Vitamin D Prevents Gestational Diabetes Mellitus via Modulating Glucose Metabolism in a Mouse Model

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Summary
Gestational diabetes mellitus (GDM) is a common disease during pregnancy that has adverse effects on both the mother and fetus. There are currently rare researches on the effect of vitamin supplementation on GDM pregnant mother and their offspring on animal and cell levels systematically. This work supplemented the GDM pregnant mouse model with vitamin D and found that vitamin D can effectively alleviate the hyperglycemia in GDM pregnant mice, increase blood insulin and adiponectin concentrations, and improve GTT and ITT in pregnant mice. In addition, vitamin D can reduce the incidence of death and high birth weight of offspring caused by GDM. The offspring of GDM pregnant mice had higher blood glucose levels in the first 5 weeks after birth compared to the normal group, and then returned to normal levels. Vitamin D can alleviate abnormal glucose metabolism in newborn mice. The therapeutic effect exhibited by vitamin D may be due to their anti-inflammatory effects, as vitamin D supplementation significantly reduces the levels of TFN-α, MCP-1, IL-1β and IL-8 in the blood. Vitamin D also regulates liver lipid metabolism, resulting in a decrease in liver lipid accumulation and a decrease in blood triglycerides (TG) and cholesterol (CHO). The results of this study demonstrate that vitamin D supplementation can serve as an effective treatment strategy for alleviating GDM symptoms.

Keywords
Gestational diabetes mellitus • Vitamin D • Glucose metabolism • Anti-inflammatory

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Introduction

Gestational diabetes mellitus (GDM) is one of the most common metabolic complications in pregnancy women with development of glucose intolerance (1]. Up to 2-25 % of pregnancies are diagnosed with GDM all round the world and about 11.9 % of pregnancies in China [2-5]

The large variation in incidence is probably due to the broad criteria of hyperglycemia for diagnosis. Women with GDM demonstrate glucose intolerance and insulin insufficiency during pregnancy with onset or first recognition [6, 7], especially in the second or third trimester. Insulin production ability by pancreas of women with GDM cannot resistant to the insulin-inhibiting effects of placental hormones such as oestrogen, human placental lactogen and cortisol[8], which could be resolved along delivery.

Many risk factors are associated with GDM including obesity, non-white ethnicity, increased maternal age, family history of diabetes and history of giving birth to large infants [9,10]. As up high to 50 % of GDM women develop T2DM within 5 years after pregnancy [11,12], and about 70 % within 22–28 years [13,14]. Moreover, pregnant women with GDM provide a hyperglycemic environment in utero to fetus. Long time of exposure to hyperglycemic environment increased the tendency of obesity, metabolic syndrome, and other cardiometabolic disorders in the off-spring [15,16]. And the risk of adverse mother, developing fetus, and offspring shows continuously increase along the

change of maternal glycemia in the final stage at 24–28 weeks [17], which may cause the occurrence of preeclampsia, increased newborn percent body fat, fetal macrosomia, obstructed shoulder delivery, cesarean delivery, postpartum hemorrhaging, neonatal asphyxia or death [7].

The precise mechanisms underlying the occurrence of GDM are not clear currently. The secretion of several anti-insulin hormones by the placenta gradually increases along with the pregnancy progress, for example, estradiol, placental growth hormone, progesterone, and prolactin. Those secreted hormones may cause an elevated insulin demand from pancreatic β cells [7]. In the normal pregnancy progress, β-cell number increases via proliferation and thereby the secretion of insulin is up-regulated to adapt to elevated insulin resistance [18]. However, Women with GDM demonstrate abnormally more anti-insulin hormones secretion and/or β-cell dysfunction causing the imbalance of glucose metabolism and insulin secretion [19]. Recombinant insulin administration for Gestational Diabetes Mellitus mouse model showed protection glucose intolerance and obesity in offspring, but failed to protect offspring when challenged with a high-fat diet [20]. Besides, normal pregnancy is also characteristic by increased total adipose mass and gain of about 30 % of recommended weight [21]. Gain of adipose tissue is mechanistically associated with systemic glucose homeostasis in the nonpregnant situation. In this scenario, modulating the systemic glucose homeostasis during pregnancy, especially in the second and third trimester may provide an option for prevention of GDM. The first line of therapeutic approach for GDM currently relies mainly on nutrient supplementation and exercise intervention. For pregnant woman who still cannot restore glucose homeostasis to control hyperglycemia despite improving their lifestyle, insulin therapy should be often prescribed [22]. However, there are several drawbacks relating to insulin treatment, such as poor treatment outcomes, lack of universal compliance, and/or increasing levels of anxiety [20]. Therefore, new therapeutic strategies with potential to improve insulin sensitivity and decrease side effects is significant important for the short- and long-term prognosis and prevention of GDM.

The deficiency of vitamin D in blood is one of the symptoms of GDM. Vitamin D is a kind of fat-soluble secosteroids hormone, which is produced in the body under sunlight exposure and response to parathyroid hormone [23]. 7-dehydrocholesteroln is photolysied under sunlight exposure into previtamin D3, followed by transformation to vitamin D3 rapidly. After two sequential hydroxylation in liver and kidney respectively, vitamin D3 is activated into physiological active form, 1a,25-dihydroxyvitamin D3 (1,25(OH)2D3), which is able to be recognized by vitamin D receptor (VDR) [24, 25]. The most reported function of vitamin D is to maintain calcium homeostasis and bone integrity. Vitamin D is also reported to facilitate to immunomodulatory and anti-inflammatory effects, which regulates the insulin secretion to modulate glucose metabolism [26-28]. The function of anti-inflammation via NF-κB signaling pathway has been approved in adult mouse-derived adipose tissue and adipocytes [29]. One of the vitamin D3 metabolites lactone-vitamin D3 is identified to selectively bind to the hydroxyacylCoA dehydrogenase trifunctional multienzyme complex subunit alpha (HADHA) which catalyzes β-oxidation of long-chain fatty acids on the mitochondria. These reports suggest the function of vitamin D in lipid metabolism. A case-control study evaluated the association of 25(OH)D concentrations in pregnant women blood with risk of GDM [30].

Daily intake of vitamins D is a highly compliant strategy for pregnant women to prevent vitamin D deficiency (VDD), thereby to decrease the risk of GDM. Li et al. estimated the effects of administration of vitamin D3-supplemented yogurt on glucose metabolism and lipid concentrations in pregnant women with GDM [31]. After 16 weeks supplementation, the fasting plasma glucose and lipid concentration in vitamin D3-supplemented yogurt intervention group were both significantly decreased compared to control group [31]. All above reports support that vitamin D acts as an important factor to regulate glucose and lipid metabolism homeostasis. However, there has been still seldom studies regarding the systematically effects of vitamin D on insulin resistance in pregnant women with GDM and even the offspring.

In this study, we systematically evaluated the function of vitamin D supplementation in pregnant mice with GDM and their offspring. 2 weeks vitamin D intervention prevented the GDM syndromes and significantly alleviated plasma glucose and lipid levels, as well as improved the outcomes of offspring. This study provides support for vitamin supplementation to alleviate GDM in clinical practice.

Methods

Human studies

Ten blood samples of normal pregnant women and ten blood samples of pregnant women with GDM were collected and detected of serum 25-hydroxyvitamin D3 concentration.

GDM model establishment

Eight weeks old C57BL/6J mice were used in this study. Mice were fed at 25℃ with 12 hours light and 12 hours dark cycles. Mice were fed with high-fat diet (HFD; 60 %kcal) for 4 weeks before pregnancy and then continually maintained the diet until the delivery. Normal pregnant (NP) mice fed with normal chow diet all the process were used as control. 2 female mice were mated with one male mouse overnight to cross. The presence of vaginal plugs is as the start time of pregnancy and is defined as gestational day 0. To simulate the high glucose environment in the uterus in which fetus live, 30 mg/kg streptozotocin (STZ) was injected intraperitoneally each day from gestational day 1 to 4. The control mice were injected with same volume of citrate buffer (which was used to resolve STZ). 3 days later after last time STZ induction, blood glucose was tested at the same time from the tail by glucometer (Roche, USA). Mice with blood glucose concentration more than 12 mmol/L were used to the following experiments. Then mice with STZ induction were randomly divided into two groups: the GDM group and the GDM with vitamin D treatment (GDMt) group. The GDMt group was given vitamin D 1.0 IU/g body weight each day by the oral administration [29,32]. The GDM and NP groups were administered intragastrically with the same volume of filled physiological saline. On gestational day 18, part of mice was sacrificed to collect the blood and liver. Other mice were maintained to give birth naturally to estimate the outcomes in offspring. From STZ induction, high fat diet was maintained for all mice. All the animal experiments conformed to guidelines for the Care and Use of Animals published by Institutional Animal Ethical Committee.

GTT and ITT

Mice were firstly fasted for 16 hours, followed by intraperitoneal injection of 1.5 g/kg body weight of glucose dissolved in saline for the glucose tolerance test (GTT). Then the tail vein blood glucose levels were tested at 0, 15, 30, 60, 90, and 120 min after injection with a glucometer. Mice were firstly fasted for 6 hours, followed by intraperitoneal injection of 0.5 U/kg body weight of insulin for the insulin tolerance test (ITT). Then the tail vein blood glucose levels were tested at 0, 15, 30, 60, 90, and 120 min after injection with a glucometer.

Insulin, adiponectin, TNF-α, MCP-1, IL-1β and IL-8 levels measurement

After fasting for 16 hours, 100-200 μL tail vein blood was collected at gestational day 18. The insulin, adiponectin, TNF-α, MCP-1, IL-1β and IL-8 levels of blood were tested by ELISA kits (CUSABIO, China) according to the manufacturer's guidance.

HE staining

Liver tissues were immediately fixed with 4 % paraformaldehyde, embedded in paraffin and cut into sections with 5 μm thickness, followed by dehydration. The sections were then stained with hematoxylin dye for 3–5 minutes, washed with PBS, dehydrated in alcohol for 5 minutes and last stained in eosin dye for 5 minutes. After that, slices were sealed and observed.

Oil red O staining

Frozen sections were cut off with the thickness of 6-10 μm and soaked in 60 % isopropanol for 2 minutes. Then the slices were washed with ice distilled, followed by staining with the oil red O staining solution for 10-15 minutes, avoiding light during this process. Wash with ice distilled water once. Add 60 % isopropanol and wash slightly to remove the dye solution. Add ice distilled water and rinse slightly. The nucleus was re-stained with hematoxylin staining solution for 5 minutes. Wash with ice distilled water once. The slices were sealed with glycerol gelatin and observed.

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). The differences between two groups were analyzed using a two tailed Student’s t-test. The difference among groups 3 or more groups were analyzed using one-way ANOVAs for multiple comparisons. The data analysis was performed using GraphPad Prism. P-values < 0.05 were considered statistically significant.

Results

Correlation between GDM and VDD during pregnancy

According to literature reports, pregnant women with GDM have a decrease in vitamin D levels in their blood [33, 34]. We selected blood samples from 10 GDM pregnant women and 10 normal pregnant women for vitamin D testing, and found that the serum vitamin D concentration of GDM pregnant women decreased significantly compared to the control group (Fig. 1A), consistent with literature reports. In order to investigate whether supplementing vitamin D can alleviate the symptoms of gestational GDM and explore its therapeutic mechanism, we constructed a GDM mouse model through a high-fat diet and multiple low-dose injections of STZ. The mouse model reproduced VDD and hyperglycemia symptoms (Fig. 1B, C), and vitamin D plasma concentration showed negative correlation with blood glucose (Fig. 1D).

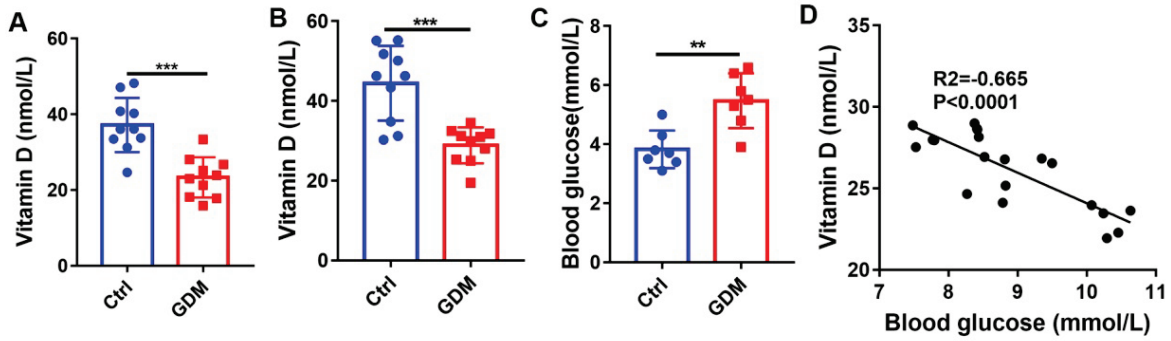

Fig. 1. Analysis of the correlation between GDM and VDD during pregnancy. **A**) Vitamin D concentration in the blood of pregnant women with GDM; **B**) Vitamin D concentration in the blood of pregnant mice with GDM; **C**) Fasting blood glucose concentration in pregnant mice; **D**) Analysis of the correlation between vitamin D and blood glucose concentration. statistics were performed using the two-tailed unpaired t-test. **P<0.01, ***P<0.001. For each experiment, the number of animals in each group is 10. GDM: Gestational diabetes mellitus.

Supplementation of VD alleviates GDM symptoms

To verify the therapeutic effect of vitamin D on GMD, we supplemented GDM pregnant mice with 1.0 IU/g of vitamin D daily. Firstly, we measured the blood glucose levels of mice on a high-fat diet for 4 weeks before and after injection of STZ (Fig. 2A, B), and the result showed that we successfully established a GDM mouse model by combining STZ administration with a high-fat diet. Then, the mice were supplemented with 1.0 IU/g of vitamin D daily (after which GDMt was defined as the vitamin D treatment group) and treated continuously for 2 weeks. After the first week, the blood glucose concentration was tested and the results showed that vitamin supplementation did not change the blood glucose statistically (Fig. 2C). However, after 2 weeks of continuous treatment, there was a significant difference between the treatment group and the untreated group (Fig. 2D). The blood glucose concentration of pregnant mice in the treatment group decreased to the similar level of the control pregnant mice, indicating that vitamin D can effectively alleviate the hyperglycemia of GDM pregnant mice.

To further evaluate the sustained effect of vitamin D on blood glucose maintenance, we conducted GTT analysis on pregnant mice. The results showed that vitamin D treatment can maintain blood glucose at relatively low levels and enhance blood glucose clearance efficiency (Fig. 2E). Because blood glucose concentration is directly related to serum insulin concentration, we conducted ITT analysis on pregnant mice. The results showed that vitamin D treatment can effectively enhance insulin response (Fig. 2F). Next, we analyzed fasting blood glucose levels and found that vitamin D treatment decreased the fasting blood glucose levels (Fig. 2G). Then we tested the levels of insulin and adiponectin, which are directly involved in glucose metabolism, and found that vitamin D supplementation can effectively increase the levels of serum insulin and adiponectin (Fig. 2H, I), indicating that vitamin D alleviates GDM symptoms in pregnant mice by regulating glucose metabolism.

Vitamin D supplementation improves the outcomes of offspring of GDM pregnant mice

Pregnant mothers with GDM provide a high glucose environment for the fetus for a long time, which

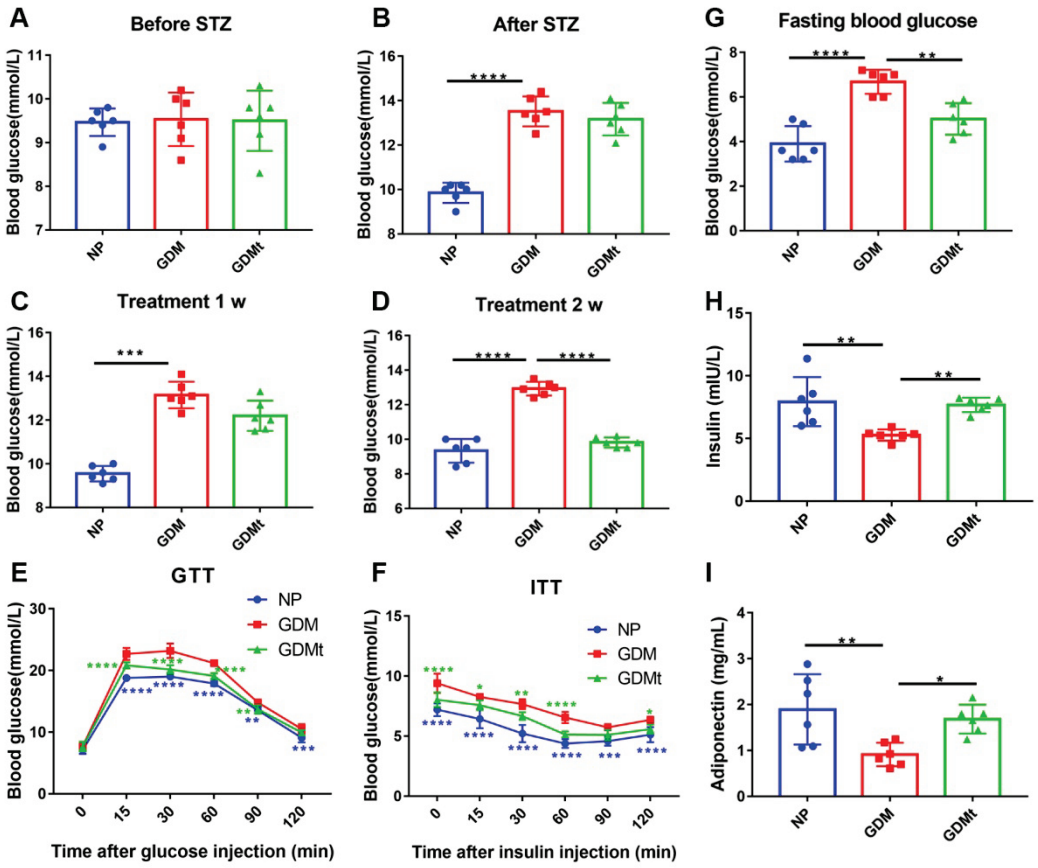

Fig. 2. Vitamin treatment alleviates hyperglycemia in GDM pregnant mice. **A, B**) Random blood glucose concentration before and after STZ induction; **C, D**) Random blood glucose concentration after vitamin D treatment for 1 or 2 weeks; **E**) GTT analysis. Mice fasted for 16 hours on gestation day 14-15 were subjected to GTT; **F**) ITT analysis. Mice fasted for 6 hours on gestation day 14-15 were subjected to ITT; **G)** Fasting blood glucose concentration of mice on gestation day 14-15; **H, I**) Insulin and adiponectin concentration of mice on gestation day 18-19. statistics were performed using the one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. For each experiment, the number of animals in each group is 6. NP: normal pregnancy; GDM: Gestational diabetes mellitus; GDMt: Gestational diabetes mellitus with vitamin D treatment.

could affect the fetal growth directly or even irreversibly. Therefore, we compared the outcomes of offspring of GDM pregnant mice treated with vitamin D with those without treatment. GDM or vitamin D supplementation did not significantly alter the number of the born offspring (Fig. 3A). However, vitamin D supplementation significantly reduced the increased number of stillbirths and neonatal weight (Fig. 3C) caused by GDM (Fig. 3B). Then we evaluated whether vitamin D treatment had long-term effects on offspring. We tested the blood glucose levels of mice before and after weaning, and found that the trend of blood glucose levels of offspring before weaning was similar to that of the mother's blood glucose. Offspring of mice with GDM demonstrated the highest blood glucose levels, but offspring of vitamin D treated mothers showed a decrease in blood glucose levels compared to the untreated group (Fig. 3D). However, after weaning, the blood glucose levels in GDM mice showed the lowest trend, while the blood glucose levels in offspring of vitamin D treated and control mice showed a relatively stable trend (Fig. 3E), which may be due to the incomplete recovery of blood glucose regulation ability in newly weaned offspring of GDM mice. Then we continued to monitor the weight changes of the newborn mice and found that the offspring of the vitamin D treated mother had lower weight than the untreated group before weaning. This trend persisted until the 5th week after birth, and by the 6th week, the weight of each group was no longer significantly different (Fig. 3F). After the offspring reached adulthood (8 weeks old), we conducted GTT and ITT analysis on them and found no significant differences in GTT and ITT among each group. This indicates that mothers with GDM may have an impact on the metabolic homeostasis of offspring for a period of time, but this effect is not lifelong. The above data indicates that supplementing

GDM mothers with vitamin D can effectively protect the development and growth healthy of the fetus and offspring.

Vitamin D supplementation diminished the systemic inflammatory response

According to literature reports, vitamin D mainly regulates glucose metabolism via modulating immune responses [29]. Therefore, we evaluated the levels of inflammatory factors in plasma at pregnant day 18 after supplementing pregnant mice with GDM with vitamin D. The results showed that vitamin D supplementation significantly reduced the level of the inflammatory factor TFN-α, MCP-1, IL-1β and IL-8 in the plasma compared to the untreated group (Fig. 4A-D), indicating that supplementing GDM mothers with vitamin D can systematically reduce the body's inflammatory response.

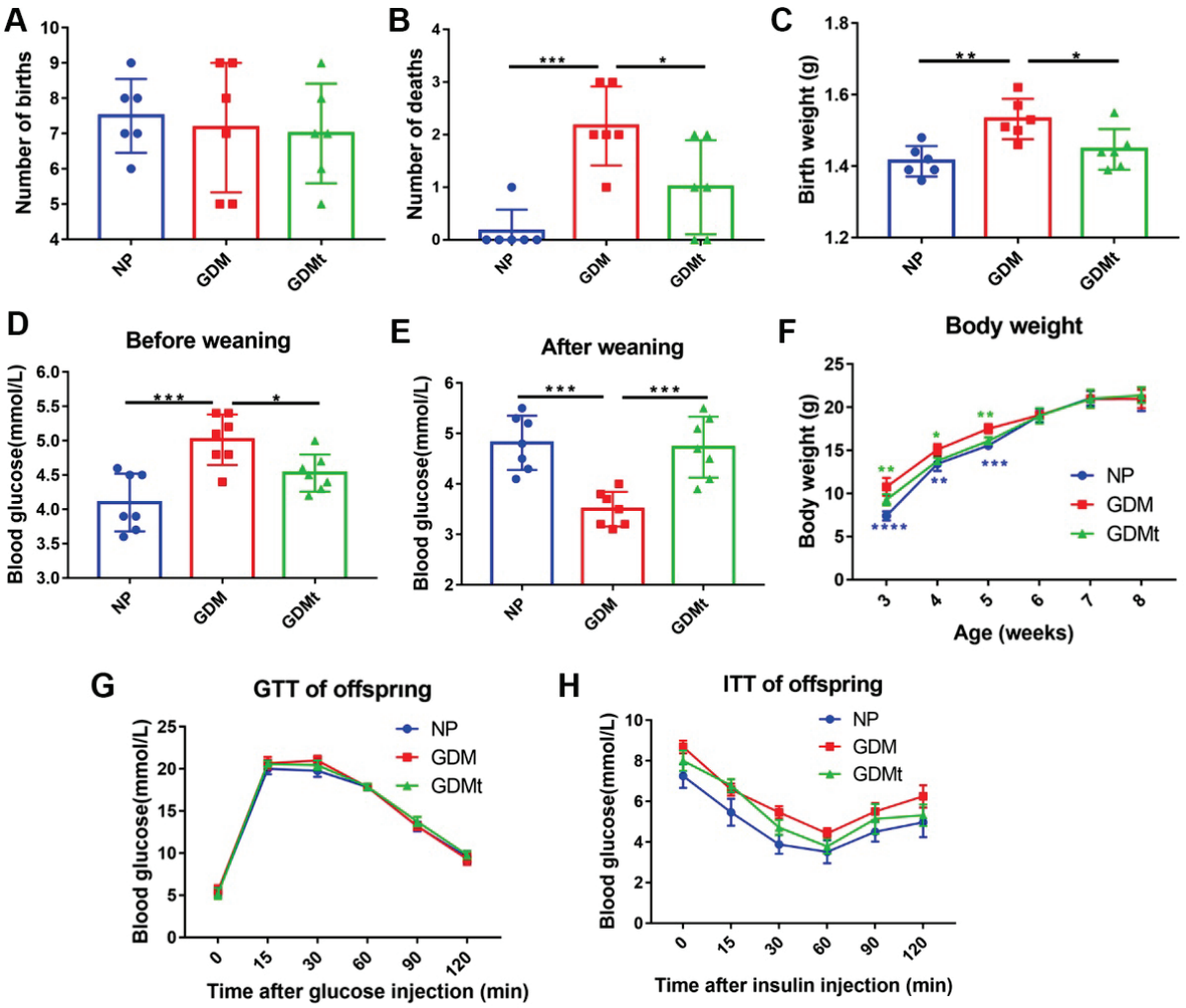

Fig. 3. Vitamin D improves outcomes of GDM offspring. **A**) Number of births; **B**) Number of deaths; **C**) Body weight of offspring; **D**) Random blood glucose concentration before weaning; **E**) Random blood glucose concentration After weaning; **F**) Change of body weight from weaning to adult; **G**) GTT analysis. Mice fasted for 16 hours at 8-week-old were subjected to GTT; H) ITT analysis. Mice fasted for 6 hours at 8-week-old were subjected to ITT. Statistics were performed using the one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. For each experiment, the number of animals in each group is 6 or 7. NP: normal pregnancy; GDM: Gestational diabetes mellitus; GDMt: Gestational diabetes mellitus with vitamin D treatment.

Vitamin D treatment improves liver fat accumulation

The liver is the most important organ that regulates blood glucose concentration. The glucose metabolism in the liver is closely related to the lipid metabolism. We observed significant improvements in blood glucose levels, GTT, and ITT in GDM mice treated with vitamin D, therefore we speculated that vitamin D treatment may also have a subsequent regulatory effect on liver fat metabolism. We collected the livers of mice after 2 weeks of vitamin D treatment for HE staining and

oil red O staining, and found that vitamin D treatment could effectively reduce the steatosis and lipid droplet accumulation in hepatocytes (Fig. 4E,F). However, the liver function indicators ALB, ALT, and AST in the GDM and GDMt groups demonstrated no statistical differences (Fig. 4G-I). Then we speculated that the lipid concentration in the blood of pregnant mice. In order to verify it, we collected the blood of pregnant mice at the pregnant day 18, and found that vitamin D supplementation effectively reduced the levels of plasma TG and CHO (Fig. 4J,K).

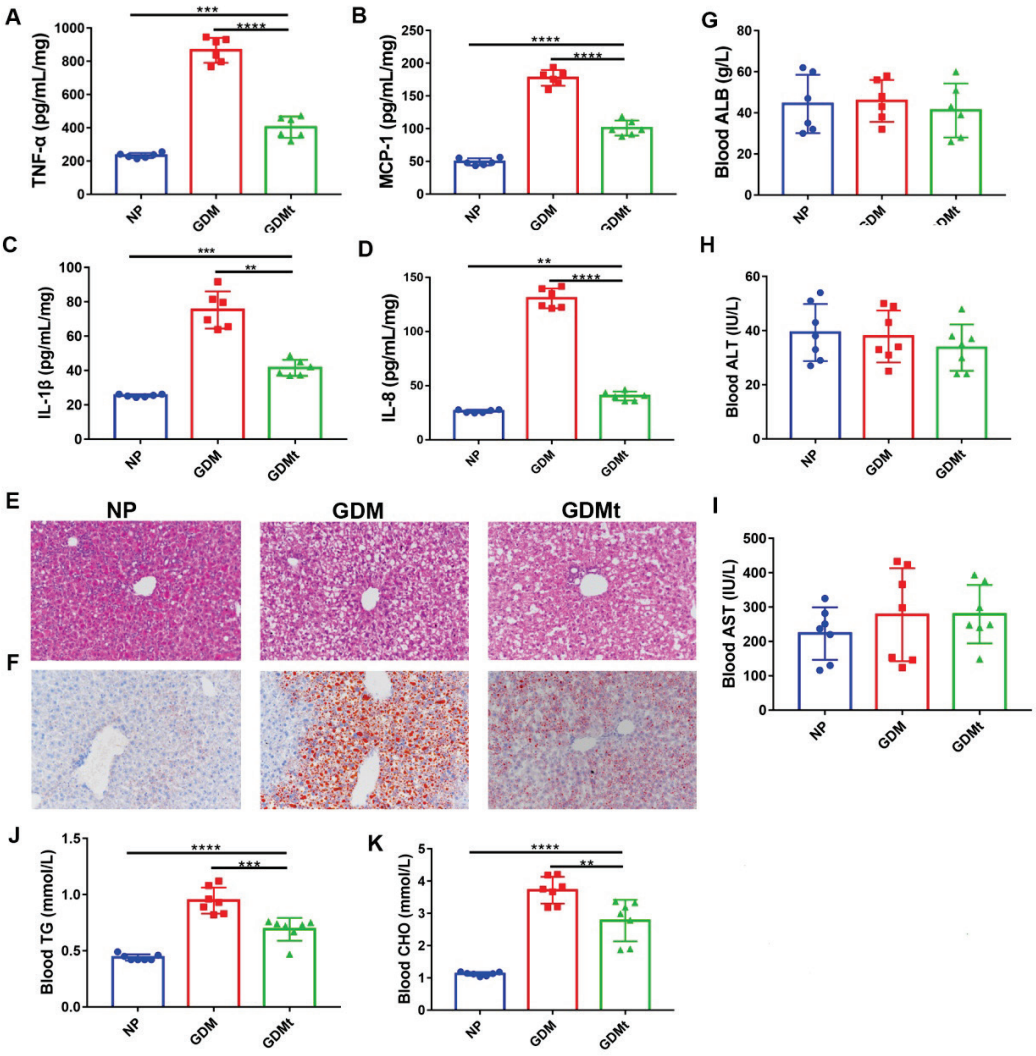

Fig. 4. Vitamin D treatment alleviates systemically inflammation and liver lipid accumulation. A-D: Inflammatory factor levels in the blood, **A**) TNF-α, **B**) MCP-1, **C**) IL-1β, **D**) IL-8; **E**) HE staining of liver at pregnant day 18; **F**) Oil Red O staining of liver at pregnant day 18; **G**) Blood ALB level; **H**) Blood ALT level; **I**) Blood AST level; **J**) Blood TG level; **K**) Blood CHO level. Statistics were performed using the one-way ANOVA. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. For each experiment, the number of animals in each group is 6. NP: normal pregnancy; GDM: Gestational diabetes mellitus; GDMt: Gestational diabetes mellitus with vitamin D treatment.

Discussion

GDM is one of the pregnancy complications with a very high incidence rate. If it is not properly treated, it will cause serious consequences to both the mother and the fetus, which may cause the occurrence of preeclampsia, increased newborn percent body fat, fetal macrosomia, obstructed shoulder delivery, cesarean delivery, postpartum hemorrhaging, neonatal asphyxia or death. Even worse, the risk of GDM mothers and their offspring to develop type 2 diabetes mellitus, obesity or other metabolic diseases later in life shows higher than those who has normal poignancy [35]. Hence, GDM contributes to a vicious intergenerational cycle from mother to child and impacts the health of the entire global population.

The published data shows that multiple factors are correlated with the occurrence of GDM, including vitamin D, Folic Acid, Omega 3 Fatty Acids, Resveratrol and Vitamins C and E [36]. It has been many reports about how vitamin D regulate glucose metabolism in disordered metabolism diseases [37, 38]. However, there is currently only correlation analysis of clinical data on whether supplementing vitamin D can alleviate GDM symptoms. There are still few reports on the long-term effects of vitamin D on glucose and lipid metabolism in GDM pregnant women and on the fetus. In order to systematically explore the effects of vitamin D supplementation on GDM mothers and offspring, a GDM mouse model was established. After STZ induction, vitamin D treatment was administered continuously for 14 days. We found that vitamin D supplementation can effectively reduce blood glucose levels and improve GTT and ITT in GDM mice, as well as increase the levels of insulin and adiponectin in the blood. This indicates that vitamins can regulate insulin sensitivity, thereby regulating the body's glucose metabolism. Then we conducted a long-term analysis of the impact of GDM pregnant mice on their offspring and found that vitamin D supplementation can effectively reduce the number of stillbirths and birth weight. The impact of GDM on the weight of offspring mainly sustained in the first 5 weeks, and the impact is no longer statistically significant by the 6th week. After reaching adulthood, there was no significant difference in GTT and ITT between the offspring of each group. However, there were significant differences in blood glucose levels between offspring mice before and after weaning. The above results indicate that GDM pregnant mice have a short-term impact on their offspring, which is very obvious before weaning, and after weaning, the offspring can gradually return to normal levels, which means that the impact of GDM pregnant mice on their offspring is not lifelong.

Understanding the etiology of GDM is important for designing appropriate preventive and therapeutic strategies. We found that vitamin D supplementation can reduce the levels of inflammatory factors in the blood of pregnant mice, which is consistent with the mechanism of action of vitamin D reported in literature, indicating that vitamin D may improve insulin sensitivity and its regulatory effect on blood glucose by regulating inflammatory factors. In addition, in the body, glucose metabolism and lipid metabolism interact each other. We also analyzed the lipid metabolism of pregnant mice, and found that vitamin D supplementation can reduce the lipid accumulation in the liver and the steatosis of hepatocytes. GDM did not significantly damage the liver function of pregnant mice, but could significantly increase TG and CHO in the blood which can be reversed by vitamin D supplementation. The above results indicate that vitamin D supplementation can regulate glucose and lipid metabolism in GDM pregnant mice, thereby systematically improving the hyperglycemia and hyperlipidemia.

Our findings systematically illustrate the impact of vitamin D on GDM mothers and offspring, and briefly explore the treatment mechanism, providing theoretical support for the clinical use of vitamin D to treat GDM symptoms. Of course, there are also areas for improvement in this study. We did not specifically analyze how the vitamin D impact on glucose metabolism. Additionally, although we observed that vitamin D supplementation can reduce liver lipid accumulation, we cannot explain whether the β-oxidation or lipid synthesis is the main pathway to result it. We will continue to research those in future studies. In summary, this study demonstrates that vitamin D supplementation is an effective measure to alleviate GDM symptoms.

Author Contributions

S. G, performed most of the experiment, wrote and edited the manuscript. X. C, performed part of the experiment and generated Fig. s. Y. L, guided the design of the project and edited the manuscript.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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The animal experiments were approved by the Animal Ethics Committee of The Fourth Hospital of Shijiazhuang (No.20230156).

The data in this study are available from the corresponding author upon request.

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