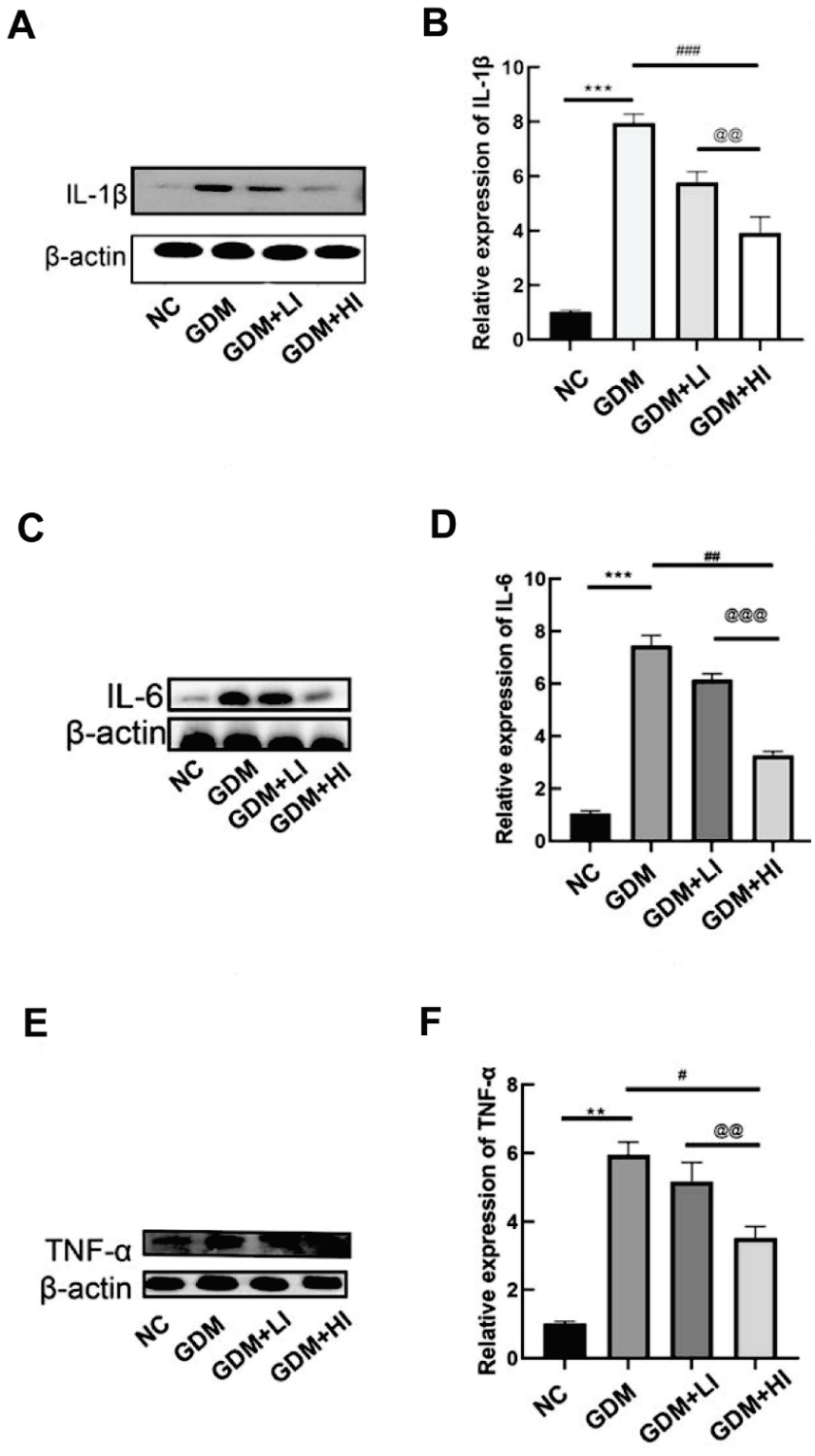

Fig. 4. Comparison of IL-1β, IL-6 and TNF-α levels in placenta tissues among groups. (**A-F**) Western blot detections of IL-1β, IL-6, TNF-α and relative expressions in each group. One-way ANOVA followed by Tukey's *post hoc* test was used for the analysis. Data are presented as mean ± SD. ** P<0.01, *** P<0.001 vs. NC group; # P<0.05, ## P<0.01, ### P<0.001 vs. GDM group; @@ P<0.01, @@@ P<0.001 vs. GDM+LI group.

Effect of EPA intervention on TLR4-NF-κB pathway in placenta tissues

NF-κB, especially NF-κB p65 is a central regulator of the inflammatory response, and blocking the TLR4-NF-κB pathway contributes greatly to inhibit the inflammatory factors release [10]. The expression levels of TLR4 on the placental surface were detected by immunohistochemical staining, and the brown color represents TLR4 protein expression. According to the results in Figure 5A-B, TLR4 protein level increased distinctly in the GDM group compared to the NC group (P<0.001), while this level was decreased in the GDM+LI group and the GDM+HI group (P<0.001). GDM induced significantly up-regulation of NF-κB p65 protein level, while the low-dose (GDM+LI) or high-dose EPA intervention (GDM+HI) decreased this level, showing more reduction in GDM+HI group (Fig. 5C-D, P<0.05). Collectively, these data indicates that the TLR4-NF-κB pathway is activated after GDM and that EPA intervention inhibits this activation, which may be a key reason for the reduced inflammatory response.

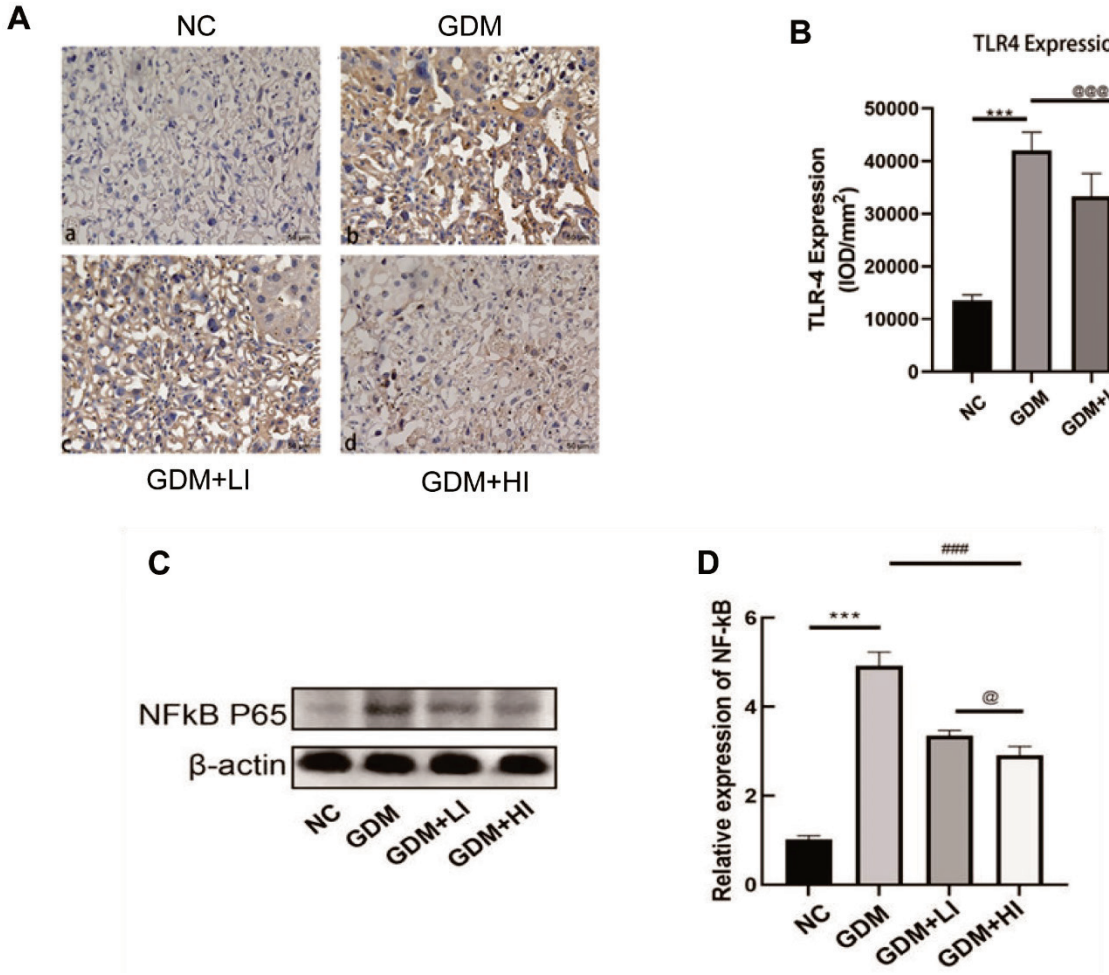

Fig. 5. Comparison of TLR4 and NF-κB expression levels in placental tissues. (**A**) Immunohistochemical staining of anti-TLR4 in placental tissues of each group. (**B**) Mean optical density measurements of immunohistochemical positive staining. (**C-D**) Western blot detection of NF-κB p65 in each group. One-way ANOVA followed by Tukey's *post hoc* test was used for the analysis. Data are presented as mean ± SD. *** P<0.001 vs. NC group; ### P<0.001 vs. GDM group; @ P<0.05 vs. GDM+LI group.

Discussion

The survey finds the prevalence of GDM is already as high as 18-20 % in pregnant women worldwide [17]. The aim of early diagnosis and intervention is to reduce the adverse outcome of the pregnant woman and the offspring. Although some measures have been taken to intervene and control gestational diabetes, the control has not been as effective as intended [18]. Previous evidence has demonstrated that EPA play an important role in alleviating inflammation and IR in humans and animals [19,20]. A former study

clarified that omega-3 fatty acid supplementation containing EPA and docosahexaenoic acid (DHA) had beneficial effects on insulin resistance in women with GDM, and the DHA-enriched fish oil had no effect on prevention of GDM [21]. Another study indicated that omega-3 supplementation reduced inflammation in PBMCs of women with GDM [22]. These provided basis suggested the protective role of EPA on insulin resistance and inflammation in women with GDM. Consistently, this study found that the levels of blood glucose, FINS and ISI in pregnant mice were significantly higher in the GDM group, while the low or high dose of EPA intervention decreased these levels, and the high dose of EPA showed more pronounced reduction effect.

The PI3K-AKT pathway is suggested to be crucial in regulation of IR [23]. Here, we detected the expressions of signaling proteins including IRS-1, AKT2, GLUT-4 and pIRS-1^{Tyr}, $pAKT2^{Ser}$. It was observed that pIRS-1^{Tyr} and $pAKT2^{Ser}$ expressions, the ratios of pIRS-1^{Tyr}/total IRS-1 and $pAKT2^{Ser}$/total AKT2, and GLUT4 expression were significantly reduced in gastrocnemius muscles of the GDM group compared to the NC group, while these levels were increased in GDM+LI group and GDM+HI group, and the GDM+HI group displayed more incremental effect. These data indicate that EPA facilitates the expression levels of insulin signaling proteins, which may explains the reason why EPA can improve the glucose metabolism. However, it is still not clear how EPA affects the insulin signaling proteins, which may refer to the upstream molecules.

The pathogenesis of GDM is complex and influenced by various factors, including genetic factors, inflammatory response and oxidative stress, etc. [24]. Extensive epidemiological data, clinical measurements and research findings collectively confirm that type 2 diabetes is a chronic inflammatory disease [25]. The main inflammatory mediators such as TNF-α, IL-6 and IL-1β are associated with GDM [26]. This study found that the IL-1β, IL-6 and TNF-α levels in placenta tissues were significantly increased in the GDM group, while these levels were down-regulated in GDM+LI group when compared to the GDM group, and reduced more in the GDM+HI group. These data suggest that the EPA intervention markedly alleviates the inflammatory response in GDM. A previous research has confirmed the inflammatory cytokines production in the placenta during GDM, and the inflammatory cytokines such as IL-6 and TNF-α can induce tyrosine phosphorylation of IRS-1, thus inhibiting PI3K-AKT pathway and leading to IR [27]. This study confirmed the inhibitory effect of EPA on inflammatory cytokines production, which may be a promising strategy for preventing IR in GDM. A recent study reported that EPA treatment reduced total lipid content by a significant amount in 3T3-L1 pre-adipocyte cells, reversed oxidative stress in mitochondria, and up-regulated the ATP synthase 6 gene, suggesting an anti-diabetic effect [28].

Recent studies have identified the Toll-like receptors and inflammatory factors are closely associated with gestational diabetes [29]. The TLR4-mediated enhancement of the inflammatory response in the paternal tissues during pregnancy leads to increased IR, which is considered the major cause of GDM [30]. It is also found that TLR4 links the innate immunity to fat-induced IR, and the low levels of TLR4 prevents IR [31]. In the present study, the protein levels of TLR4 and NF-κB p65 were distinctly elevated in the GDM group compared to the NC group, while this level was decreased after treatment of low or high dose EPA treatment, and the high dose EPA showed more decline. These data indicates that the TLR4-NF-κB pathway is activated after GDM and the EPA intervention inhibits this activation. Dasu *et al.* [32] demonstrated that the TLR4, NF-κB and IL-1β expressions in the peripheral blood mononuclear cells of patients with type 2 diabetes were enhanced, and has close association with levels of FINS and ISI, which is consistent to our study. Based on these previous work, this study further identified the ameliorative effect of EPA on inflammatory response and insulin resistance in GDM, and the potential regulatory mechanism. Additionally, some studies found other interesting discoveries, Nakanishi *et al*. demonstrated that EPA inhibited the accumulation of visceral fat in male patients with type 2 diabetes, suggesting some sex differences in EPA affecting type 2 diabetes [33].

Given that only 2 doses of EPA were administered in this study, we considered that the usage of EPA within a certain range could alleviate the inflammatory response and insulin resistance in GDM. In addition, almost all the high dose of EPA showed more pronounced effect on GDM when compared to the low dose of EPA, suggesting that the effect of EPA is dose-dependent. This study has some limitations. The modeling method of STZ with high fat diet is practical, while have some difference in the pathogenesis of GDM. The data have no sufficient clinical application basis, and the mechanism is preliminary and needs further

investigation. In addition, there is a significant gap linking decrease in inflammation and insulin resistance as they were in different tissues.

In conclusion, this study demonstrated that EPA alleviated IR in pregnant mice with GDM and this effect may be related to the reduced inflammatory response on the placental surface. This work may provide a promising intervention method and theoretical basis for the clinical study of GDM.

Conflict of Interest

There is no conflict of interest.

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