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Research Article
Hesperidin Exerts the Gestational Diabetes Mellitus via AGEs-RAGE Signalling Pathway

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Abstract

Background and Objective: Gestational diabetes mellitus (GDM) are known as the increased risk of postpartum type 2 diabetes. It is believed that the efficient maternal glycemic control of GDM could not decrease the neonatal morbidity from serious complications. Thus, this study was aimed to investigate the protective effect of hesperidin against streptozotocin (STZ) induced gestational diabetes mellitus (GDM) rat models and its possible mechanism. **Materials and Methods:** The STZ was used for the induction of diabetes mellitus for the pregnant rats and received the hesperidin to 19 days. On the 19th days (pregnant rats), the blood samples and fetal rats of all group rats were collected, weighted the fetal rats and placental was estimated. Serum advanced glycation end products (AGEs) was estimated in the heart and brain of pregnant rats at regular interval. The mRNA expression of NOX2, MCP-1, RAGE, p65, VEGF, VCAM-1 and RAGE were estimated in the placenta. **Results:** Dose-dependent treatment of hesperidin significantly ($p<0.001$) altered the placental weight, fetal rats weight, serum blood glucose, lipid level, glycogen and serum insulin level. Hesperidin treatment slightly, but not considerably reduces the probability of fetal development defects as compared to GDM group. **Conclusion:** Hesperidin showed the potential effect on fetal development in pregnant rats with gestational diabetes mellitus via AGEs-RAGE signalling pathway.

Key words: Gestational diabetes mellitus, hesperidin, plasma insulin, lipid profile, serum advanced glycation end products (AGEs)

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The International Diabetes Federation has estimated that the 382 million adults suffered from diabetes mellitus Worldwide[1]. The figure of DM almost double till the 2035 and its reached near the 592 million[2]. Gestational diabetes mellitus (GDM) connected with a lot of metal complications and fetal abnormalities that primarily arise from GDM induced placental lesions[3]. The GDM occurs almost 4% of pregnancies and it's also defined as the glucose intolerance with onset or first diagnosis during the pregnancy[4]. The GDM known as the increased risk of postpartum type 2 diabetes. It is believed that the efficient maternal glycemic control of GDM could not decrease the neonatal morbidity from serious complications such as; dysplasia, preeclampsia and asphyxia[5,6]. The GDM explained as carbohydrate intolerance that resultant in hyperglycemia, starting or identified during the 1st time during the pregnancy. The GDM increases the prevalence of complications for both (mother and embryo) during the pregnancy, childbirth and beyond. Previous studies suggested that the early detection and treatment of GDM could be improving the outcomes for child and mother. Previous evidence suggested that the early detection and exact metabolic control of diabetic pregnant women should reduce the intensity and repetition of short and long complications in the offspring of the diabetic mother[7,8]. The most common report about complications of gestational diabetes in neonatal are premature birth, congenital anomalies, neonatal hyperglycemia, infant death and macrosomia. Recent research suggested that various teratogen agents can act via boosting the oxidative stress during the expansion of embryo and down-regulated the antioxidant defense system. The oxidative stress described as the imbalance between the generation of reactive oxygen species and antioxidant defense system of the body which closely linked with the number of diseases such as; cardiovascular, cancer, diabetes and its complications. Furthermore, a severe embryonic injury may occur if exposure with these teratogens induced oxidative stress during the early stage of angiogenesis[9,10].

It is believed that diabetes condition and consequently increase the glucose concentration induces the generation of hydroxyl and oxygen free radicals customarily. It well known that hyperglycemia directly enhances the oxidative stress in embryo tissue and reduced the oxidative stress could be helpful for the expansion of manifestations linked with diabetes. Recent studies suggested that during the oxidative stress condition such as; allostatic overload, start the production of nitrogen/reactive oxygen species (RNS/ROS) via lipid peroxidation, mitochondria, RNA and DNA damage[10,11]. Various studies suggested that oxidative stress play an important role in the expansion of both types of diabetes and its complications[12,13]. Researcher suggested that the flavonoids rich food reduced diabetes, oxidative stress and its complications[14]. Hesperidin (flavonoids) also proved their antioxidant potential in various diseases[15,16].

The various researcher suggested that the receptor for advanced glycation end products (RAGE) and its ligands such as advanced glycation end products (AGEs) act together to fabricate the AGEs-RAGE signalling pathway and start the oxidative stress and inflammatory reaction that play an important role in the pathophysiology of diabetes mellitus[17]. With increase the age, AGEs (modified protein) start the accumulation into different tissues of the body, particularly in the condition related to the glycemic condition or oxidative stress. Previous studies suggested that the increasing level of AGEs during the pregnant mother can be a forecaster of GDM induced adverse perinatal result and may be used as a significant indicator of GDM linked to birth defects[18]. The use of the hesperidin may be a promising agent in developing the new strategies of GDM treatment and an urgent need to scrutinize the protective effect of soluble on fetal development effect via maternal GDM. In the current study, the authors treated the GDM rats with the hesperidin and estimated the different biochemical parameters, AGEs-RAGE signaling as well as developmental defects in the newborn rats to estimate the underlying mechanism.

MATERIALS AND METHODS

Experimental animals: Swiss albino Wistar rats (both sex, weight 180-200 g, 10-12 week old) were used for the current experimental study. The rats were received from the Institutional Animal House and kept in the polyethylene cages. All the female and male rats were kept separately and maintained the body weight of female rats until 12 weeks. All the experimental study was performed in accordance with the protocols approved by the Institutional Ethical Committee (XMU/18/011/06). The current experimental study was performed in the month of November, 2018- January, 2019 in China.

Experimental protocol: The rats having a higher blood glucose level (6.7 mmol L^{-1}) were excluded from the experimental study. After that, the rats were allowed to free for mating and female rats lacking pessary next day were considered pregnant for 0.5 days[19]. Single intraperitoneal injection of streptozotocin (STZ) was used for the induction of Gestational diabetes mellitus (GDM).

Evaluation of fetal development in the rat model of GDM: After successfully induction of GDM, the blood glucose level, plasma insulin, AGEs and RAGE level in heart and brain tissue of pregnant rats were estimated at regular interval. The AGEs and RAGE level were estimated via using the available commercial ELISA kits. On the end of the experimental study (19 days), the fetuses and placenta were successfully removed. The weight and number of fetuses and placenta weight were recorded. Macroscopically all the fetal rats were scrutinized for the estimation of defects and malformations of each organ.

At the end of the experimental study, all group rats were sacrificed and the tissue, fetus, placental and serum were collected. For the biochemical parameters, the serum samples were centrifuged at 3000 rpm for 15 min at 4°C and preserved in the refrigerator at -20°C.

Biochemical parameters: The lipid parameters such as; total cholesterol (TC), low-density lipid-protein (LDL), triglycerides (TG), very low-density lipid-protein (VLDL) and high-density lipid-protein (HDL) were estimated via using the standard kits. Fasting free fatty acid (FFA) were also estimated by using the standard kits. Glycosylated hemoglobin A1c (HbA1c) was estimated via using the standard kits (Sigma Aldrich, USA). The hepatic glycogen content in the hepatic tissues was estimated via using the anthrone method with minor modification.

Antioxidant parameters: Antioxidant parameters such as; superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GPx) were also estimated via using the available kits (Span Diagnostics, India).

In order to estimation the secretory function of pancreatic islet β-cells, endogenous insulin level in blood and serum C-peptide (CP) content were also estimated via using the 125I-radio immunoassay method.

Real-time PCR: For the real-time PCR study, the total RNA was successfully extracted from the fetal heart fetal brain and placentas via using the Trizol reagent and for the synthesis of cDNA using the Promega RT kits and oligo primers (Promega, Madison, WI, USA). The following RNA expressions showed in Table 1 were estimated[20].

Western blot analysis: For estimation of the mechanism extracted the total protein from the placenta, heart and brain tissues by using the lysis buffer. Equal amounts of proteins were resolved on a polyacrylamide gel, transferred onto nitrocellulose membrane and blocked with 5% skim milk. The anti-rage antibody was used for the estimation of RAGE protein level and anti-rabbit HRP-conjugated IgG.

Statistical analysis: The result was presented as the mean±SD and analyzed with one-way analysis of variance (ANOVA) followed by Dennett using the GraphPad Prism software (version 7). The $p<0.05$ was considered as the statistical significant.

RESULTS

Effect of hesperidin on body weight of pregnant rats: The body weight of rats before pregnancy and during pregnancy was presented in Fig. 1. It is showed that no difference in the body weight of all group rats before pregnancy, but after the pregnancy, the body weight of all group rats increased. Normal control group rats showed increased body weight as compared to the initial body weight and other group rats. The STZ induced group rats showed increased body weight (319.23±9.12) as compared to initial body weight (261.34±8.93). The STZ induced group rats treated with hesperidin significantly ($p<0.001$) increased body weight in a dose-dependent manner.

Effect of hesperidin on blood glucose level: During the protestations, all group pregnant GDM rats exhibited the almost similar blood glucose level. At the day 3, normal control pregnant GDM rats showed the blood glucose level (5.45±0.83 mmol L^{-1}) and end of the experimental study

Table 1: List of primers

	Sequence	
Primers	Forward	Reverse
RAGE	5' CCTGAGACGGGACTCTTCACGCTTCGG 3'	5'CTCCTCGTCCTCCTGGCTTTCTGGGGC3'
VCAM-1	5' GAGAATTCGGCCCCAACCATGAACTTTCTGCT3'	5' GACGGTCACCCTTGAACAGTTC3'
VEGF	GAGAATTCGGCCCCAACCATGAACTTTCTGCT	G AGCATGCCCTCCTGCCCGGCTCACCGC
Nox2	CCCTTTGGTACAGCCAGTGAAGAT	CAATCCCAGCTCCCACTAACATCA
MCP-1	CCCCAGTCACCTGCTGTTAT	TGGAATCCTGAACCCACTTC
P65	AGCACCATCAACTATGATGAGTTTC	GAGTTATAGCCTCAGGGTACTCCAT
β-actin	TGTGCTATGTTGCCCTAGACTT C	CGGACTCATCGTA CTCCTGCT

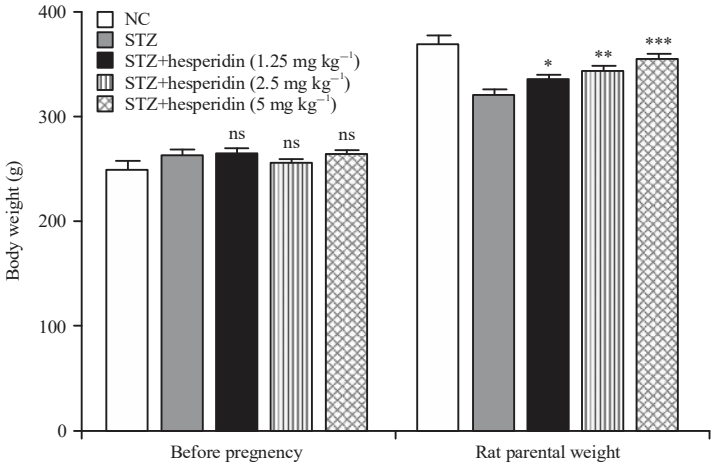

Fig. 1: Effect of hesperidin on the body weight of different group of rats

Values are expressed as Mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 compared with diabetic rats, ns: Non-significant

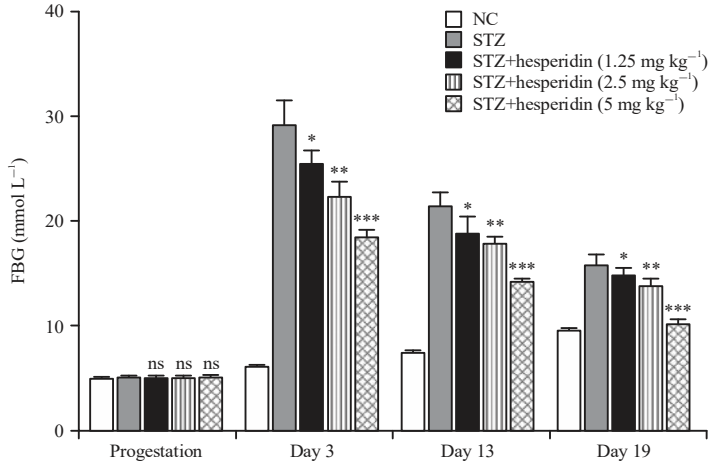

Fig. 2: Blood glucose level in pregnant rats of different group of rats

Values are expressed as Mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 compared with diabetic rats, ns: Non-significant

(day 19) the blood glucose level (9.57±1.23 mmol L^{-1}). The STZ induced group rats demonstrated the blood glucose level 29.13±1.26 mmol L^{-1} on day 3 and 15.94±1.04 mmol L^{-1} on day 19. Hesperidin showed the blood glucose level 25.12±1.14, 22.34±1.23, 18.72±1.09 mmol L^{-1} on day 3 and 14.34±0.92, 13±0.98, 10.02±0.87 on day 19 at a dose of 1.25, 2.5 and 5 mg kg^{-1}, respectively (Fig. 2).

Effect of hesperidin on serum AGEs level: Normal control group rats showed the unchanged level of AGEs till the end of the experimental study. The STZ induced GDM group rats showed the increased serum AGEs level at the end of the experimental study. The STZ induced GDM group rats treated with hesperidin significantly ($p<0.001$) reduced the serum AGEs level in a dose-dependent manner (Fig. 3).

Effect of hesperidin on fetuses: The STZ induced group rats showed the reduced fetuses weight as compared to the normal control group rats and dose-dependent treatment of hesperidin significantly ($p<0.001$) increased the fetus weight (Fig. 4a).

The placental weight and normal control group rats showed the placental weight. The STZ induced group GDM rats showed the increased placental weight as compared to normal control and dose-dependent treatment of hesperidin significantly ($p<0.001$) reduced the placental weight (Fig. 4b).

A similar trend was observed in the placental index of normal control and STZ induced GDM group rats. The STZ induced group rats showed the increased placental index and dose-dependent treatment of hesperidin showed the reduction of the placental index (Fig. 4c).

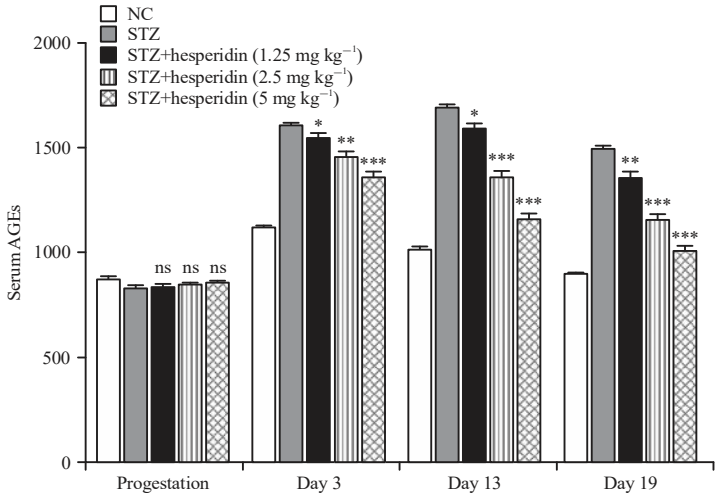

Fig. 3: Serum AGEs in pregnant rats of different group of rats

Values are expressed as Mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 compared with diabetic rats, ns: Non-significant

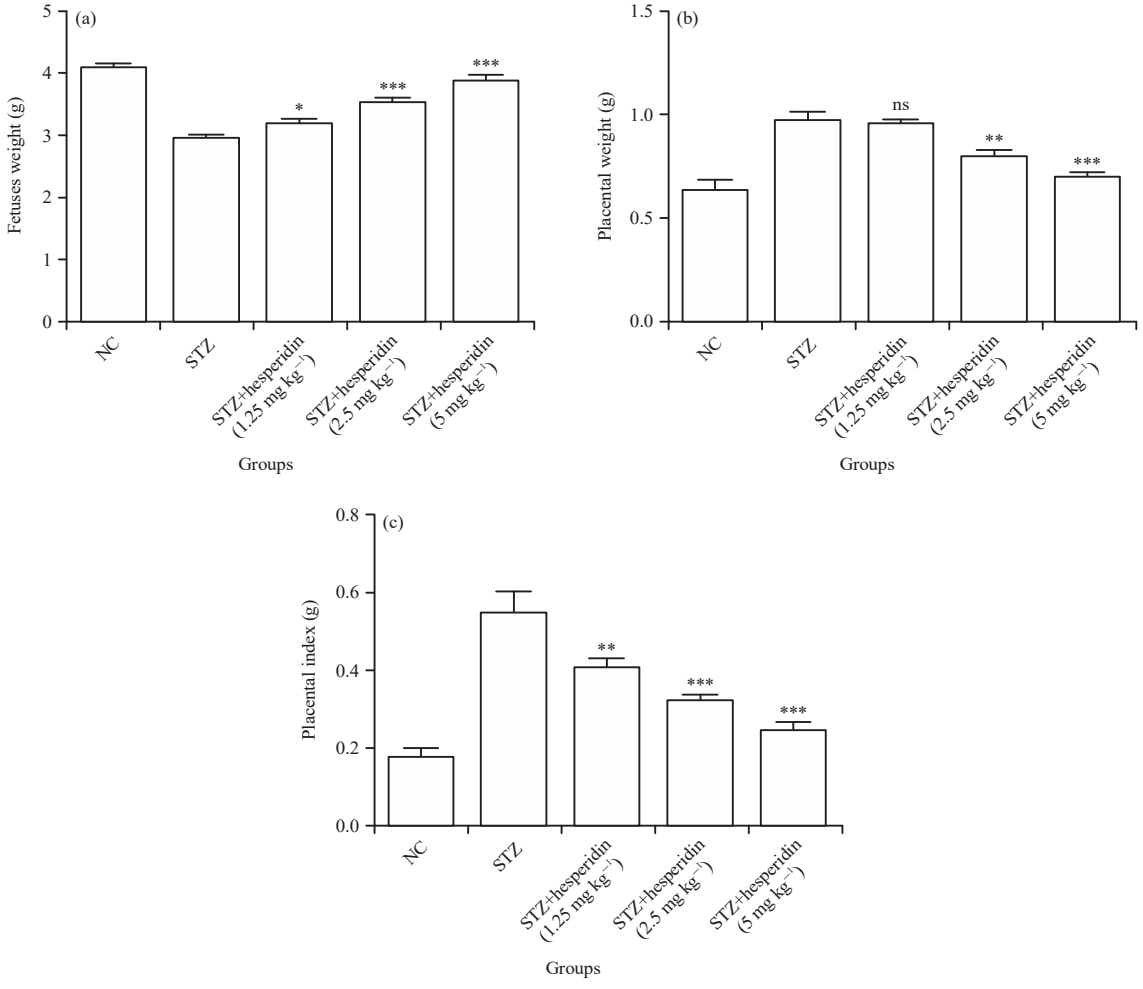

Fig. 4(a-c): Effect on the placental weight, (a) Fetuses weight, (b) Placental weight and (c) Placental index

Values are expressed as Mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 compared with diabetic rats, ns: Non-significant

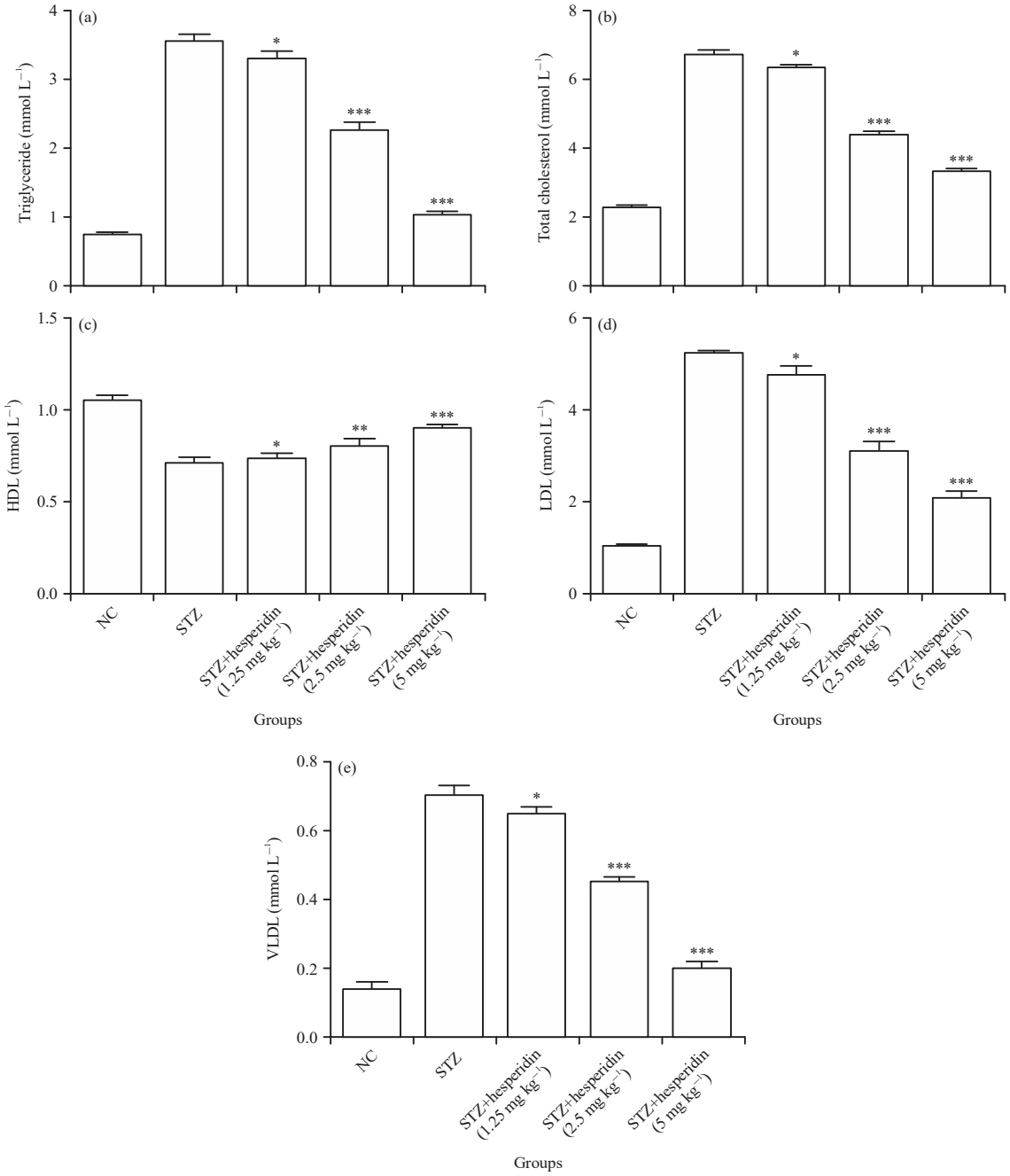

Fig. 5(a-e): Effect on the lipid profile of different group of rats, (a) Triglycerides, (b) Total cholesterol, (c) HDL, (d) LDL and (e) VLDL
Values are expressed as Mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 compared with diabetic rats, ns: Non-significant

Effect of hesperidin on lipid profile: The effect of hesperidin on the lipid profile of normal and STZ induced GDM rats presented in Fig. 5. The STZ induced group GDM rats showed the increased level of TC, TG, LDL, VLDL and reduced level of HDL at the end of the experimental study. Hesperidin treated group rats showed the reduction of TC, TG, LDL, VLDL and increased the level of HDL in a dose-dependent manner.

Effect of hesperidin on biochemical parameters: The STZ induced GDM rats exhibited an increased level of HbA1c, FINS, free fatty acid, serum C-peptide and reduced level of hepatic glycogen at the end of the experimental study (Fig. 6). Dose-dependent treatment of hesperidin significantly ($p<0.001$) reduced the HbA1c, FINS, free fatty acid, serum C- peptide level and increased the hepatic glycogen level as compared to the STZ induced GDM rats.

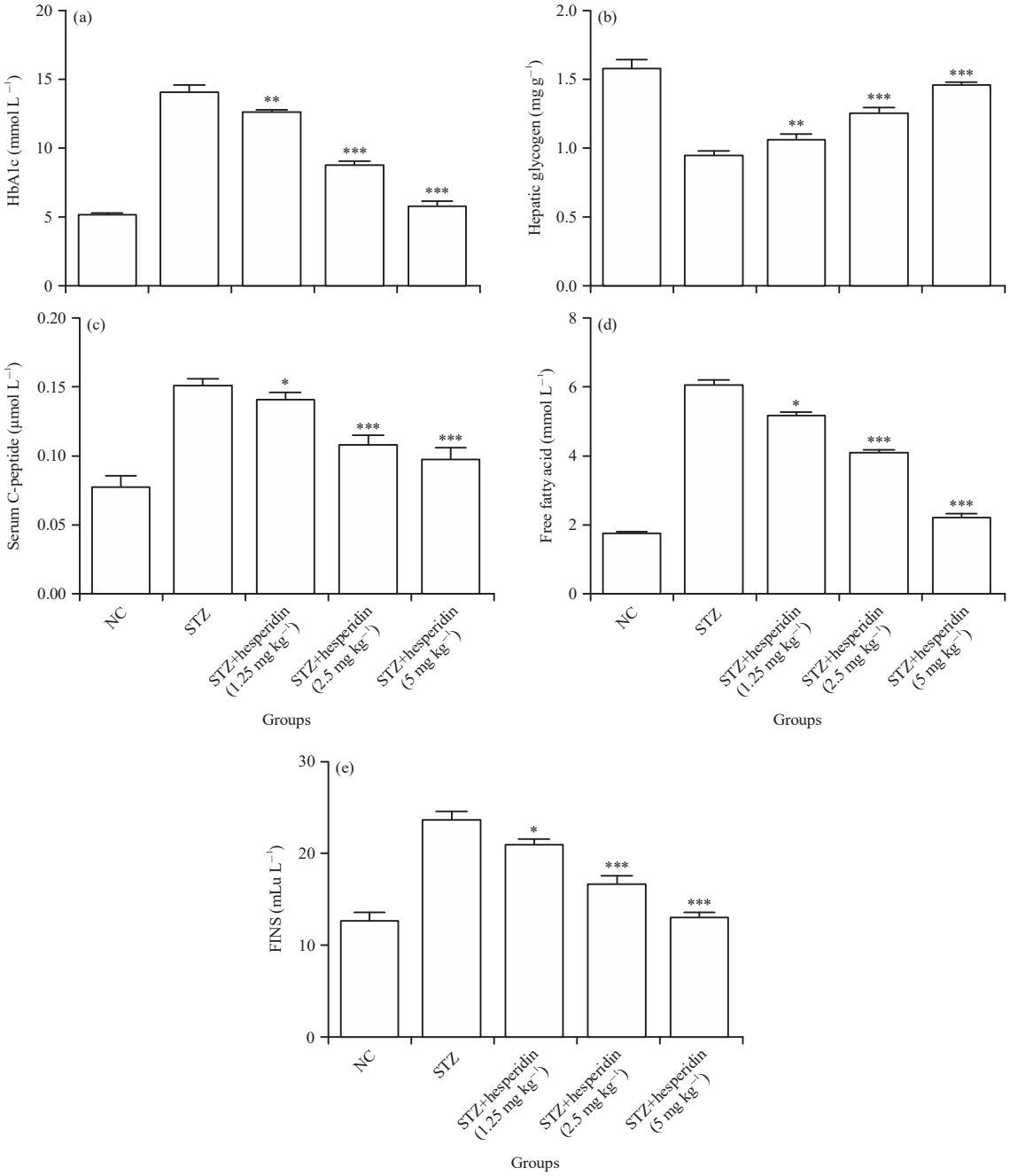

Fig. 6(a-e): Effect on the biochemical profile of different group of rats, (a) HbA1c, (b) Hepatic glycogen, (c) Serum C-peptide, (d) Free fatty acid and (e) FINS

Values are expressed as Mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 compared with diabetic rats, ns: Non-significant

Effect of hesperidin on antioxidant parameters: The effect of hesperidin on the antioxidant parameters of STZ induced GDM rats showed in Fig. 7. The STZ induced GDM rats showed the reduced level of SOD, GSH, GPx and CAT and dose-dependent treatment of hesperidin showed the increased level of SOD, GSH, GPx and CAT in a dose-dependent manner.

Effect of hesperidin on the expression levels of placental, heart and offspring brain genes: The effect of different gene expression in the placenta, heart and offspring brain tissue showed in Fig. 8. In the current study, estimated the level of mRNA expression of RAGE as well as expression of genes expressions such as; NOX_2, proinflammatory cytokines EGFR, MCP-1, p65 and adhesion molecule VCAM-1, that are known

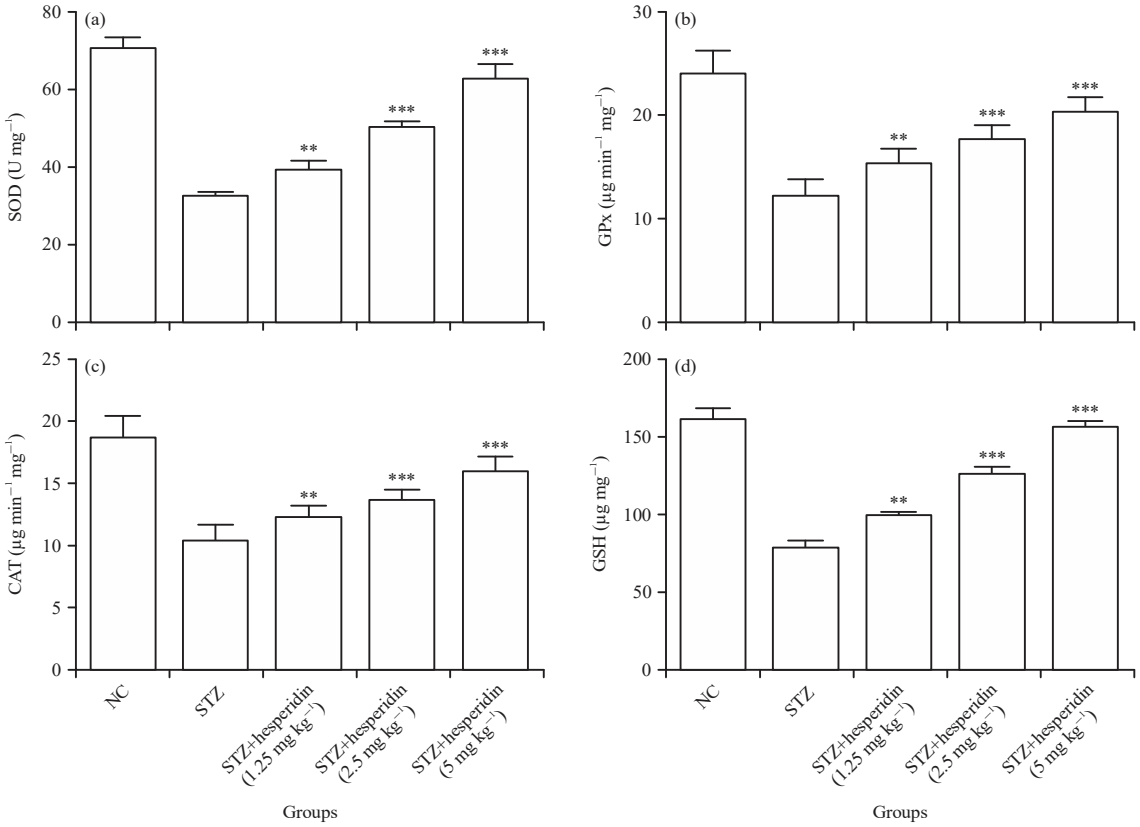

Fig. 7(a-d): Effect on the antioxidant profile of different group of rats, (a) SOD, (b) Gpx, (c) CAT and (d) GSH
Values are expressed as Mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 compared with diabetic rats, ns: Non-significant

to be affected via diabetes and hyperglycemia. The placentas from all groups of rats were collected after the cesarean section and the mRNA expression levels were estimated via real-time PCR. Figure 8 exhibited a significantly increased mRNA expression of NOX2, RAGE, MCP-1, NOX2, p65 and VCAM-1 in the STZ induced GDM group rats as compared to normal control. The result suggested that the hesperidin significantly ($p<0.001$) partially decrease the mRNA expression and bring near to the normal control group rats.

DISCUSSION

Previous studies suggested that the GDM develop during the early stage of DM and insulin resistance considered as the common pathogenesis of DM and GDM[21,22]. The GSM linked with the number of maternal complications[23,24] as well as the fetal abnormalities which are arising during the GDM induced placental lesions[25,26]. In the current study, scrutinized the protective effect of hesperidin against the chemically induced GDM rats via alteration of serum glucose level, insulin and lipid level. Previous research suggested that the ROS play a significant role in the pathology of various congenital anomalies, which produced via gestational diabetes, cocaine intake, alcohol and radiation[27,28]. Some drugs such as; thalidomide and phenytoin also induced the teratogenicity[29]. Hyperglycemia often induces the increase in the generation of free radical in the body[30]. Several researchers suggested that the non-enzymatic glycation, glucose oxidation and consequent degradation of glycated proteins are responsible for the generation of free radicals during diabetes[31,32]. An endogenous antioxidant such as; GPx, SOD, CAT and vitamins (A, C and E) play an important role to scavenge the free radicals and prevents their adverse effects[33,34]. Also, previous studies suggested that the expansions of embryos are very sensitive to increase the level of ROS, especially during the organogenesis stage[35]. The SOD, first line antioxidant enzymes and takes part to reduce the ROS from the body[36,37]. The SOD indirectly reflected the body's capability to remove the oxygen free radicals and giving GDM rats the treatment of hesperidin could keep away from the extreme utilization of SOD and other antioxidant enzymes[38,39], which is favourable to efficiently and timely eradicate the ROS in the body[40]. The ROS (signal molecule), it almost similar to the second messengers and could generate the many redox-sensitive signal pathways.

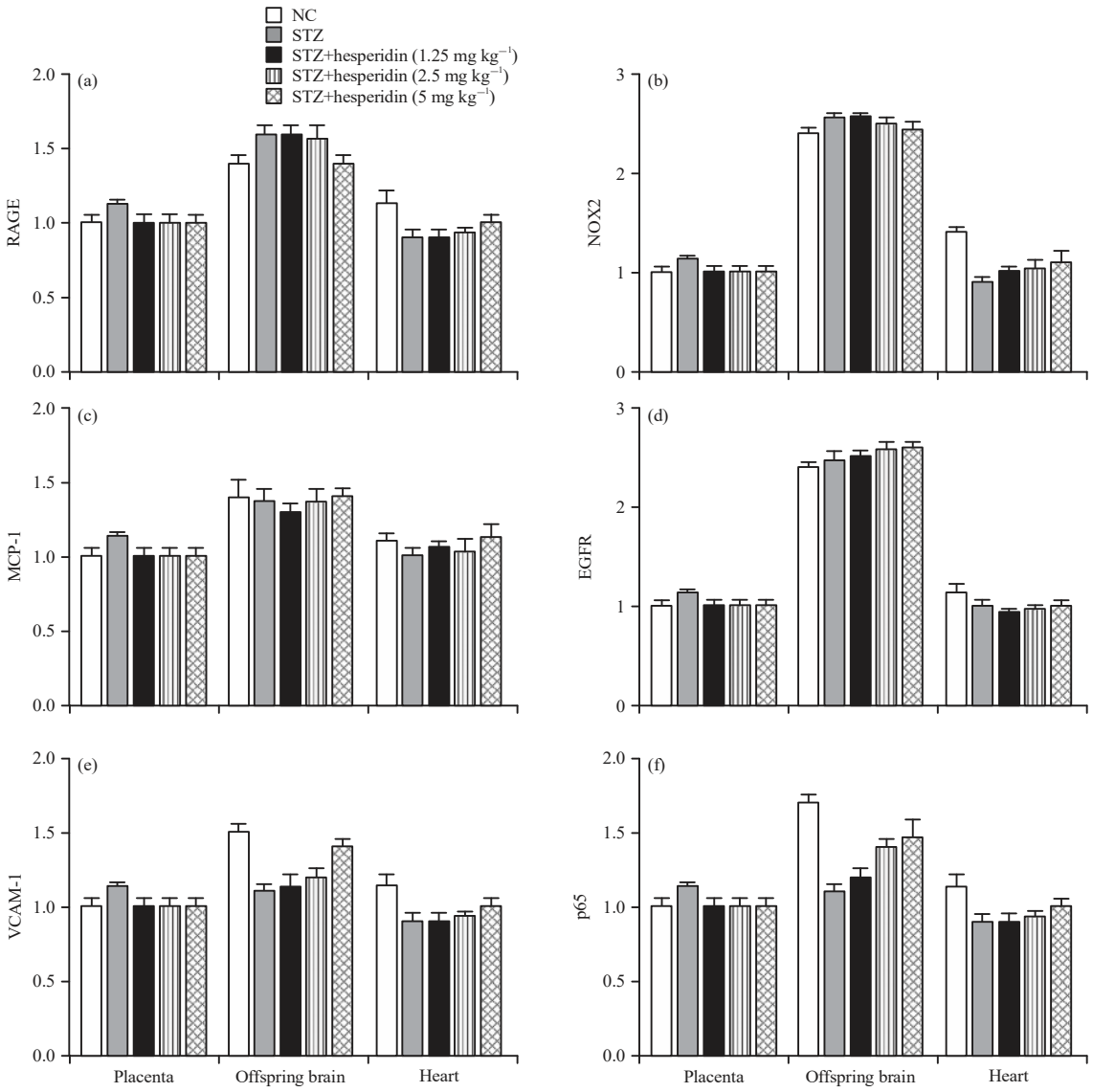

Fig. 8(a-f): Effect on the related gene expression of placenta, offspring brain and heart, tissues, (a) RAGE, (b) NOX2, (c) MCP-1, (d) EGFR, (e) VCAM-1 and (f) p65

Values are expressed as Mean±SEM. *p<0.05, **p<0.01 and ***p<0.001 compared with diabetic rats, ns: Non-significant

Further, they induced the phosphorylation of the insulin receptor and substrate in the insulin signal pathways, consequently leading to the decreased activity of downstream signal molecules and the weakened insulin-sensitizing effect and eventually insulin resistance occurred[41,42]. In the current experimental study, hesperidin significantly eliminated the ROS effect via improved the endogenous antioxidant enzyme, insulin-sensitizing effect and reduced the insulin resistance, this may be induced of blood glucose level in rats of interference group could be regulated and improve. Studies suggested that the deposition of AGEs induced via persistent hyperglycemia during pregnancy, one of the significant factors that induce fetal abnormalities[20,43].

During the hyperglycemia condition, excess deposition of glucose started the combines with the free amino acids on proteins, nucleic acids and lipids in conditions of nonenzymatic glycation to form AGEs that deposit in the tissue[44]. The accumulation of AGEs leads to a series of toxic/side effects such as; cross-linking of matrix proteins and collagen, enhanced mononuclear cell infiltration, vascular permeability which linked with the microvascular and renal complications[45]. The relationship between the AGEs and its RAGE (full-length cell surface receptor), significant pathway that caused the undesirable effects in the cells via boosted the oxidative stress, nuclear factor kappa-B activation and inflammatory mediators[46,47]. On the other hand, hesperidin,

RAGE and RAGE receptor (C-turecated variant) that lack of transmembrane and effector domains act both as competitive inhibitor of legends and scavenger of soluble AGEs which bind to and activated the RAGE.

The AGEs-RAGE induced the intracellular pathway such as; pro-inflammatory and pro-atherogenic mediators such as NFkB-dependent mediators IL-6, VCAM-1, IL-1α, TNF-α, endothelin-1, E-selectin tissue factor and RAGE[20]. In the current study, estimated the expression of EGFR, MCP-1, VCAM-1, p65 and Nox-2 in the STZ induced GDM rats and placentas of pregnant rats. In the current experimental study, the increased expression of Nox2 was observed in the placentas correlated with the previously published study that addition of NF-kB dependent pathways, binding to the AGEs to RAGE also activated the NADPH oxidase pathway, which resultant increased the generation of ROS.

Hesperidin treated group rats showed the reduced expression of VCAM-1, MCP-1, NADPH, EGFR, Nox2 and p65 in the pregnant GDM rats suggested the potential antioxidant effect. Several researchers suggested that the oxidative stress induced via AGEs-RAGE signaling pathway play a significant role in diabetes and also induced the various complications such as diabetic retinopathy, diabetic nephropathy, cardiovascular disease and pathogenesis of fetal malformations[48,49]. The STZ induced group rats showed the increased blood glucose level and AGEs in the serum of pregnant rats with GDM and dose-dependent treatment of hesperidin showed the reduced blood glucose level and AGEs in the serum of pregnant women and frequency of fetal abnormalities in the pups. Hesperidin significantly down-regulated the total number of development defects in the offsprings of GDM rats and in future performed the experimental study on the molecular level and identify the particular gene for providing the protective effect.

CONCLUSION

In the current experimental study, STZ induced GDM rats showed the increased blood glucose level and AGEs, which associated with up-regulation of the frequency of expansion of fetal malformations in offsprings. Hesperidin significantly reduced the oxidative stress, inflammatory signaling pathways and RAGE-related gene expression in the GDM induced rats.

SIGNIFICANCE STATEMENT

This study discovered the beneficial effects of hesperidin on Fetal Development in Pregnant Rats with Gestational Diabetes Mellitus via AGEs-RAGE signalling pathway. Further, this study will help the researcher to uncover the critical areas of pregnancy with gestational diabetes mellitus where AGEs-RAGE signalling pathway. Thus a new theory on gestational diabetes mellitus during pregnancy may be arrived at.

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