Effects of Stevioside on the Expressions of GLUT 1, GLUT 3, and GLUT 4 Proteins in Diabetic Rat Placenta

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ABSTRACT

The placenta provides maternal–fetal nutrient transport. The primary source of energy for fetus development is glucose and maternal–fetal glucose transport occurs through glucose transporters (GLUTs). Stevioside, a component of *Stevia rebaudiana* Bertoni, is used for medicinal and commercial purposes. We aim to determine the effects of stevioside on GLUT 1, GLUT 3, and GLUT 4 proteins expressions in diabetic rat placentas. The rats are divided into four groups. A single dose of streptozotocin (STZ) is administered to form the diabetic groups. Pregnant rats receive stevioside to form the stevioside and diabetic + stevioside groups. According to immunohistochemistry results, GLUT 1 protein is found in both the labyrinth and junctional zones. GLUT 3 protein is limited in the labyrinth zone. GLUT 4 protein is detected in trophoblast cells. According to Western blotting results, on the 15th and 20th days of pregnancy, there is no difference in the expression of GLUT 1 protein between groups. On the 20th day of pregnancy, the expression of GLUT 3 protein in the diabetic group is statistically higher compared to the control group. On the 15th day and 20th day of pregnancy, the expression of GLUT 4 protein in the diabetic group is statistically lower compared to the control group. Insulin levels in blood samples derived from rat abdominal aorta are determined by the ELISA method. According to the ELISA results, there is no difference in insulin protein concentration between groups. Stevioside treatment reduces GLUT 1 protein expression under diabetic conditions.

Introduction

The placenta is a specialized organ that supports the normal development and growth of the fetus. The placenta is responsible for delivering nutrients and oxygen to the fetus. Moreover, the placenta produces hormones that regulate maternal physiology throughout pregnancy and create a barrier against the maternal immune system [1].

Rat placenta consists of a labyrinth zone, a junctional zone, and a maternal decidua. The labyrinth zone is the fetal part of the placenta. Moreover, the labyrinth zone is the site of the nutrient and gas exchange found between maternal and fetal circulation. The junctional zone, which is another placental part, has three different cells: spongiotrophoblast cells, glycogenic cells, and giant cells [2]. Spongiotrophoblast and giant cells have endocrine functions and produce hormones/cytokines that are in the family of

prolactin [3–5]. The maternal decidua is the maternal part of the rat placenta.

Diabetes mellitus is a metabolic disorder characterized by high plasma glucose concentrations and is fundamentally divided into two classes; type 1 diabetes and type 2 diabetes. Type 1 diabetes is known as insulin-dependent diabetes. Type 1 diabetes is associated with insufficient insulin production as a result of disrupting the β cells found in the pancreas. On the other side, type 2 diabetes is fundamentally associated with insulin resistance. Type 2 diabetes results from a decreased sensitivity of insulin receptors to insulin.

Diabetes can affect both the mother and the developing fetus during pregnancy. If diabetes isn't controlled during pregnancy, it causes serious complications in the fetus such as macrosomia, congenital malformations, and premature births [6]. For these
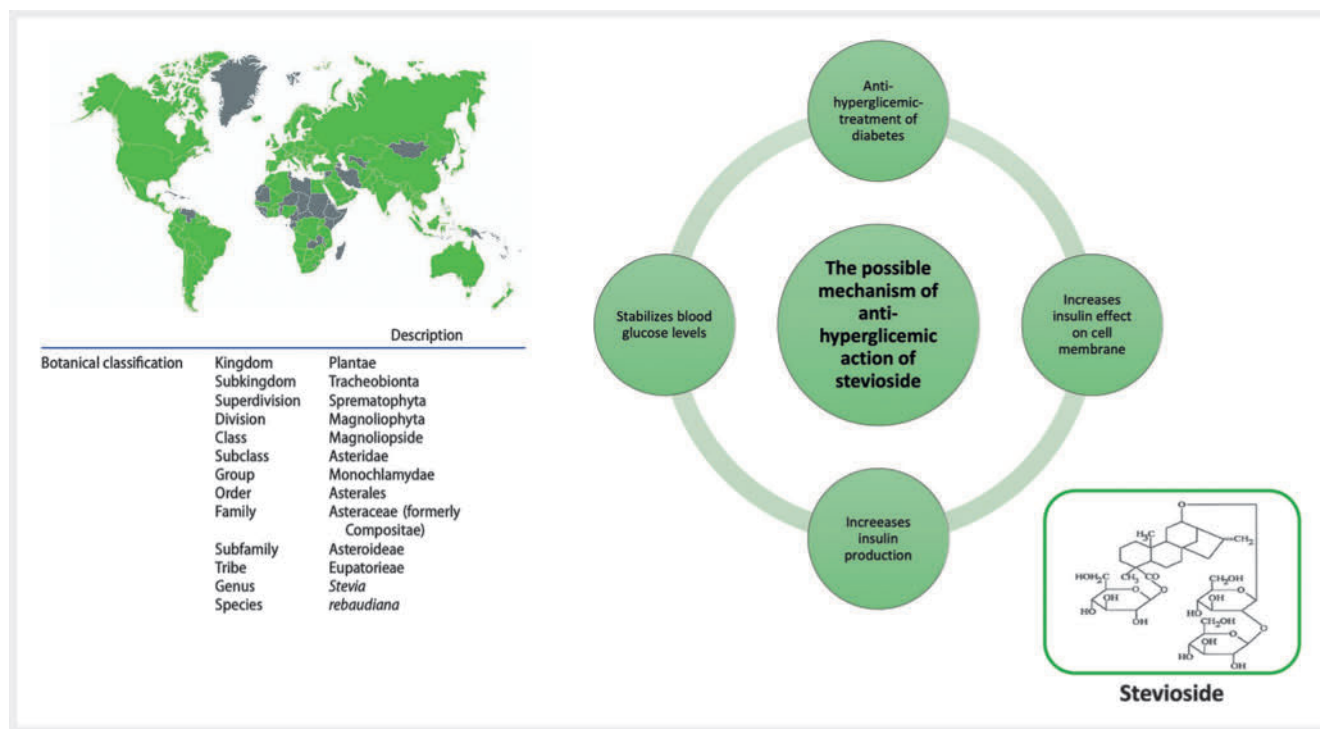
▶ **Fig. 1** General information (regions where *Stevia rebaudiana* is used and its classification) about *Stevia rebaudiana*, and stevioside's chemical structure and possible anti-hyperglicemic activity (adapted from Zou et al. and Sukhmani et al.) [15, 53]. The green-colored regions on the map show the approved regions of *Stevia rebaudiana*.

reasons, treatment or management of diabetes are necessary for a successful pregnancy.

Medicinal plants are one of the current approaches for diabetes treatment and management. The *Stevia rebaudiana* Bertoni plant is a member of the Asteraceae family. The Stevia genus includes 230 species, but only *Stevia rebaudiana* provides the sweet essence. Botanically, *Stevia rebaudiana* Bertoni is described as a perennial low shrub with spreading roots, brittle stems, and small, elliptical leaves [7]. *Stevia rebaudiana* Bertoni, one of the medicinal plants, grows in South America. This plant is quite sweet because of steviol glycoside. Steviol glycosides are composed of steviol, stevioside, rebaudioside A, B, C, D, E, and F, and dulcoside. *Stevia rebaudiana* Bertoni and its components have been used for medicinal and commercial purposes for a long time as they are natural and have no calories [8]. Many studies demonstrated that *Stevia rebaudiana* Bertoni decreased plasma glucose concentrations in diabetic rats compared to control [9–11]. Moreover, it was shown that *Stevia rebaudiana* Bertoni and stevioside had important roles in the modulation of glucose transporters and the improvement in insulin sensitivity and secretion [12–14]. Therefore, the present study aimed to investigate the effects of stevioside on blood glucose and insulin levels and GLUT 1, GLUT 3, and GLUT 4 in rat placentas. To achieve these aims, control, stevioside, diabetes, and diabetes + stevioside groups were formed. A single dose of STZ was administered to form the diabetic groups. Pregnant rats received stevioside to form the stevioside and diabetic + stevioside groups.

Results and Discussion

The *Stevia rebaudiana* plant, which is a member of the Asteraceae family, is commonly used around the world. The possible mechanism of the anti-hyperglicemic action of stevioside has been shown in previous studies [15, 16] (▶ **Fig. 1**). Stevioside treatment in diabetic pregnancy didn't affect plasma glucose values and insulin levels. Maternal diabetes during pregnancy comprises unwanted conditions for embryonic and fetoplacental development. *Stevia rebaudiana* are suggested to have beneficial effects on human health, including antihypertensive, antihyperglycemic, and anti-human-rotavirus activities [17–20]. However, more clinical research is needed to prove the beneficial effects of stevioside on human health. In one study, treatment with stevioside in diabetic rats significantly reduced body weight and serum lipid profile while increasing HDL cholesterol levels, suggesting its hypocholesterolemic properties [21]. In another study, stevioside administration resulted in a significant reduction in plasma glucose and type 2 diabetes mellitus biomarkers such as DPP IV [22]. In another study, stevioside and steviol lowered lipopolysaccharide-induced pro-inflammatory cytokine productions by affecting cytokine gene expression via the IκBα/NF-κB signaling pathway [23]. Casas-Grajales S et al. reported that stevioside protected the liver from oxidative stress by upregulating the cell's endogenous antioxidant machinery, thereby counteracting rat experimental liver damage *in vivo* and *in vitro* [24].

In 2006, the World Health Organization (WHO) conducted a thorough review of recent experimental studies on stevioside
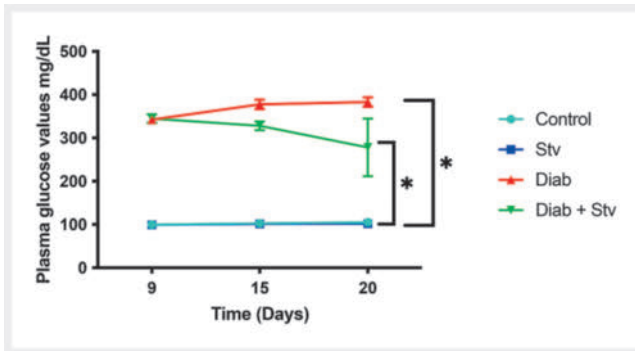
▶ **Fig. 2** Values of blood glucose on the 9th, 15th, and 20th days of pregnancy in the control, stevioside (Stv), diabetic (Diab), and diabetic + stevioside (Diab + Stv) groups (*p < 0.05). Data are presented as mean ± SD. n = 6. The experiment was repeated three times independently.

and steviol in animals and humans and concluded that stevioside and rebaudioside A are not genotoxic *in vitro* or *in vivo* and that the genotoxicity of steviol and some of its oxidative derivatives are not expressed *in vitro* and *in vivo* [25]. In the present study, we aimed to investigate the effects of stevioside on blood glucose and insulin levels and GLUT 1, GLUT 3, and GLUT 4 in rat placentas.

Blood glucose measurements were evaluated on the 15th and 20th days of pregnancy, belonging to the control, stevioside, diabetic, and diabetic + stevioside groups. Blood glucose levels were normal in experimental groups since no treatment was applied to the subjects on day 0. It was concluded that the blood glucose value was higher in the diabetic and diabetic + stevioside groups than in the control and stevioside groups on the 9th, 15th, and 20th days of pregnancy. There was no difference between groups (▶ **Fig. 2**).

Fetus, placenta weights, and number of pups were evaluated in experimental groups. In 15 days of pregnancy, there was no difference in the fetus weights and the placental weights between groups. In 20 days of pregnancy, while the fetus weights in the diabetic group were statistically lower than in the control and stevioside groups, the diabetic group had statistically higher placental weights compared to the control group (▶ **Fig. 3**). Fetus weights in diabetic pregnancy might be altered in two ways. Maternal hyperglycemia stimulates fetal overgrowth, while maternal vasculopathy can be associated with placental insufficiency leading to altered nutrient transport and subsequent intrauterine growth restriction (IUGR) [26]. Abnormal glycogen storage in the placenta is associated with maternal diabetes, thus diabetic placenta is bigger than the control [27–30]. Moreover, increased glycogen storage in diabetic placentas is associated with impaired fetal growth [29]. In this study, a decrease in fetal weight associated with the development of IUGR was observed on day 20 of the diabetic pregnancy, while an increase in placental weight was observed. It has been hypothesized that the mechanism causing the development of IUGR in the fetuses of diabetic pregnancies might be related to maternal vasculopathy, in line with previous studies. IUGR carries the risk of unwanted pregnancy, including preterm birth, intrauterine fetal death, and neonatal death. However, our results

suggest that administration of stevioside to diabetic pregnancies may be a promising option in treating IUGR development by causing an increase in fetal weight. Our results were similar to previous studies [26, 29, 30]. In 15 days of pregnancy, there was no difference in the number of pups between groups. The number of mothers for each group was determined to be six. We obtained 63 fetuses and placentas from the control groups, 61 fetuses and placentas from the stevioside groups, 59 fetuses and placentas from the diabetic groups, and 60 fetuses and placentas from the diabetic + stevioside groups. In 20 days of pregnancy, there was no difference in the number of pups between groups. We obtained 64 fetuses and placentas from the control groups, 63 fetuses and placentas from the stevioside groups, 57 fetuses and placentas from the diabetic groups, and 59 fetuses and placentas from the diabetic + stevioside groups.

Glucose is essential for fetal and placental development. Glucose production in a fetus is limited, thus the fetus is entirely dependent on the maternal supply of glucose [31]. Glucose transport of the placenta is carried out by facilitated diffusion transporter proteins called glucose transporter proteins (GLUTs) [32]. Several kinds of glucose transporters such as GLUT 1, GLUT 3, and GLUT 4 provide constant glucose transport from the maternal circulation to the fetus through the placenta. In the placenta on the 15th and 20 days of pregnancy, GLUT 1 protein was detected both in the labyrinth and junctional zones. In the labyrinth zone, GLUT 1 protein was localized in fetal endothelial and trophoblastic cells. These cells are specialized for transplacental glucose transport. In the junctional zone, GLUT 1 protein was localized in spongiotrophoblast cells, glycogenic cells, and giant cells (▶ **Fig. 4**). Our immunohistochemistry results showed that GLUT 1 protein is localized in both the junctional zone and labyrinth zone on the 15th and 20th days of pregnancy, primarily in trophoblast cells and fetal endothelial cells (▶ **Table 1**). These findings suggest that GLUT 1 expression is localized in cells known to be responsible for glucose transport across rat placenta. Moreover, the expression of the junctional zone is necessary for placental metabolism. It is unclear whether the expression of GLUT 1 is altered under diabetic conditions. Jansson et al. reported a positive relationship between fetus weight and GLUT 1 density in the placental glucose delivery in type 1 diabetic pregnancies [33]. In the placenta on the 15th and 20 days of pregnancy, GLUT 3 protein was only detected in the labyrinth zone. In the labyrinth zone, GLUT 3 protein was localized in fetal endothelial and trophoblastic cells (▶ **Tab. 1**). GLUT 3 protein is necessary for glucose transport (▶ **Fig. 5**). Our immunohistochemistry results showed that GLUT 3 protein was localized in trophoblast and fetal endothelial cells on the 15th and 20th days of pregnancy. Like GLUT 1, GLUT 3 expression is also localized in cells known to be responsible for glucose transport across rat placenta. GLUT 4 provides glucose transport in response to insulin. Our studies showed that GLUT 4 was detected in trophoblast cells on the 15th and 20th days of pregnancy (▶ **Tab. 1**, **Fig. 6**). Gutierrez-Torres et al. showed that GLUT 4 was detected in both synctiotrophoblast and stromal cells [34].

Western blots of the total GLUT 1, GLUT 3, and GLUT 4 on the 15th and 20th days of pregnancy in experimental groups are represented in (▶ **Figs. 7** and **8**). On the 15th and 20th days of pregnancy, there was no difference in the expression of GLUT 1
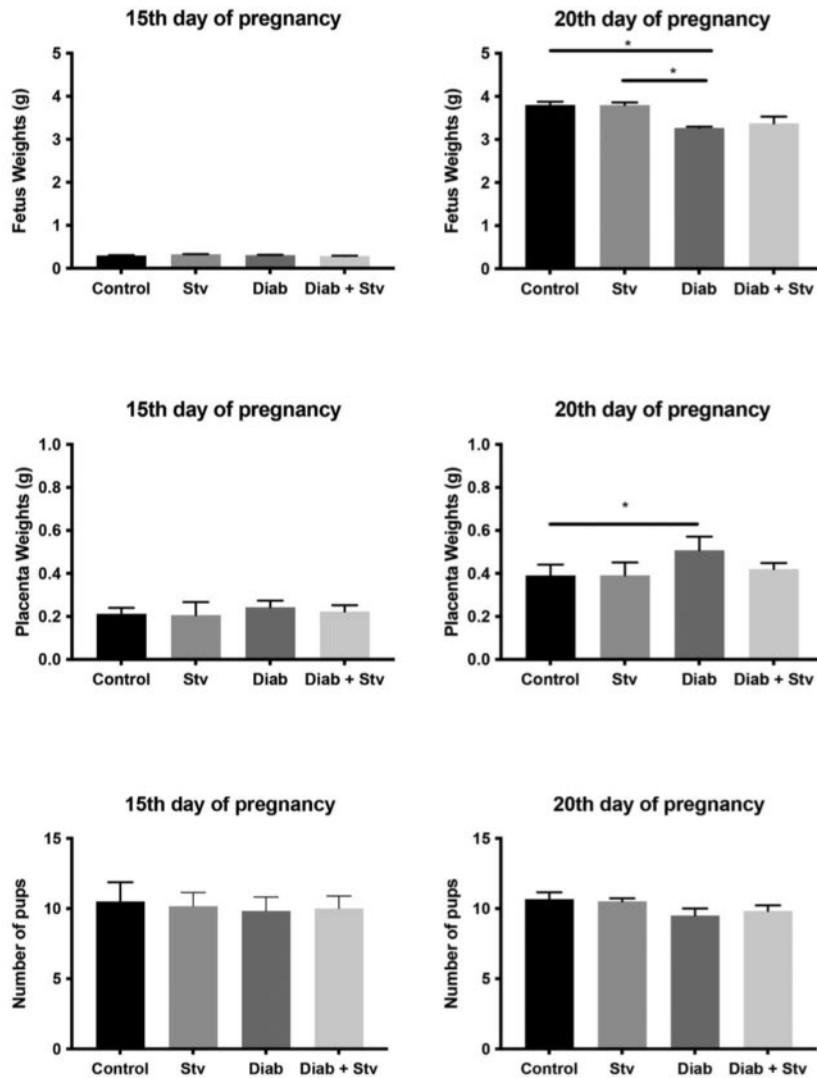
▶ **Fig. 3** Fetus, placental weights, and number of pups of the control, stevioside (Stv), diabetic (Diab), and diabetic + stevioside (Diab + Stv) groups for the 15th and 20th days of pregnancy (*p < 0.05). Data are presented as mean ± SD. n = 6. The experiment was repeated three times independently.

protein between groups (▶ **Fig. 8 a**). Ogura et al. reported that GLUT 1 protein in the placentas of diabetic mice was statistically significantly decreased compared to controls [35]. As with the results of our other study, we showed that GLUT 1 protein expression in diabetic placentas was similar to in control placentas [36]. After STZ, stevioside treatment decreased GLUT 1 protein expression under diabetic conditions. Further research is necessary to elucidate between GLUT1 and stevioside relations. Our results showed that GLUT 1 protein expression increased in all groups toward the end of pregnancy. These findings show that increased glucose requirements toward the end of pregnancy are mainly provided by GLUT 1. On the 15th day of pregnancy, there was no difference in the expression of GLUT 3 protein between groups. On the 20th day of pregnancy, the expression of GLUT 3 protein in the diabetic group was statistically higher compared to the con-

trol group. Expression levels of GLUT 3 protein in diabetic placentas statistically significantly increased compared to the control group (▶ **Fig. 8 b**). Our results were consistent with previous studies [37, 38]. In both human and rodent studies, placental levels of GLUT 3 protein decrease toward the end of pregnancy [39–43]. While GLUT 3 is primarily responsible for glucose uptake in early pregnancy, maternal glucose restriction decreases GLUT 3 protein at term. Our results are supported by the literature. On the 20th day of pregnancy, stevioside treatment in diabetic placentas didn't affect GLUT 3 protein expression. On the 15th days and 20th days of pregnancy, the expression of GLUT 4 protein in the diabetic group was statistically lower compared to in the control group (▶ **Fig. 8 c**). After STZ, insulin levels are statistically significantly decreased in diabetic groups; thus, GLUT 4 protein is also decreased in relation to insulin (▶ **Fig. 9**). Prata et al. reported
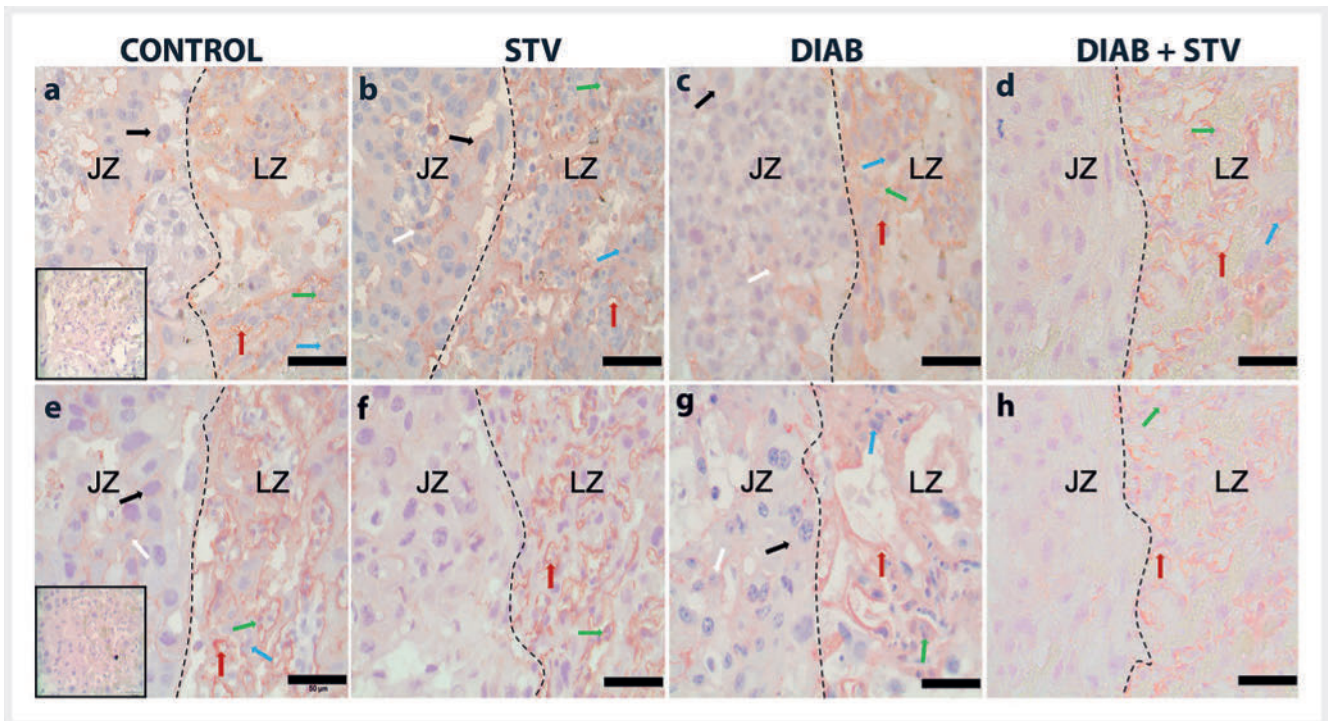
▶ **Fig. 4** GLUT 1 immunohistochemical stainings (polymer detection method with HRP, AEC chromogen for positive signaling, and Mayer's hemalum for counterstain) in rat placenta. **a–d** 15th day of pregnancy, **e–h** 20th day of pregnancy. Red arrows show the fetal endothelial cells, black arrows are giant cells, green arrows are syncytiotrophoblast cells, white arrows are spongiotrophoblast cells, orange arrows are glycogenic cells, and blue arrows indicate the cytotrophoblast cells. LZ: labyrinth zone; JZ: junctional zone. Negative staining is shown in the small squares in ''a and e''. Scale bar = 50 µm. n = 6. The experiment was repeated three times independently.
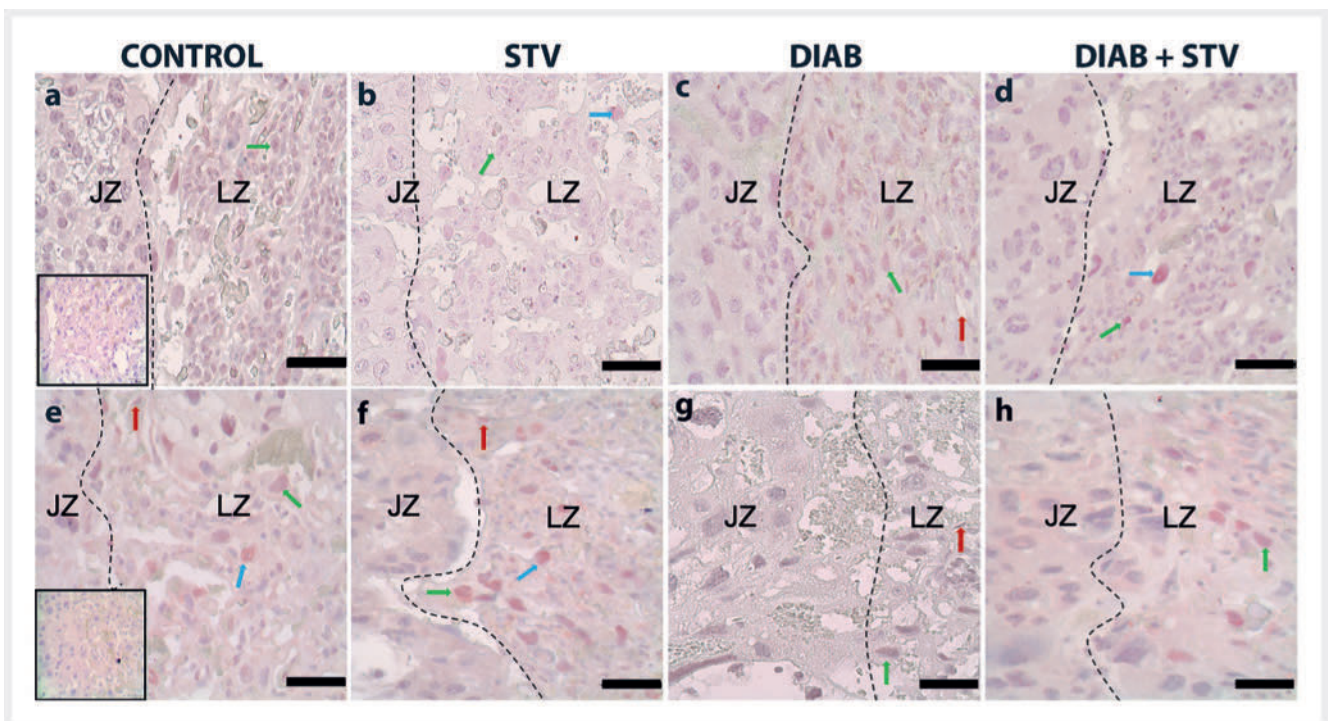
▶ **Fig. 5** GLUT 3 immunohistochemical stainings (polymer detection method with HRP, AEC chromogen for positive signaling, and Mayer's hemalum for counterstain) in rat placenta. **a–d** 15th day of pregnancy, **e–h** 20th day of pregnancy. Red arrows show the fetal endothelial cells, green arrows are syncytiotrophoblast cells, and blue arrows indicate the cytotrophoblast cells. LZ: labyrinth zone; JZ: junctional zone. Negative staining is shown in the small squares in ''a and e''. Scale bar = 50 µm. n = 6. The experiment was repeated three times independently.

▶ **Table 1** H-score evaluations of GLUT 1, GLUT 3, and GLUT 4 between experimental groups. This table was designed according to the percentage of positively stained cell counts; +++, ++, +, and – indicate the differences in signal intensities observed by optical microscopy and reflect the levels of GLUT 1, GLUT 3, and GLUT 4 proteins: – = absent; + = weak expression (10–30%); ++ = moderate expression (40–60%); and +++ = high expression (70–100%). Empty cells in the table indicate the concerned tissue is either not yet differentiated or has not been investigated on the 15th and 20th days of pregnancy. Decidual cells: DC; spongiotrophoblast cells: SC; giant cells: GC; glycogenic Cells: GlyC; labyrinth trophoblast cells: LT; labyrinth giant cells: LG; fetal endothelial cells: FEC. All cells were scored according to five different areas per placental section. Triple-blind studies were conducted.

Gestational Days	DC	SC	GC	GlyC	LT	LG	FEC	Experimental Groups	Protein
15	+	+	+	+	+	–	++	Control	GLUT 1
	+	+	+	+	+	–	++	Stv	
	+	+	+	+	+	–	++	Diab	
	+	+	+	+	+	–	+	Diab + Stv	
20	+	++	++	+	+++	–	+++	Control	
	+	++	++	+	+++	–	+++	Stv	
	+	++	++	+	++	–	+++	Diab	
	+	++	++	+	++	–	++	Diab + Stv	
15	–	–	–	–	++	–	+	Control	GLUT 3
	–	–	–	–	++	–	+	Stv	
	–	–	–	–	++	–	+	Diab	
	–	–	–	–	++	–	+	Diab + Stv	
20	–	–	–	–	+	–	+	Control	
	–	–	–	–	+	–	+	Stv	
	–	–	–	–	+	–	++	Diab	
	–	–	–	–	+	–	+	Diab + Stv	
15	–	–	–	–	+	–	–	Control	GLUT 4
	–	–	–	–	+	–	–	Stv	
	–	–	–	–	+	–	–	Diab	
	–	–	–	–	+	–	–	Diab + Stv	
20	–	–	–	–	+	–	–	Control	
	–	–	–	–	+	–	–	Stv	
	–	–	–	–	+	–	–	Diab	
	–	–	–	–	+	–	–	Diab + Stv	

that, like the insulin effect, stevioside led to an increase in glucose uptake into rat fibroblasts by activating the PI3K/Akt pathway–so activating GLUT 4 [44]. Rizzo et al. reported that stevioside was able to enhance glucose uptake in both SH-SY5Y neuroblastoma and HL-60 myeloid leukemia human cells by activating the PI3K/Akt pathway [45]. According to our study, stevioside treatment in diabetic placentas didn't affect GLUT 4 protein expression. Expression levels of GLUT 1 were increased toward the end of pregnancy, especially in trophoblast cells and fetal endothelial cells of the labyrinth zone. Expression levels of GLUT 3 were decreased toward the end of pregnancy, especially in trophoblast cells of the labyrinth zone. In contrast to GLUT 1 and GLUT 3, expression levels of GLUT 4 didn't change toward the end of pregnancy (▶ **Table 1**).

Plasma serum insulin ELISA experimental results showed that the highest insulin values on the 15th and 20th days of pregnancy were determined in the control group. It was determined that the amount of insulin in the blood serum was higher than the other groups in the control and stevioside groups and that the control group contained a high amount of insulin compared to the stevioside group (▶ **Fig. 9**). After STZ, insulin levels were statistically significantly decreased in diabetic pregnancy. Stevioside treatment in diabetic pregnancy didn't affect insulin levels. Stevioside treatment in normal pregnancy decreased insulin levels. Stevioside, which has a similar effect to insulin, may have reduced the demand for insulin in the normal pregnancy groups. As pregnancy progresses, insulin sensitivity decreases by about 50–60% in normal pregnancies [46]. Our results are consistent with these data.
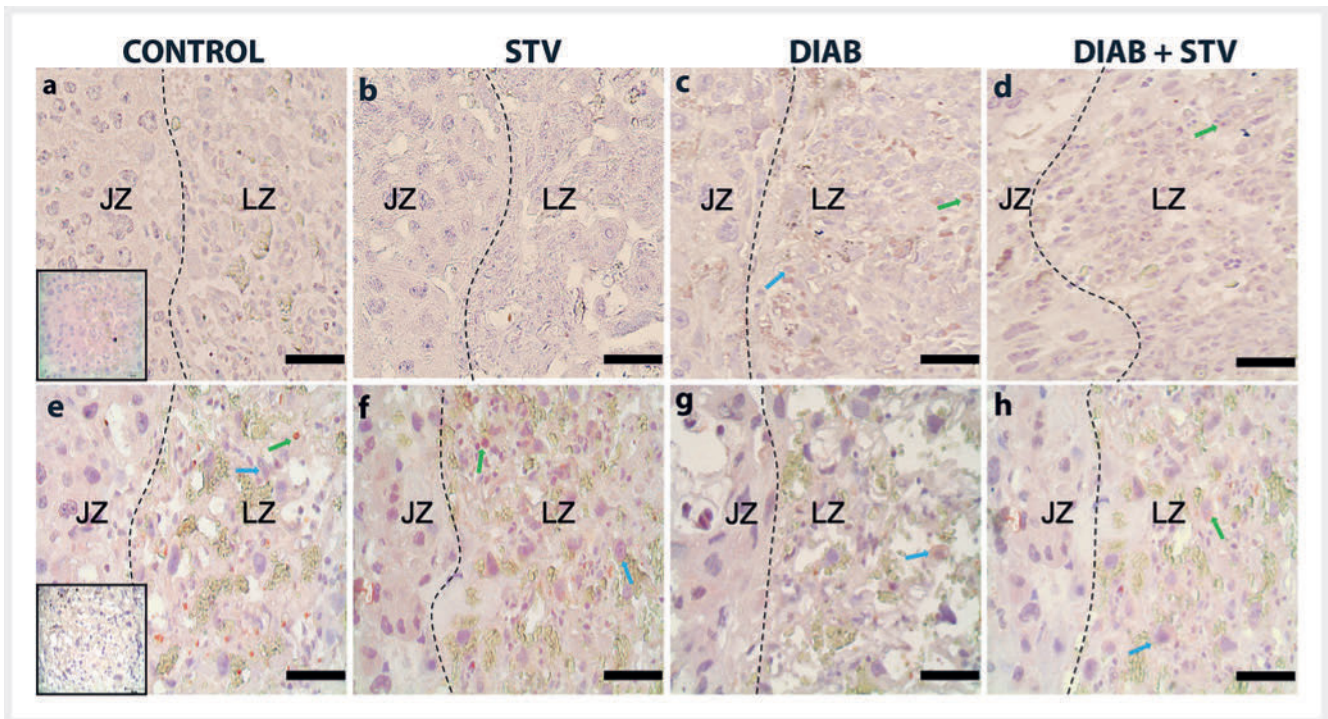
► **Fig. 6** GLUT 4 immunohistochemical stainings (polymer detection method with HRP, AEC chromogen for positive signaling, and Mayer's hemalum for counterstain) in rat placenta. **a–d** 15th day of pregnancy, **e–h** 20th day of pregnancy. Green arrows show the syncytiotrophoblast cells and blue arrows are the cytotrophoblast cells. LZ: labyrinth zone; JZ: junctional zone. Negative staining is shown in the small squares in ''a and e''. Scale bar = 50 μm. n = 6. The experiment was repeated three times independently.

Glucose transporter proteins that play an important role in the placenta are GLUT 1 and GLUT 3, and these two glucose transporters work independently of insulin. GLUT 4 is an insulin-dependent transporter [47]. Unlike GLUT 1 and GLUT 3, GLUT 4 has a limited effect on the placenta. In addition, an increase in plasma insulin is not always reflected in tissues. In conclusion, this study demonstrated that stevioside regulates GLUT 1, which is responsible for transplacental glucose transport. However, stevioside treatment had no effect on blood glucose levels, insulin levels, or GLUT 3 and GLUT 4 proteins. This is believed to be due to the dose of stevioside administered to rats. In this study, stevioside was administered to rats at physiological concentrations of 1 mg/kg [48]. Although the duration of stevioside use is similar in studies in the literature, there are studies where the dose is 25, 30, 200, and 250 times [18, 49–51].

Material and Methods

Experimental groups

Wistar rats were obtained from Animal Experiment Unit at the University of Akdeniz (date of approval: February 26th, 2015 and approval number: 2015.02.11) aged 8 weeks and weighing 150–250 grams. Wistar rats were kept under a 12 h light–dark cycle and were fed a standard diet. The rats had been kept in cages overnight, with two females per one male rat. After mating, the presence of the sperm in the vaginal smear the following morning was designated as day 0 of pregnancy. Pregnancy rats were ran-
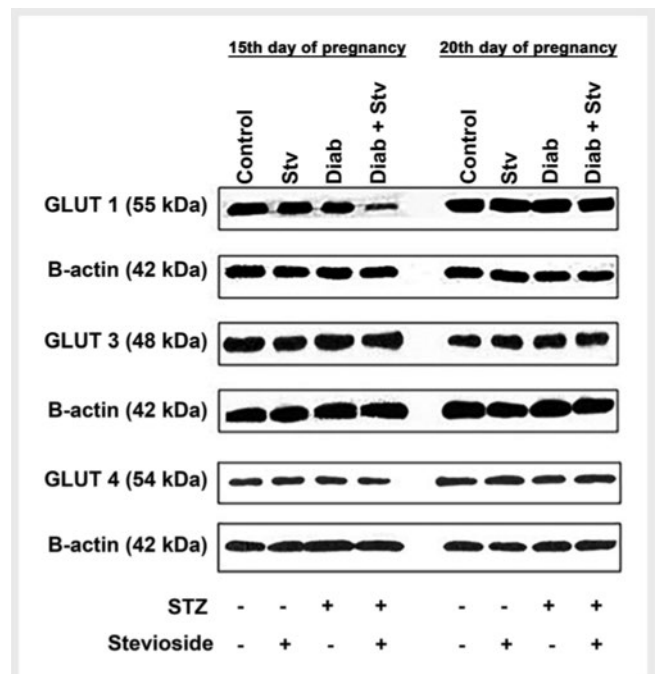
► **Fig. 7** Representative Western blots of the total GLUT 1, GLUT 3, and GLUT 4 on the 15th and 20th days of pregnancy in the control, stevioside (Stv), diabetic (Diab), and diabetic + stevioside (Diab + Stv) groups. *β*-actin was used as the loading control. n = 6. The experiment was repeated three times independently.
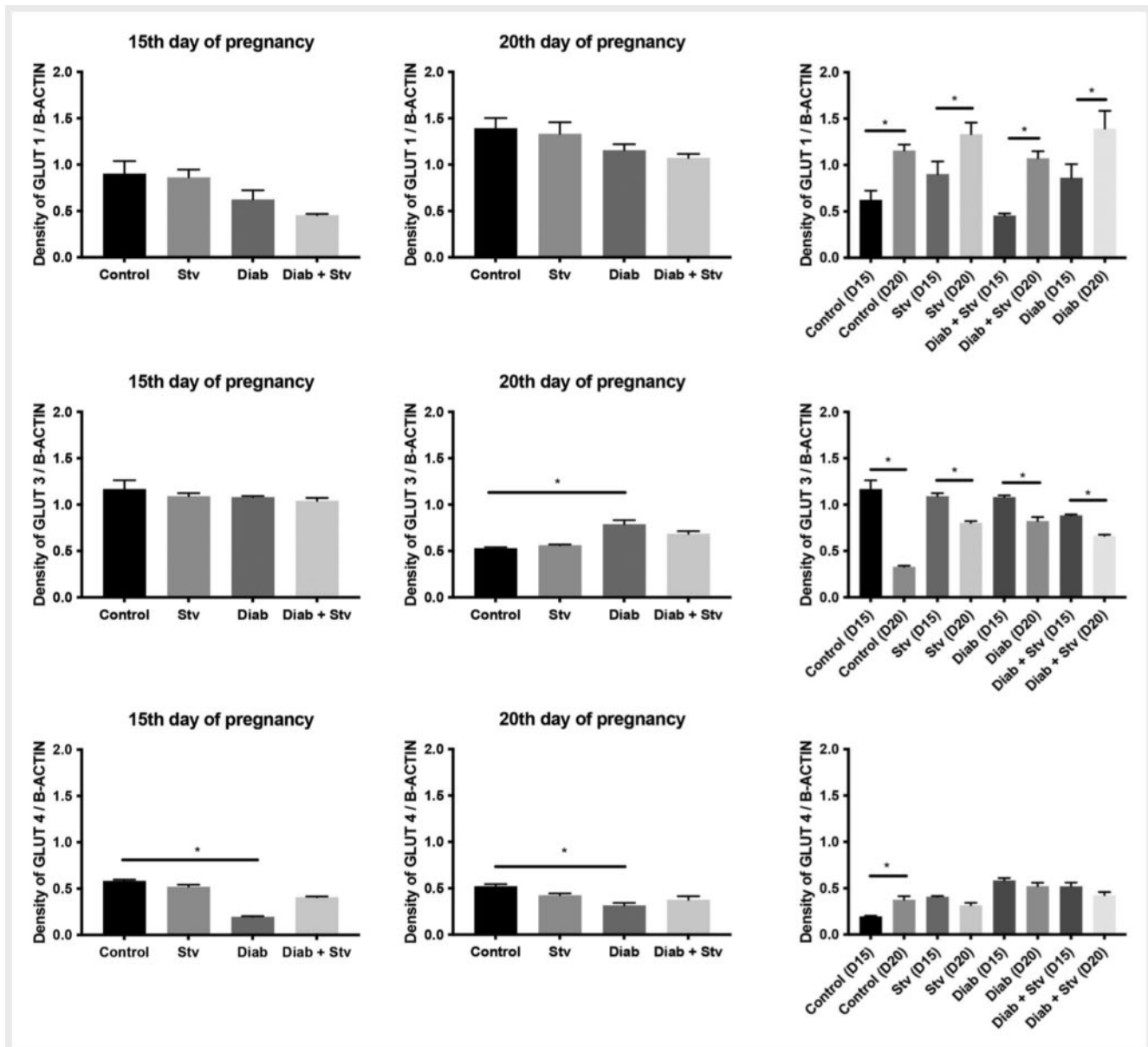
▶ **Fig. 8** Density of the total **a)** GLUT 1, **b)** GLUT 3, and **c)** GLUT 4 proteins on the 15th and 20th days of pregnancy in the control, stevioside (Stv), diabetic (Diab), and diabetic + stevioside (Diab + Stv) groups (*p < 0.05). Data are presented as mean ± SD. n = 6. The experiment was repeated three times independently.

domly divided into four groups with 6 rats for each groups. Pregnancy rats were injected intraperitoneally with a single dose of 50 mg/kg STZ on the 7th day of pregnancy. Glucose blood levels are measured in animals 2 days after STZ injection. Only rats with blood glucose levels over 200 mg/dL were admitted as diabetic and included in the study. Pregnancy rats in stevioside and diabetic + stevioside groups were injected intraperitoneally with a single dose of 1 mg/kg/day stevioside from the onset of pregnancy to the dissections process (15 days or 20 days according to groups) [48].

Blood glucose measurements

Blood samples were taken from the tail veins of rats on the 0th, 9th, 15th, and 20th days of pregnancy. Blood samples were placed on a single-use test strip. Blood glucose measurements were performed by a glucometer (accu check, ACCUA01).

Fetus-placental weights and number of pups

Rats were euthanized by the cervical dislocation method by giving a anesthesia/tranquilizer substance on the 15th and 20th days of pregnancy and their placentas and fetuses were removed. Placenta and fetuses of pregnant rats belonging to the control,
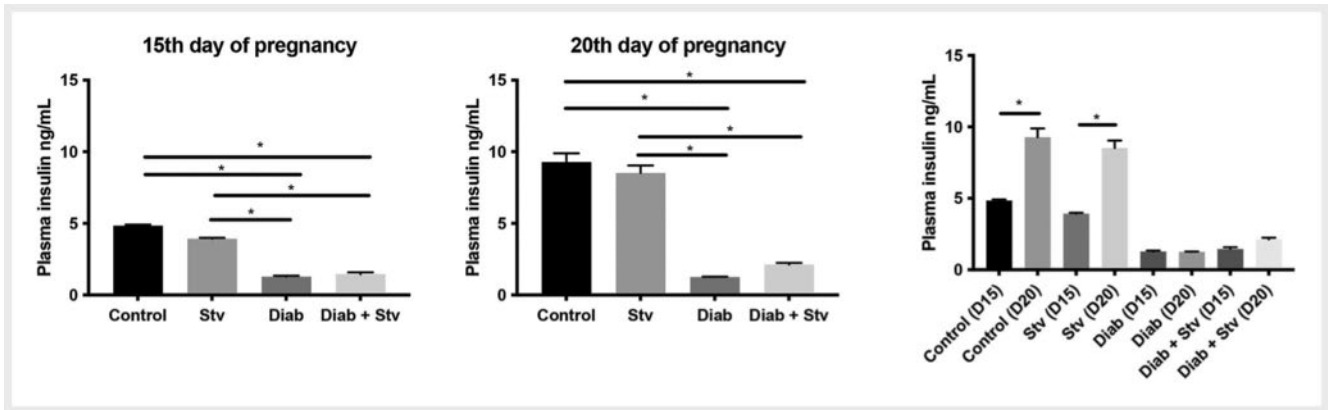
▶ **Fig. 9** Concentrations of plasma insulin protein on the 15th and 20th days of pregnancy in the control, stevioside (Stv), diabetic (Diab), and diabetic + stevioside (Diab + Stv) groups (*$p < 0.05$). Data are presented as mean ± SD. n = 6. The experiment was repeated three times independently.

stevioside, diabetic, and diabetic + stevioside groups were taken and weighed. The number of pups in each group was evaluated.

Immunohistochemistry

The placenta tissues were fixed in formalin and embedded in paraffin, and serial sections (5 µm) were collected on SuperFrost Plus slides. After the deparaffinization step, slides were transferred to 0.01 M citrate buffer (pH = 6) and subsequently heated in a microwave oven for 3 × 6 min at 804 W for antigen retrieval. After cooling for 20 min at room temperature, the sections were washed three times with PBS; 3% hydrogen peroxide in methanol was used to block endogenous peroxidase activity for 30 minutes. After the washing steps, nonspecific binding sites were blocked with ultra V block (Lab Vision) for 8 min at room temperature. Following nonspecific binding blocking, sections were incubated with primary antibodies: GLUT 1 (Abcam ab652, 1/1000 dilution), GLUT 3 (Abcam ab53095, 1/300 dilution), and GLUT 4 (Abcam ab654, 1/500 dilution) for overnight at + 4 °C temperature in a moist chamber. Slides were used for the UltraVision Quanto Detection System HRP (Thermo TL-125-QHL) according to the instructions of the manufacturer. These were washed with PBS and stained with AEC substrate. The sections were counterstained with Mayer's hemalum (Merck, 109 249) and mounted with entellan (Merck, 107 961). Negative controls were performed by substitution of the primary antibody with PBS.

Immunohistochemical h-score assessments of GLUT 1, GLUT 3, and GLUT 4 between experimental groups were performed in agreement with the previous study [36]. Decidual cells, spongiotrophoblast cells, giant cells, glycogenic cells, labyrinth trophoblast cells, labyrinth giant cells, and fetal endothelial cells in rat placenta are evaluated in ▶ **Table 1**. All cells were scored according to five different areas per placental section. This table was designed according to the percentage of positively stained cell count; +++, ++, +, and – indicate the differences in signal intensities observed by optical microscopy and reflect levels of GLUT 1, GLUT 3, and GLUT 4 proteins: – = absent; + = weak expression (10–30%); ++ = moderate expression (40–60%); and +++ = high expression (70–100%). Empty cells in the table indicate that the

concerned tissue is either not yet differentiated or has not been investigated on the 15th and 20th days of pregnancy. Triple-blind studies were conducted.

Western Blotting

Placenta samples for each group were weighed and put into a lysis buffer with a protease inhibitor cocktail. After homogenization, samples were centrifuged at 14 000×g for 15 min at + 4 °C. Supernatants were collected and stored at − 80 °C. The protein concentration was determined by Lowry assay [52] and 50 µg of protein was applied per lane. Samples were subjected to SDS polyacrylamide gel electrophoresis at 80 V for approximately 2.5 hours and were then transferred onto PVDF membranes (Amersham, RPN2020F) in a buffer containing 0.2 mol/L glycine, 25 mmol/L Tris, and 20% methanol overnight at + 4 °C under 30 V. The membranes were blocked with 5% non-fat dry milk (BioRad 1706404XTU) and 0.1% Tween 20 (Lab Vision, TA 125 TW) in 0.14 mol/L Tris-buffered saline (TBS; pH 7.2–7.4) at + 4 °C. Membranes were incubated with primary antibodies: beta-actin (Abcam ab6276, 1/5000 dilution), GLUT 1 (Abcam ab652, 1/1000 dilution), GLUT 3 (Abcam ab15311, 1/500 dilution), and GLUT 4 (Abcam ab654, 1/2000 dilution) for overnight at + 4 °C temperature. After washing with Tris-buffered Saline with Tween 20 (TBS-T; 0.05 M Tris, 0.15 M NaCl, 0.001% Tween 20), membranes were incubated for 2 h at room temperature (RT) with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Vector) antibodies. After washing with TBS-T, antibodies were detected by the chemiluminescence-based SuperSignal CL HRP substrate system (Pierce). Membranes were exposed to Hyperfilm (Amersham), which was subsequently analyzed by using Image J software. All results were normalized to beta-actin.

Enzyme-Linked Immunosorbent Assay (ELISA)

Rats were sacrificed on the 15th and 20th days of pregnancy and 4–5 ml of blood samples were taken from the abdominal aorta. Plasma centrifugation was performed at 2000–3000 g for 5 min and stored at − 80 °C until tested. Plasma insulin levels were measured using an enzyme-linked immunosorbent assay (ELISA). The

ELISA (Merck Millipore rat-insulin kit, EZRMI-13 K) kit has 100% sensitivity to rat insulin. Insulin quantification was performed using a rat/mouse-specific insulin ELISA kit. The assay and analysis were performed according to the instructions of the manufacturer.

Statistical Analysis

A Student's *t*-test and one-way ANOVA tests were applied using Graphpad Prism 7 (Graphpad Software, Inc.) to determine whether there was a statistically significant difference between the groups and the values with $p < 0.05$ being considered statistically significant.

Contributors' Statement

Ertan Katirci performed immunohistochemistry, western blotting, ELISA and blood-glucose measurements, analyzed them statistically, created all figures and drafted the manuscript. Remziye Kendirci-Katirci assisted with immunohistochemical evaluations and statistical analysis. Emin Turkay Korgun provided BAP (TYL-2015–873) funding to Akdeniz University and assisted with data evaluation. Emin Turkay Korgun formed the hypothesis of the project and assisted with both drafting the manuscript and data interpretation and evaluation (involved in immunohistochemical evaluations and statistical analysis).

Conflict of Interest

The authors declare that they have no conflict of interest.

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