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MATERNAL-FETAL MEDICINE

In vivo: maternal betaine supplementation normalized fetal growth in diabetic pregnancy

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Abstract

Purpose Diabetes alters maternal metabolism and can lead to aberrant fetal growth. In addition to insulin treatment, nutritional diet interventions are recommended for promoting fetal health against diabetes-induced adverse effects. Therefore, we conducted an in vivo study to investigate betaine efficacy on fetal development against maternal diabetes.

Methods Thirty-two dams were divided into four equal groups: control (C), betaine supplementation (BS), diabetic pregnancy (DP) and diabetic pregnancy plus betaine supplementation (DP + BS). Fasting blood sugar (FBS) and body weight (BW) were monitored during pregnancy. After physiological delivery, dams glycated hemoglobin (HbA1c) concentrations were measured, followed by fetal development indices including litter size (LS), neonatal weight (NW) and crown-rump (CR). Also, maternal oxidative status was assessed by evaluating glutathione (GSH) content, glutathione peroxidase (GSH-Px) and catalase (CAT) activities, and malondialdehyde (MDA) concentration in the erythrocytes.

Results Betaine supplementation significantly alleviated FBS and tended to recover BW loss. It also significantly decreased HbA1c values in dams of DP + BS compared to DP group. Normalized fetal indices such as LS, NW and CR under betaine supplementation were associated with a significant increase in GSH content and GSH-Px activity, as well as decreased MDA concentrations in erythrocytes of dams in the DP + BS versus the DP group, indicating improved redox balance in the dams.

Conclusion We indicated for the first time that betaine supplementation improved the maternal glucose metabolism and redox balance associated with normalized fetal growth. Nevertheless, further studies are required to investigate the mechanisms through which betaine protects fetal growth in diabetic pregnancy.

Keywords Diabetic pregnancy · Betaine · Oxidative stress · Intrauterine growth restriction · Uteroplacental insufficiency

Introduction

Offspring developmental stages including embryonic and fetal are very sensitive to alterations in intrauterine environment. Diabetes during pregnancy either type I or type II exerts debilitating impacts on maternal intrauterine milieu thereby increasing the risk of aberrant fetal growth include large for gestational age (LGA) and small for gestational age (SGA) [26]. It also predisposes the neonates to develop metabolic syndromes later in life which is suggested to be correlated with diabetes epidemic. Extensive evidence suggests that women with higher levels of maternal fasting blood sugar (FBS) or gestational diabetes mellitus are more susceptible to deliver LGA infants [14]. Recently, a cohort study in a pollution of mothers with pre-pregnancy or gestational diabetes revealed that SGA incidence was more frequent [11]. On the other hand, mothers with poorly controlled type I diabetes are at increased risk of delivering a microsomic infant [16]. Intrauterine growth restriction (IUGR), a term for fetus inability to gain its genetically growth potential, is associated with incidence of SGA in all types of diabetic pregnancies both in human and animal models [28]. Although maternal glycemic control has shown to be beneficial for promoting fetal development against diabetes complications and despite the current treatments, these adverse outcomes are still prevalent [6]. In this regard, lifestyle modifications such as dietary interventions are suggested to be a good approach to promoting maternal and fetal health against diabetes-induced disorders [14].

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Betaine is a micronutrient first discovered in the juice of sugar beets (*Beta vulgaris*) in the 19th century it can be obtained endogenously by choline catabolism and exogenously at high concentrations in whole grains [8]. According to data obtained from animal models, betaine increases cellular glucose utilization in metabolic disorders such as obesity and nonalcoholic fatty liver disease [10, 20]. In this regard, recent papers have emphasized the possible association between decreased plasma concentration plus increased urinary excretion of betaine and glucose metabolism in gestational diabetes mellitus and diabetes type II [7, 23]. Human and animal studies imply that maternal adequate betaine content during pregnancy might provide a normal milieu for fetal development against mother metabolic alterations [24, 36]. In this regard, a previous study in our laboratory demonstrated positive nutritional effects of betaine on the maternal health and fetal development in pregnant rats stressed by ethanol administration [1]. Also, a couple of attempts made by Joselit et al. indicated that betaine supplementation ameliorated maternal glucose metabolism and fetal growth in animal models of maternal obesity induced by high fat feeding in pregnant rodents [18].

The aforementioned studies provide proof of concept that betaine may be further explored as an adjuvant therapy in the clinical setting for improving fetal outcomes in diabetic pregnancies. Thus, we conducted an in vivo study to investigate betaine potential efficacy on fetal development against maternal diabetes.

Materials and methods

Materials

Streptozotocin (STZ) was purchased from Sigma© Chemical Company (St Louis, Missouri, USA). Betaine (Betafin® 96%) was prepared from Biochem (Lohne, Germany). HbA1c detection kit was purchased from BioSystems© (Barcelona, Spain). Glutathione peroxidase (GSH-Px), catalase (CAT), glutathione (GSH) and malondialdehyde (MDA) kits were supplied from Kiazist© Company (Kiazist Life Sciences, Iran).

Animals

A total of 90 male and female Sprague–Dawley rats (60 virgin female rats weighing 180–200 and 30 male rats weighing 220–250) were purchased from Animal laboratory of Razi Herbal Medicines Research Center (Lorestan University of Medical Sciences, Khorramabad, Iran) and maintained in the same place in a controlled environmental condition (23 ± 1 °C, 50 ± 10% humidity, and 12:12 light–dark cycle) with standard laboratory diet and tap water ad libitum.

Cyclicity checking and mating

The estrous cycle of rats was characterized by modification of the methods previously reported by Aziz et al. (Supplementary Fig. 1). The rats in the estrus stage were allowed to mate overnight with males at a 2:1 ratio. In the following morning, pregnancy was confirmed by the presence of a vaginal plug (mucus plug) or spermatozoa in vaginal smear respectively, considered as day 0 of gestation (Supplementary Fig. 2). Then, each pregnant female was transferred into a separate cage and followed up toward the parturition [5].

Experimental design

A total of 32 pregnant rats were obtained from preparation steps and distributed into 4 equal groups: Control (C), Diabetic pregnancy (DP), Betaine supplementation (BS, 1.5% w/w of the daily diet), and diabetic pregnancy plus betaine supplementation (DP + BS) [2]. The dams in all groups were fasted for 12 h, and diabetes was induced (day 1 of gestation) by injecting a single dose of STZ intraperitoneally (65 mg/kg) freshly dissolved in cold sodium citrate buffer (0.1 M, pH 4.4) in the DP and DP + BS groups while the other pregnant rats only received an equal volume of the vehicle [17]. Diabetic condition was observed 48 h post STZ injection by some signs such as polyuria, polydipsia, and polyphagia. Then, FBS was measured using Glucometer (ACCU-CHEK® Active glucometer, Roche Diagnostics, Germany) where the rats with blood glucose values greater than 350 mg/dl were considered as diabetic dams. Once the diabetic condition was confirmed, betaine supplement was added to animal's water in the mentioned groups from 3rd day toward the 3rd week (day 21) of gestation. Also, a graphical scheme briefly demonstrates the experimental design (Fig. 1).

FBS and BW screening

FBS and body weight (BW) of dams were measured and recorded using the glucometer and a digital weight scale (Sartorius, Germany) during pregnancy on days 7, 14, and 21 of the experiment.

Blood sample collection

After delivery, all the dams were anesthetized by light diethyl ether and blood samples drawn through cardiac puncture and collected into heparinized tubes. Whole blood was used for hemoglobin and glycated hemoglobin (HbA1c)

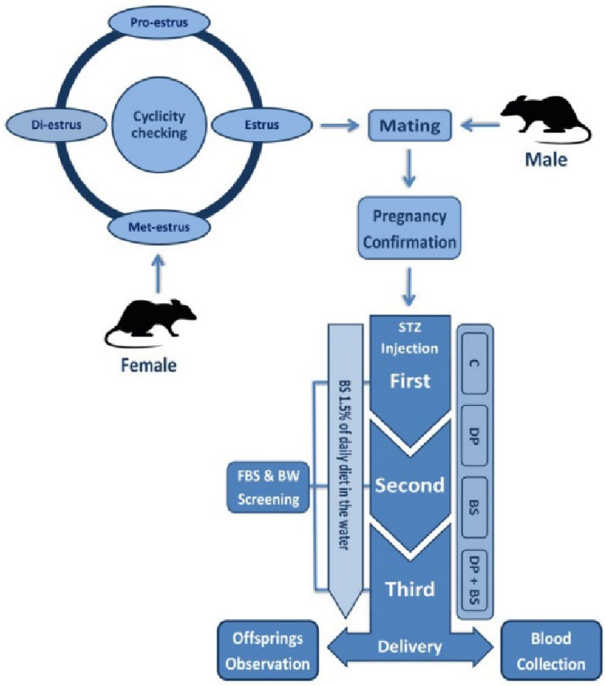

Fig. 1 Experimental design graphical scheme: C (control), DP (diabetic pregnancy), BS (betaine supplementation), DP + BS (diabetic pregnancy plus betaine supplementation); first, second and third (weeks of pregnancy)

measurements and washed erythrocytes for oxidative stress assessment as fresh.

HbA1c measurement

Glycated hemoglobin was measured by BioSystems (Barcelona, Spain) HbA1c (%) detection kit according to the manufacturer's instruction using ion-exchange chromatography approach spectrophotometrically (S2000 UV model; WPA, Cambridge, UK) as previously described [3]. HbA1c concentration was calculated based on the percentage of total hemoglobin as HbA1c % and then converted to mmol/mol as described by the National Glycohemoglobin Standardization Program (NGSP).

Oxidative stress biomarkers

Enzymatic and non-enzymatic antioxidant capacity as well as lipid peroxidation status was evaluated in washed erythrocytes as a proxy of dams redox condition. GSH-Px and CAT activities, GSH and MDA content were measured by Kiazist life Science Company kits according to the manufacturer's instruction using BioTek ELx808 absorbance reader (BioTek Instruments, Inc. Winooski, Vermont, USA).

Offspring observation

After physiological delivery, the litter size (LS) was recorded. Also, the neonatal weight (NW) and crown-rump (CR) were measured using the digital weight scale and a millimeter graded meter (Tokyo, Japan), respectively.

Statistical analysis

Statistical analysis was performed using the statistical package GraphPad PRISM version 6 (GraphPad Software, San Diego, CA, USA) with all the results being presented as mean ± S.E.M. The statistical differences were applied across all groups by one-way analysis of variance (ANOVA) and supported by Tukey's post hoc analysis.

Results

FBS and BW

As observed in Table 1, FBS levels in the C and BS groups were significantly elevated in the 2nd versus 1st week of gestation ($p < 0.05$). Also, a significant increase was seen in the 3rd versus 1st week and 3rd against 2nd of gestation ($p < 0.01$). STZ administration induced hyperglycemia in the DP and DP + BS from 1st week of gestation toward

Table 1 Fasting blood sugar and body weight during gestation from different groups

Groups	Fasting blood sugar (mg/dl)				Body weight (g)			
	Pre gestation	1st week	2nd week	3rd week	Pre gestation	1st week	2nd week	3rd week
C	93.1 ± 1.4	103.0 ± 2.1	125 ± 3.1[a]	154 ± 3.0[b]	189.3 ± 2.9	196.4 ± 2.9	215.3 ± 2.3	246.1 ± 3.7
DP	91.8 ± 1.3	590.0 ± 8.0*	600.0 ± 8.3*	612.0 ± 10.0*	192.1 ± 2.5	183.5 ± 3.8	201.6 ± 3.2[α]	226.6 ± 3.2[α]
BS	91.8 ± 1.9	98.6 ± 1.2	115.0 ± 2.4[a]	128.8 ± 1.98[b]	192.0 ± 3.9	197.6 ± 2.7	217.3 ± 2.9	253.0 ± 5.9
DP + BS	90.6 ± 1.8	545.6 ± 11.2*,#	499.8 ± 16.6*,#	518 ± 7.9*,#	191.6 ± 2.5	193.9 ± 5.5	211.1 ± 1.6	241.9 ± 2.1

Data presented as mean ± SEM ($n = 8$) [a]$p < 0.05$ vs. 1st; [b]$p < 0.01$ vs. 1st and 2nd; *$p < 0.0001$ vs. C and BS; #$p < 0.0001$ vs. DP; [α]$p < 0.001$ vs. C and BS

C control, *DP* diabetic pregnancy, *BS* betaine supplementation, *DP + BS* diabetic pregnancy plus betaine supplementation

the parturition, while the betaine treatment reduced FBS values in DP + BS rats in 2nd compared to 1st week of pregnancy ($p = 0.06$). In spite of an apparent increment for FBS levels of DP + BS group in the 3rd compared to 2nd week of pregnancy, it was not significantly different ($p = 0.82$). FBS diminished in DP + BS group in the 3rd against 1st week of gestation ($p = 0.051$). When summing up the betaine effects on FBS during pregnancy, we observed a significant reduction compared to DP group ($p < 0.0001$) on all days. There was not a significant difference for BW between the C, BS and DP + BS groups across all days. The dams in the DP group indicated a significant reduction for BW versus the C and BS groups ($p < 0.01$), though no statistical difference was seen in the 1st week of pregnancy. Betaine supplementation tended to prevent the weight loss of dams in DP + BS against DP group in 2nd ($p = 0.071$) and 3rd week ($p = 0.056$).

HbA1c

As indicated in Fig. 2, STZ administration induced a significant rise of HbA1c values in DP and DP + BS groups compared to C and BS ($p < 0.0001$). Under betaine supplementation, this parameter dropped significantly in DP + BS rats in comparison with DP group's ($p < 0.0001$).

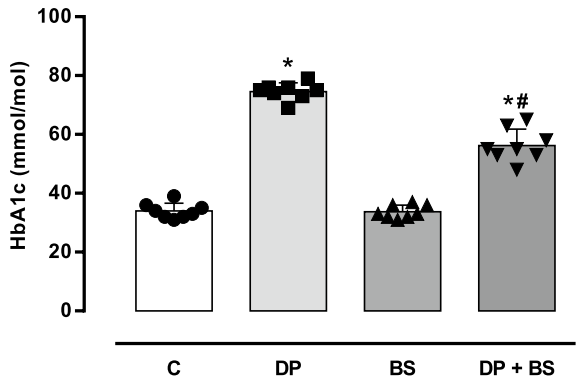

Fig. 2 HbA1c concentration among the groups. *C* control, *DP* diabetic pregnancy, *BS* betaine supplementation, *DP + BS* diabetic pregnancy plus betaine supplementation. Data are expressed as mean ± SEM ($n = 8$) and geometrical shapes indicates data scattering $^{*}p < 0.0001$ vs. C and BS groups, $^{\#}p < 0.0001$ vs. DP group

Oxidative stress biomarkers

The GSH-Px activity of erythrocytes significantly decreased in DP group compared to C and BS ($p < 0.0001$). Betaine supplementation corrected GSH-Px activity and brought it close to the normal level in DP + BS compared to DP ($p < 0.05$), and there was no statistical difference between C, BS and DP + BS groups. In contrast, CAT activity indicated no significant difference between the groups. Erythrocytes GSH content in DP group decreased significantly against C and BS ($p < 0.0001$). Although betaine supplementation significantly elevated the GSH content in DP + BS compared to DP ($p < 0.01$), it was not sufficient to compensate the reduction compared to C and BS groups ($p < 0.001$). Regarding lipid peroxidation, MDA significantly increased in DP group when compared to the other groups ($p < 0.001$). Betaine supplementation completely recovered MDA level back to normal values in DP + BS and there were no statistical difference between C, BS and DP + BS groups (Table 2).

Offsprings observation

Although LS decreased in DP group dams compared to C and BS ($p < 0.0001$), betaine supplementation significantly prevented LS reduction in DP + BS versus DP group ($p < 0.0001$). NW significantly declined in the dams of DP group versus the other groups ($p < 0.0001$). In spite of many pathophysiological alterations in the diabetic state, betaine treatment during pregnancy prevented NW loss in dams of DP + BS against DP group's ($p < 0.001$). There was no significant difference for NW between C, BS, and DP + BS groups. CR decreased significantly in DP group offspring compared to the other groups ($p < 0.0001$). Also, under betaine supplementation CR restored to the normal range in DP + BS against DP group ($p < 0.0001$). Meanwhile, none of the differences for LS, NW and CR between the C, BS and GD + BS groups were statistically significant (Fig. 3).

Table 2 Erythrocytes antioxidant and lipid proxidation biomarkers from different groups

Groups	C	DP	BS	DP + BS
GSH-Px (UI/g Hb)	336.1 ± 27.6	183.8 ± 6.2[a]	341.4 ± 17.7	273.0 ± 19.6[b]
CAT (mUI/g Hb)	6.2 ± 0.4	5.2 ± 0.2	6.1 ± 0.7	5.9 ± 0.3
GSH (mmol/g Hb)	7.5 ± 0.5	1.5 ± 0.1[c]	7.2 ± 0.5	4.1 ± 0.3[d]
MDA (mmol/g Hb)	1168 ± 62.9	1949 ± 171.9[e]	1228 ± 108.1	1199 ± 128.9

Data presented as mean ± SEM ($n = 8$) [a]$p < 0.0001$ vs. C and BS; [b]$p < 0.05$ vs. DP; [c]$p < 0.0001$ vs. C and BS; [d]$p < 0.01$ vs. DP; [e]$p < 0.0001$ vs. Other groups

C control, *DP* diabetic pregnancy, *BS* betaine supplementation, *DP + BS* diabetic pregnancy plus betaine supplementation, *GSH-Px* glutathione peroxidase, *CAT* catalase, *GSH* glutathione, *MDA* malondialdehyde

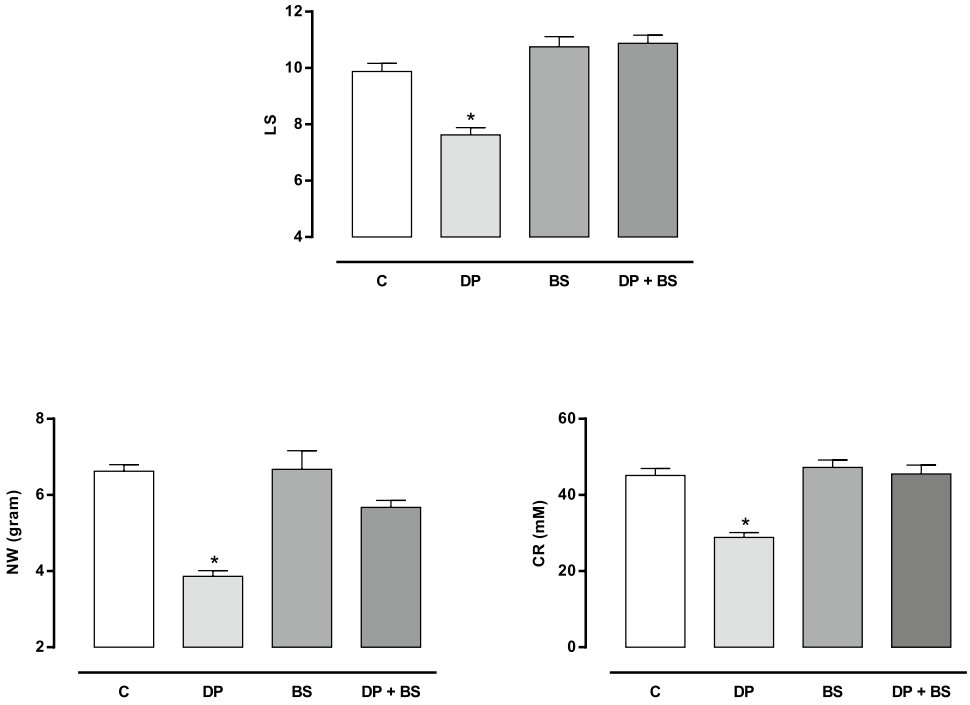

Fig. 3 Offspring developmental indices among the groups. C (control), DP (Diabetic pregnancy), BS (Betaine supplementation), DP + BS (Diabetic pregnancy plus betaine supplementation), Litter size (LS), Neonatal weight (NW), Crown-rump (CR). Data presented as mean ± SEM ($n = 8$) *$p < 0.0001$ vs. the other groups

Discussion

We observed that impaired glucose metabolism, BW loss, and redox imbalance were associated with IUGR in diabetic dams. Interestingly, betaine supplementation improved FBS, tended to recover BW loss, increased GSH content and GSH-Px activity, and also alleviated MDA which was followed by normalized fetal indices.

Hyperglycemia has been introduced as the basic cause of IUGR and subsequent SGA in diabetic pregnancies [35] and significant improvement was obtained since insulin discovery [9]. Also, a low BW due to maternal diabetes has been associated with low NW and LS in human and multiparous species [11, 22]. Accordingly, we perceived that improvement in maternal FBS and BW were accompanied by normalized LS, NW, and CR. However, Eriksson et al. [12] reported that in spite of a normoglycemic state under insulin treatment, maternal and fetal body weight, as well as, the number of viable offsprings were significantly lower in diabetic pregnant rats compared to control groups. In contrast, another animal model indicated that insulin treatment failed to affect the infant's body weight [19]. Also, human studies well have proven that insulin treatment alone is not efficient to completely prevent fetal adverse outcome in all types of diabetic pregnancies [13]. This major discrepancy in fetal outcomes correlated with maternal blood sugar previses another underlying pathophysiological mechanism, as evidenced by our result in which glucose-lowering effect was not the main mechanism by which betaine protected fetal development because maternal blood glucose levels remained far higher than normal values.

Its been suggested that maternal oxidative stress disturbs normal placental development by altering angiogenesis and trophoblast invasion, also, causing erythrocyte aggregation and increases non-functional form of hemoglobin (ferric-Hb). This, in turn, leads to low utero-placental perfusion followed by impaired fetal nutrient and oxygen availability which is characterized by IUGR [27, 28, 30]. In this regard, Sinzato et al. [31] reported that increased lipid peroxidation and decreased antioxidant capacity have been detected in maternal and umbilical cord plasma, as well as placental and fetal tissue in association with IUGR. In consensus, our results demonstrated that fetal growth retardation was associated with a low GSH content, decreased GSH-Px activity, and increased MDA concentrations in erythrocytes of diabetic dams, thereby supporting the existing data suggesting the involvement of maternal oxidative stress in the mechanism of IUGR. In diabetic pregnancies, hyperglycemia is responsible for oxidative stress through several routes such as polyol pathway, increased advanced end glycated products (AGEs), and diacylglycerol (DAG) production [34]. Herein, BW loss implies on the release of high mass of DAG into the bloodstream as a result of fat and protein catabolism, also, high levels of HbA1c as a proxy for AGEs proved to be well correlated with IUGR [5, 19]. Howbeit, we indicated that without favorable improvement in BW and HbA1c of diabetic pregnant rats, redox imbalance was regulated under betaine supplementation followed by normalized fetal indices. As well as, a recent cohort notified that good glycemic

control could not prevent the redox imbalance in GDM women [29]. This could shed light on the missing key of the non-integrated association between maternal blood sugar and fetal outcomes as was questioned in the first paragraph. Indeed, both in vivo and in vitro studies found that antioxidant supplementation diminishes embryonic maldevelopment in diabetic pregnancy (Eriksson). Cederberg et al. [6] gave notice that maternal supplementation with Vitamins E and C could provide a normal intrauterine milieu for fetal development in diabetic pregnancy. Also, low level of these antioxidant vitamins has been reported to be associated with fetal maldevelopment [15]. In contrast, a meta-analysis demonstrated that supplementation with these antioxidant vitamins did not improve fetal growth [28]. However, it seems no clinical trial evaluated the impact of antioxidant supplementation on IUGR in diabetic pregnancies. Recently, King et al. [21] reported that supplementation with choline (betaine precursor) increases maternal betaine levels and regulates fetal growth characterized by increased NW and CR in a mouse model of placental insufficiency. Previous studies in our laboratory revealed betaine positive impacts on enzymatic and non-enzymatic antioxidant systems. Also, we found that betaine supplementation improves fetal development in pregnant rats stressed by ethanol administration [1, 2]. Herein, our result demonstrated that betaine supplementation enlivened redox balance in diabetic pregnant rats which were followed by normalized LS, NW, CR. Thus, it could be comprehended that betaine regulated fetoplacental blood flow in downstream pathways of its antioxidant properties followed by improved fetal growth.

In another perspective, its been reported that redox imbalance, maternal–fetal one-carbon metabolism alterations, and low methyl donor availability are correlated with IUGR in association with epigenetic regulation such as DNA methylation of the fetal genome [25]. Pancreatic and duodenal homeobox 1 (Pdx1) is a gene required for pancreatic development, β-cell differentiation, and insulin secretion. Malregulated methylation of Pdx1 alters it's expression, subsequently causes fetus inability of glucose uptake followed by growth retardation [4, 33]. Sulaiman et al. [32] noted that maternal methyl donor supplementation partially restored the number of pancreas β-cells and tended to improve insulin sensitivity compared to untreated IUGR lambs. Consistently with this, decreased pancreatic β-cells is observed in human IUGR newborns [28]. Currently, B_{12} and folate supplementation is commonly prescribed to pregnant women to promote fetal growth [26]. Betaine is a modified glycine, a pivotal molecule in one-carbon metabolism, providing methyl groups for many cellular methylation reactions including DNA methylation [8]. In this regard, Li et al. [24] declared that betaine supplementation to gestational sows modulated DNA methylation in newborn piglets. Hence it can be hypothesized that betaine might prevent β-cells dysfunction by providing adequate methyl groups for optimal DNA methylation of Pdx1 in downstream of increased antioxidant capacity. This, in turn, would maintain normal glucose metabolism in fetal circulation characterized by promoted fetal growth.

Conclusions

Taken together, we reported for the first time that betaine normalized fetal growth correlated with alleviating maternal glucose levels and boosting antioxidant capacity. In spite of improved maternal glucose metabolism and antioxidant capacity under betaine supplementation in this study, it did not reflect the absolute mechanism by which betaine protected and normalized fetal growth in impaired intrauterine milieu by diabetes. Herein, we set the ground for future studies of evaluating betaine as a dietary intervention in the clinical setting for ameliorating maternal–fetal health in diabetic pregnancies. In this regard, further research is required to investigate the role of hyperglycemia in pathophysiological mechanism of LGA and SGA, as well as, primary constitution of the mechanism through which betaine normalized fetal growth in diabetic pregnancy.

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Author contributions PS performed the experiments, collected and analyzed the data and wrote the manuscript. MG performed the experiment and collected the data. AR helped with the project management, edited the manuscript and has done native English edit. MA helped with the project management, performed the quality control of data and algorithms and analyzed the data, also edited the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethics approval All rats were treated humanely and in compliance with the recommendations of Animal Care Committee for the Lorestan University (Khorramabad, Iran) with approval number: LU.ECRA. 2017.4.

Informed consent This report does not contain any studies with human participants performed by any of the authors.

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