Beneficial effects of walnut (Juglans regia L.) oil-derived polyunsaturated fatty acid prevents hyperlipidemia and oxidant status in pregnant rats with diabetes

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Walnut oil-derived PUFA, Dyslipidemia, Oxidative stress, Gestational diabetes
Abstract

Background: Gestational diabetes mellitus has a long-term effect on pregnant women. Walnut (*Juglans regia* L.) oil-derived polyunsaturated fatty acid (PUFA) possesses multifarious pharmacological activities. This study investigated the beneficial effects of PUFA on the gestational diabetes mellitus (GDM) and to study the underlying mechanism in pregnant rats with diabetes.

Methods: The GDM rat model was generated by intraperitoneal injection of streptozotocin (40 mg/kg) on gestational day (GD) 6, GD7 and GD8. The differences between groups were estimated using one-way ANOVA followed by the Tukey’s multiple comparison test for post-hoc analysis.

Results: The results indicated that PUFA could mitigate GDM in pregnant diabetic rats, as embodied by the decrease of fasting blood glucose and the increase of plasma insulin and hepatic glycogen levels. Also, PUFA could suppress oxidative stress in pregnant diabetic rats, as reflected by the decrease of malondialdehyde (MDA) content, an increase of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities. PUFA could also mitigate the abnormal changes of lipid profiles in plasma and hepatic tissue. Moreover, the relative mRNA expression of sterol regulatory element-binding transcription factor-1 (SREBP-1), stearoyl-CoA desaturase-1 (SCD-1), fatty acid synthase (FAS), and acetyl-coenzyme A carboxylase (ACC), was suppressed by PUFA in pregnant diabetic rats.

Conclusions: These results suggested that PUFA supplementation during the pregnancy might be beneficial in preventing diabetic complications in the mother.

Introduction

Gestational diabetes mellitus, a frequent metabolic disorder in pregnancy, being present in 1–18% of all pregnancies. It has been defined as gestational diabetes mellitus is characterized by hyperglycemia or glucose intolerance with onset during pregnancy resulting from defects in insulin secretion or insulin action. The chronic hyperglycemia of pregnancies is associated with long-term dysfunction and damage of different organs for the mother and offspring as well as a possibility of increased fetal-maternal morbidity. Also, the offspring of women with gestational diabetes mellitus are more likely to develop obesity, impaired glucose tolerance and metabolic disorders in later life.
And part of gestational diabetes patients may even accompany an increased risk of impaired glucose tolerance and with an increased risk of maternal preeclampsia in the years after pregnancy. A previous study has reported that the intrauterine oxidative stress environment contributed to adverse outcomes and metabolic diseases influenced fetal programming in pregnancies. Superfluous oxidative stress has been implicated in the pathogenesis and development of gestational diabetes mellitus. Although the underlying molecular mechanisms of diabetes complications are complicated and remain unclear, however preclinical and clinical researches indicated that gestational diabetes is related to oxidative stress, leading to a decline in the antioxidant defense system and an increased production of reactive oxygen species. Enhanced oxidative stress after hyperglycemia is induced mainly because of increased glycation end-product formation. Thus, it has been speculated that the usage of the antioxidant agent may exert a protective effect against diabetes complications via suppression of reactive oxygen species. Thus, it is urgent to develop a novel treatment for gestational diabetes.

The usage of traditional medicine and food derived from natural antioxidants is regarded as an alternative therapy for improving oxidative stress in diabetes. The green husks, leaves, and seeds of the walnut (Juglans regia L.) are the main source of these functional ingredients which have been used as folk medicine for the prevention and treatment of some diseases including anorexia, diabetes mellitus, cancer, thyroid dysfunction and infectious diseases. Polyunsaturated fatty acids (PUFA), phenolic acids, and flavonoids are considered as major active compounds in Juglans regia L. seeds. Recently, clinical researches have indicated that the addition of walnut oil in the daily diet may serve as a helpful remedy for hyperlipidemic patients with diabetes mellitus type 2. However, whether walnut oil-derived PUFA exhibited beneficial effects against gestational diabetes mellitus is not yet clear.

The present research was performed to investigate the beneficial effects of walnut oil-derived PUFA on gestational diabetes mellitus and the possible underlying mechanism in streptozotocin-induced
diabetic pregnant rats.

Materials And Methods

Plant material and analysis of walnut oil

Walnut (Juglans regia L.) nuts were purchased from the local market in Shandong province. Walnut oil was extracted from Shandong walnuts according to the cold-press method with minor modification\textsuperscript{15}. The composition of PUFA in walnut oil was measured by converting them into free fatty acids by saponification\textsuperscript{16}. The free fatty acids were separated and determined by HPLC-UV equipped with Lichrosorb RP-18 column (particle size 5 µm; 150×4.6mm, Merck). The HPLC conditions were as follows: The column temperature was maintained at 30 °C with a flow rate of 0.9 mL/min, the UV absorption at a wavelength of 192nm and the mobile phase composition was water/acetonitrile (1:9) isocratic for 15 min. The free fatty acid peaks were identified by matching them with fatty acid standards (Sigma-Aldrich, USA). All other chemical reagents were purchased from Aladdin Reagent Co. (Shanghai, China).

Animals and treatment

Animal procedures in this experiment were approved by the Animal Care and Use Committee of the Central hospital of Linyi, by the guiding principles for the care and use of animals published by the National Institute of Health. Wistar rats (10 weeks old female rats: 180–240g; adult male rats: 300–340g) were purchased from the Experimental Animal Center of Shandong Province. All rats were housed in a controlled room with a temperature of (22–24°C), a relative humidity of (40–60%) with a light cycle (12/12 h light/dark) and fed with a basic diet and water. After 7 days of adaptation, female rats and male rats were permitted to mate. Pregnancy was confirmed by the presence of a copulatory plug in the next morning, and the day was defined as gestational day (GD) 0.

The GDM rat model was induced by intraperitoneal injection of 40 mg/kg streptozotocin on GD6, GD7, and GD8, respectively\textsuperscript{17}. The rats with a fasting blood glucose value of more than 16.7 mmol/L were considered diabetic and used for further researches. The blood glucose levels in rats were measured at GD0, GD9, and GD18. The present study was performed in five groups of eight pregnant rats each: pregnant control group (PC), gestational diabetes mellitus group (GDM) and gestational diabetes rats.
fed a diet supplemented with a low dose of polyunsaturated fatty acids (225mg/kg body weight, LPUFA), a middle dose of polyunsaturated fatty acids (450mg/kg body weight, MPUFA), and a high dose of polyunsaturated fatty acids (900mg/kg body weight, HPUFA) The doses of PUFA were selected according to previous study 18.

The blood samples were obtained from the orbital venous plexus after overnight fasting. Plasma samples were obtained from blood after centrifugation at 10,000 rpm for 15 min at 4˚C. Samples were stored at -80˚C until analysis. Rats were euthanized on GD18 by light ether anesthesia after overnight fasting, fetuses, placentas, and liver tissues were immediately weighed and stored at -80˚C until further assay.

Determination of insulin concentration, blood glucose levels, and lipids parameters

Plasma insulin concentration was measured by a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (Thermo Scientific). Blood glucose levels, plasma triglycerides (TG), total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and hepatic levels of triglycerides (TG), cholesterol (TC), were measured, respectively, using commercial kits obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China) according to the supplier’s instructions.

Estimation of hepatic glycogen levels and oxidative stress

Hepatic tissues were homogenized in an ice-cold saline solution. After that, the hepatic homogenates were centrifuged at 10,000 rpm for 20 min at 4˚C. Hepatic glycogen levels were assayed with commercial kits obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China) according to the supplier’s protocols. The oxidative stress was also examined by the measurement of MDA level and SOD, GSH-Px and CAT activities using commercial kits obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China).

RNA isolation and real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from liver tissues using a commercial reagent (Invitrogen, CA, USA) according to the manufacture’s protocol. The cDNA synthesis was performed by reverse transcription of 1μg total RNA using Frist Strand cDNA Synthesis Kit (Thermo, USA). RT-PCR amplification was
carried out with an SYBR Green qPCR Master Mix kit (Thermo, USA) according to the manufacturer’s protocol. The qPCR was carried out in duplicate, the condition of RT-PCR amplification reaction as follows: 45 cycles of 95°C for 10 s, 60°C for 30 s and 72°C for 30 s with the primer sequences (Table 1). The expression of target gene transcripts was related to the reference gene (GAPDH). Results were expressed as folds of control.

The data statistical analysis

All experimental results were reported as the means ± SD. The differences between groups were estimated using one-way ANOVA followed by the Tukey’s multiple comparison test for post-hoc analysis using GraphPad Prism software (GraphPad software, Inc., La Jolla, USA); *P* < 0.05 was usually considered as statistically significant.

Results

Component analysis of PUFA in the walnut oil

The PUFA in walnut oil was quantitated by HPLC-UV and qualitative estimation of walnut oil indicated the presence of PUFA about 90.64%. As showed in Table 2, the major fatty acids were linoleic acid (62.47%), followed by oleic acid (15.36%) and linolenic acid (12.81%).

PUFA intervention ameliorates gestational diabetes mellitus in pregnant rats

As showed in Figure 1, on gestation day 18, fasting plasma insulin level and hepatic glycogen content of GDM decreased in comparison with the PC group (*P* < 0.01). However, the administration of MPUFA and HPUFA to pregnant diabetic rats inhibited the decline of plasma insulin level and hepatic glycogen content in GDM rats (*P* < 0.05, *P* < 0.01). As displayed in Figure 2, on gestation day 18, blood glucose levels of GDM increased in comparison with the PC group (*P* < 0.01). However, the administration of MPUFA and HPUFA to pregnant diabetic rats inhibited the increase of blood glucose levels in GDM rats (*P* < 0.05, *P* < 0.01). These results indicated that PUFA could ameliorate gestational diabetes in pregnant rats.

PUFA intervention ameliorates fetal growth restriction and reduces the incidence of embryo lethality under gestational diabetes mellitus condition

The growth of fetal was generally normal in the PC group. As showed in Figure 3, compared with the
PC group, GDM fetuses suffered an obvious decrease in fetal body weight and increase in the placental weight. However, these characteristics mostly ameliorated by the administration of HPUFA to pregnant diabetic rats ($P < 0.05$). As showed in Table 3, although the incidence of embryo lethality was greater in the GDM group compared with the PC group ($P < 0.01$), PUFA administration of diabetic rats reduced the incidence of dead fetuses ($P < 0.05, P < 0.01$).

PUFA intervention ameliorates lipids metabolism under gestational diabetes condition

Abnormal change of lipid metabolism is a major contributor to gestational diabetes. In this study, the changes of lipid profiles of each group are displayed in Figure 4, plasma levels of TC, TG, and LDL-C were significantly increased while HDL-C was declined in the GDM group compared to the PC group ($P < 0.01$). However, the disorder of lipid metabolism was ameliorated by the administration of MPUFA and HPUFA to pregnant diabetic rats ($P < 0.05, P < 0.01$). Besides, the hepatic levels of TC and TG were also measured in the present study, and results were consistent with the trends in plasma (Figure 4E and Figure 4F). These data indicated that PUFA could improve the disorder of lipid profiles under gestational diabetes pathological conditions.

PUFA intervention ameliorates hepatic oxidative stress under gestational diabetes condition

Oxidative stress is closely related to the disorder of lipid profiles and another major contributor to the development of gestational diabetes. In the present study, we investigated the effect of PUFA on oxidative stress in gestational diabetes rats. As showed in Figure 5, hepatic SOD, GSH-Px, and CAT activities were significantly decreased while MDA content was increased in the GDM group compared to the PC group ($P < 0.01$). However, oxidative stress ameliorated by the administration of MPUFA or HPUFA to pregnant diabetic rats ($P < 0.05, P < 0.01$). The results showed that PUFA suppressed oxidative stress under the gestational diabetes pathological condition.

PUFA intervention suppresses SREBP-1 and its target gene expression under gestational diabetes condition

In this study, the mRNA expression of SREBP-1 was measured to investigate the underlying mechanism of the protective effect of PUFA against gestational diabetes. As displayed in Figure 6A, mRNA expression of SREBP-1 was increased in gestational diabetes rats, compared with that of
normal pregnant rats ($P < 0.01$). The administration of MPUFA or HPUFA suppressed the increase of SREBP-1 mRNA expression in gestational diabetes rats ($P < 0.05$, $P < 0.01$). Besides, the mRNA expression of SREBP-1 target genes (SCD-1, ACC, and FAS) was also measured in the present study. As displayed in Figure 6B-D, the mRNA expression of SCD-1, ACC and FAS were increased in gestational diabetes rats, compared with that of normal pregnant rats ($P < 0.01$). The administration of HPUFA suppressed the increase of SCD-1, ACC and FAS mRNA expression in gestational diabetes rats ($P < 0.05$).

**Discussion**

The use of traditional medicine and food derived from natural products is increasing in the management of multifarious diabetes-associated complications, largely because of the general notion that traditional medicine and food are less adverse effects compared with synthetic drugs. About a significant biological activity, recent studies have indicated that a walnut oil-rich diet improved type 2 diabetes. However, its protective effects in STZ-induced diabetes rats during pregnancy have not been investigated so far. Hence, the major purpose of the present research was to investigate the effects of PUFA supplementation to ameliorate gestational diabetes associated the possible mechanisms in an STZ-induced gestational diabetes model.

In the present study, the typical symptoms of diabetes were induced by the administration of STZ to pregnant rats, such as hyperglycemia and fetal growth restriction, which was consistent with previous studies. What is more, our result indicated that PUFA could attenuate gestational diabetes in STZ-induced diabetes rats, as reflected by the decline of fasting blood glucose and the increase of plasma insulin level and hepatic glycogen content. This result was consistent with the previous study of the hypoglycemic effects of flax and sesame seed mixture in diabetic pregnant rats. Previous literature has indicated that experimental induction of gestational diabetes was associated with obvious increases in the incidence of embryo lethality. In the present study, the PUFA administration afforded obvious protection against STZ-induced embryo lethality under gestation diabetic conditions. As far as we know, this was the first time study that investigated the protective
The effect of PUFA from walnut oil against STZ-induced embryopathy in pregnant rats. Besides, the obvious decline in fetal weight was observed following the STZ-induced GDM. The previous study has reported similar results in STZ-induced GDM, and more importantly, the improvement effects were achieved by the antioxidant Ipomoea Aquatica (whole leaf powder) supplementation. This result suggested that the efficacy of antioxidants in ameliorating diabetic embryopathy.

Oxidative stress is seen as overwhelming of the endogenous defense systems by the excessive production of reactive oxygen species that given rise to cell dysfunction. It also plays a vital role in the development of complications of GDM. In the present study, the protective effects of PUFA administration were demonstrable in terms of obvious amelioration of gestational diabetes-associated oxidative stress in pregnant rats. The increased levels of hepatic oxidative stress markers in diabetic rats were in agreement with a previous study. Our results indicated that PUFA administration counteracted STZ-induced oxidative stress in maternal liver tissue, clearly implying its antioxidative effect in vivo. A previous study has indicated the antioxidant activity of walnut oil in mice aging model. However, this was the first study that indicated the protective ability of PUFA from walnut oil against oxidative injury associated with STZ-induced GDM during pregnancy. These findings showed that the antioxidant activities of PUFA may be involved in the anti-diabetic effect of PUFA in GDM rats.

It has been reported that abnormal alterations of lipid metabolism were associated with the development of GDM and oxidative stress. And PUFA possessed lipid-lowering activities under different pathological conditions. Consistent with several previous studies, our present results also showed that GDM induced abnormal changes in lipid metabolism both in plasma and in the liver. These results were connected with an increase in hepatic cholesterol and triglyceride levels, possibly due to increased secretion and synthesis of lipoprotein. In the present study, we observed that walnut oil-derived PUFA could ameliorate the abnormal changes of lipid metabolism in pregnant rats, as reflected by the increase of HDL level and the decrease of TG, TC and LDL levels. These findings suggested that amelioration of abnormal alterations of lipid metabolism may take part in anti-diabetic activities of walnut oil-derived PUFA in GDM rats.
To further investigate the underlying mechanism of PUFA lipid-lowering effect in GDM rats. The mRNA levels of genes involved in fatty acid metabolism in hepatic tissue were examined. SREBP-1 was a nuclear transcription factor played an important role in the regulation of cholesterol, triglyceride, and fatty acids biosynthesis by managing its target genes participated in fatty acid synthesis, including SDC-1, ACC and FAS. Besides, abnormal mRNA expression of SREBP-1 was in connection with the pathogenesis of diabetes. In the present study, we discovered that PUFA declined SREBP-1, SCD-1, ACC, and FAS mRNA expression in STZ-induced GDM rats. Thus, our results suggested that PUFA may also mediate disorders of lipid metabolism through down-regulation of SREBP-1, SCD-1, ACC, and FAS, thereby declining the serum levels of TC, TG, and LDL-C in STZ-induced GDM rats.

Conclusion
Several abnormalities were found in GDM mothers. Our results clearly stated that walnut oil-derived PUFA as a therapeutic reagent to prevent GDM in pregnant rats. Walnut oil-derived PUFA administration improves the disorders of lipid metabolism and oxidative stress and ameliorates insulin resistance and hyperglycemia in GDM rats, as well as decreased embryo lethality and improved reproductive outcome. Further analysis reveals that SREBP-1 may be of the foremost target of PUFA that mediated its anti-diabetic activities in GDM rats.

Abbreviations
polyunsaturated fatty acid (PUFA); gestational diabetes mellitus (GDM); gestational day (GD); malondialdehyde (MDA); superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GSH-Px); sterol regulatory element-binding transcription factor-1 (SREBP-1); stearoyl-CoA desaturase-1 (SCD-1); fatty acid synthase (FAS); and acetyl coenzyme A carboxylase (ACC); triglycerides (TG); total cholesterol (TC); low density lipoprotein cholesterol (LDL-C); high density lipoprotein cholesterol (HDL-C).

Declarations
Authors’ contributions
BMS and HY were responsible for performing the experiments, collecting data, and preparing the manuscript draft; CL, LLY, and FL were responsible for analyzing the data and editing the manuscript; LXZ and XQH was responsible for conceiving the research goals, writing, and editing the manuscript.
All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All the animal experiments in this study were by the ethics guidelines of institutional animal care and use committee (IACUC) and were approved by IACUC of the Central hospital of Linyi.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Tables

Table 1. Sequences of primers used quantitative real-time PCR.

| Gene     | Forward primer          | Reverse primer          |
|----------|-------------------------|-------------------------|
| SCD-1    | 5’- TGCTGATCCCCACAATTCCC-3’ | 5’- CTTTGACGGCTGGGTGTTTG-3’ |
| SREBP-1C | 5’-CCCTGCGAAGTGCTCACAA-3’ | 5’-GCATTCTACCACCTACGTTTCA-3’ |
| ACC      | 5’-ACACTGGCTGGCTGGACAG-3’ | 5’-CACACAACTCACAACATGGTG-3’ |
| FAS      | 5’-GGCCACCTCAGTCTGTAT-3’ | 5’-AGGTCCAGCTGAGGTAC-3’ |
| GAPDH    | 5’-GAACGGGAAGCTCAGTCG-3’ | 5’-GCATGTCAGATCCACAACGG-3’ |

Table 2. Composition of PUFA in the walnut oil.

| Component                       | Percentage of total fatty acids (%) |
|---------------------------------|-------------------------------------|
| Oleic acid (C18:1n9c)           | 15.36 ± 0.76                        |
| Linolenic acid (C18:3n3)        | 12.81 ± 0.56                        |
| Linoleic acid (C18:2n6c)        | 62.47 ± 1.06                        |
Table 3. Effect of PUFA on the total number of fetuses, the number of live and dead fetuses, and the percentage of dead fetuses in pregnant rats.

| Group     | Total | Live | Dead | % Dead fetuses |
|-----------|-------|------|------|---------------|
| PC        | 104   | 101  | 3    | 2.88          |
| GDM       | 78    | 48   | 30   | 38.46*        |
| LPUFA     | 82    | 54   | 28   | 34.15         |
| MPUFA     | 86    | 62   | 24   | 27.91*        |
| HPUFA     | 91    | 72   | 19   | 20.88**       |

The data are expressed as the mean ± SD (n = 8/group). PC, pregnant control; GDM, gestational diabetes model group. #, P < 0.01 (vs. the PC group); *, P < 0.05 (vs. the GDM group); **, P < 0.01 (vs. the GDM group).

Figures

Figure 1

Walnut oil-derived PUFA alleviates gestational diabetes symptoms in GDM rats. Plasma insulin levels (A) and hepatic glycogen levels (B) were measured in GD 18. Data are expressed as the mean ± SD (n =8/group). #P < 0.01 (vs the PC group), **P < 0.01 (vs the GDM group), *P < 0.05 (vs the GDM group).
Figure 2

Walnut oil-derived PUFA effectively normalizes hyperglycemia caused by GDM. Data are expressed as the mean ± SD (n = 8/group). #P < 0.01 (vs the PC group), **P < 0.01 (vs the GDM group), *P < 0.05 (vs the GDM group).
Figure 3
Walnut oil-derived PUFA effectively improves the fetal growth restriction caused by GDM. Fetal body weights (A) and placental weights (B) were recorded in pregnant rats. Data are expressed as the mean ± SD (n = 8/group). #P < 0.01 (vs the PC group), *P < 0.05 (vs the GDM group).
Figure 4
Walnut oil-derived PUFA alleviates lipid metabolism in GDM. Plasma TC levels (A), plasma TG levels (B), plasma LDL-C levels (C), plasma HDL-C levels (D), hepatic TC levels (E) and hepatic TG levels (F) were measured in GD 18. Data are expressed as the mean ± SD (n =8/group). #P < 0.01 (vs the PC group), **P < 0.01 (vs the GDM group), *P < 0.05 (vs the GDM group).
Walnut oil-derived PUFA alleviates oxidative stress in GDM. Hepatic SOD (A), hepatic GSH-Px (B), hepatic CAT (C), hepatic MDA (D) were measured in GD 18. Data are expressed as the mean ± SD (n = 8/group). #P < 0.01 (vs the PC group), **P < 0.01 (vs the GDM group), *P < 0.05 (vs the GDM group).
Figure 6

Effect of walnut oil-derived PUFA on the mRNA expression of SREBP-1 and its target genes in pregnant rats. The mRNA expression of SREBP-1 (A), SCD-1 (B), ACC (C) and FAS (D) were examined. Data are expressed as the mean ± SD (n =8/group). #P < 0.01 (vs the PC group), **P < 0.01 (vs the GDM group), *P < 0.05 (vs the GDM group).