FULL PAPER *Anatomy*

Quantitative Expression and Immunohistochemical Detection of Glucose Transporters, GLUT1 and GLUT3 in the Rabbit Placenta during Successful Pregnancy

Hamayun KHAN[1)], Ken Takeshi KUSAKABE[1,2)]*, Shoichi WAKITANI[1)], Masato HIYAMA[2)] and Yasuo KISO[1,2)]

[1)]Laboratory of Basic Veterinary Science, The United Graduate School of Veterinary Science and [2)]Laboratory of Veterinary Anatomy, Faculty of Agriculture, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, Japan

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ABSTRACT. Glucose is essential for the development of the fetus. We address here the quantitative expression and immunohistochemical localization of glucose transporter (GLUT1 and GLUT3) in the rabbit placenta during successful pregnancy. Blood glucose level showed a significant decrease at the gestation period in comparison with non-pregnancy. Maternal serum glucose was gradually increased according to fetal development. Quantitative RT-PCR results showed that expression of GLUT1 was significantly increased from day 13 to day 18, while GLUT3 mRNA level was significantly decreased during the same periods. Western blot analysis demonstrated that GLUT1 protein did not change significantly in the placenta during pregnancy when compared to non-pregnant uteri. Immunohistochemistry indicated that distribution of GLUT1 was observed mainly to the surface of the outer trophoblasts, whereas GLUT3 mainly localized to the basal site of the inner trophoblasts and fetal blood vessels. These results suggest that glucose is transported through GLUT1 from the maternal blood stream for use as a placental fuel and for further transport through GLUT3 to the fetal circulation, thus signifying the distinct anatomical localization of GLUT1 and GLUT3 in the rabbit placenta during successful pregnancy.
KEY WORDS: GLUT1, GLUT3, immunohistochemistry, mRNA, rabbit placenta.

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Glucose, being the important source of carbon for placental and fetal metabolism, is one of the essential nutrients for tissue synthesis during fetal development [4, 12, 26]. Fetal consumption of glucose rapidly increases toward term due to the almost 20-fold increase in fetal weight during the second half of gestation [18]. Since fetuses are not able to generate glucose *de novo* until late in gestation, fetal glucose usage is critically dependent upon the transfer system across the placenta [4, 21]. Hence, glucose transfer from the maternal circulation is a vital feature of mammalian fetal development and organogenesis [1, 11, 26].

The facilitative glucose transporters (GLUTs) and their several isoforms constitute D-glucose transport system, in the energy-independent and the glucose concentration gradient-dependent manner. The GLUT molecule is composed of about 500 amino acids, which are highly conserved between isoforms, and contains twelve membrane-spinning domains that are thought to form a functional channel for glucose transportation [2]. Each isoforms of GLUTs have distinct antigenic difference in the carboxyl terminal but overlapping in the tissue distributions. Among GLUT isoforms, GLUT1 was first detected in erythrocytes [27], and subsequent findings showed widespread distribution in kidney, brain and placenta, which form generally blood-tissue barrier, regarded GLUT1 as a basic factor for glucose uptake. GLUT3 was firstly identified as a neuronal glucose transporter located in axon and dendrites [37, 41]. Expression of GLUT 3 is also observed in the brain, sperm, placenta, pre- and post-implantation embryo, involving in the specific glucose requirements [37, 47].

Fetal growth is very rapid during the last third of gestation in the rabbit [20]. Fetuses at the third trimester strongly require placental glucose supply, mediated by a family of membrane–spanning glycoprotein glucose transporters [16, 44]. The transportation of maternal glucose across the placenta into the fetal circulation occurs through facilitative diffusion, mainly by GLUT1 and GLUT3, which mediate basal glucose uptake [6–8, 10, 31]. Neither GLUT1 nor GLUT3 alone is sufficient for the well being of the early embryo. Both GLUT1 and GLUT3 are crucial for normal intrauterine development in mice as deletion of either gene results in intrauterine-grown restriction (IUGR) [19, 42]. GLUT1, known as a predominant placental glucose transporter [3], is responsible for supplying glucose for use as a placental fuel. GLUT3 is important for early embryonic development in neurulation and contributes to optimal fetal growth during late gestation [19, 30, 33, 37, 45].

GLUT1 and GLUT3 have been an important subject of extensive studies in mammalian placentas [15, 17, 23, 25, 35, 36, 46, 47]. GLUT1 represents major glucose transporter in the human placenta that controls glucose transportation from the maternal to the fetal compartment under physiological conditions, and its expression is maximal at the term, while GLUT3 expression is highest in early gestation, showing reduction in the mid and full-term placenta [25, 35]. Furthermore, GLUT1 is expressed in syncytiotrophoblasts and endothelial cells in the human term placentas [23, 25, 40] and GLUT3 is mostly found in endothelial cells

* CORRESPONDENCE TO: KUSAKABE, K. T., Laboratory of Veterinary Anatomy, Faculty of Agriculture, Yamaguchi University, 1677–1 Yoshida, Yamaguchi 753–8515, Japan.
e-mail: kusakabe@yamaguchi-u.ac.jp

of the fetal vessels [23]. In the hemochorial placenta of rats and mice, GLUT1 protein is localized both in the junctional and labyrinthine regions [15, 47] and GLUT3 is found in the labyrinthine region [36].

Rabbit placentas show bidiscoidal, labyrinthine and hemochorial structure. The placental-fetal blood barrier is formed by 2 layers, namely outer and inner trophoblast layers composed by syncytiotrophoblasts. Trophoblasts of both types have an activity for placental transportation; particularly outer trophoblasts have typical membranous pores that act as an important channel for molecular transport. The functional differentiation of rabbit trophoblasts was completed up to at early placentation (day 13) of pregnancy. Trophoblast differentiation is completed up to day 18 of pregnancy from the view points of placental barrier and transport route formation. In rabbits, no previous literature is available about glucose transporters, GLUT1 and GLUT3 through placental barrier during successful pregnancy except the one study [28], which was carried out in the rabbit placenta under restricted evaluation for the impact of maternal hypercholesterolemia on placental glucose transfer with feeding the cholesterol-enriched diet throughout gestation. Accordingly, our investigation has been conducted to study in detail the quantitative expression and localization of GLUT1 and GLUT3 in the normal rabbit placenta during successful pregnancy.

MATERIALS AND METHODS

Animals: Total of 17 female rabbits (Japanese White) bred under normal condition with the animal facility of Kyudo (Saga, Japan) was purchased at day 13, 18, 25 and 28 of pregnancy and non-pregnancy. After blood sample collection from the auricular artery, rabbits were sacrificed by the intravenous injection of 130 mg/kg pentobarbital sodium. Non-pregnant uteri, placentas and intact fetuses were collected. The placenta samples were immediately frozen and kept at –80°C until usage, and/or fixed with Zamboni's solution (0.21% picric acid and 2% paraformaldehyde in 130 mM phosphate buffer). Fetal blood was collected from the heart at day 25 and 28 of pregnancy. Fetal livers were collected at day 18 instead of blood samples. Serums and liver samples were kept at 4°C and –80°C respectively until glucose assay. The experimental protocols and animal use were approved and followed on the Yamaguchi University Animal Experimental Guidelines.

Glucose Assay: Glucose concentration was measured in the serum from maternal and fetal blood and fetal liver. Fetal livers were used for glucose assay in compensatory for inadequate volume of fetal blood. Liver samples were homogenized and the supernatants were collected. Measurement was conducted using Glucose C2 kit (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instructions.

Real-time RT-PCR: Total RNA was extracted from placental tissues on day 13, 18, 25 and 28 of pregnancy as well as non-pregnant uteri using Isogen RNA extraction reagent (Nippon Gene, Tokyo, Japan). cDNA was synthesized for 1 μg of total RNA with ReverTra Ace (Toyobo, Osaka, Japan). Real time PCR was conducted using SYBR Green PCR Master Mix (Applied Biosystem, Foster city, CA, U.S.A.) and the following primers, GLUT1: 5'-GCCCTGCATGTCCTATCTGA-3' and 5'-TGAAATTCGAGGTCCAGTTGG-3'; GLUT3: 5'-CGTCATCTTTGCCGTCTTC-3' and 5'-ACATGGGTGGTGGTCTCAA-3'; and glyceraldehydes-3-phosphate dehydrogenase (GAPDH): 5'-TCACCATCTTCCAGGAGCGA-3' and 5'-CACAATGCCGAAGTGGTCGT-3'. GAPDH was used as reference for normalization of quantification. Data were quantified by $\Delta\Delta C_T$ method of relative quantification using the StepOne Software and StepOne Plus Real-Time PCR system (Applied Biosystem).

Western blot: Rabbit placentas at day 13, 18, 25, 28 of pregnancy and non-pregnant uteri were homogenized in the extraction buffer (125 mM Tris-HCL pH 8.0, 2 mM $CaCl_2$, 1.4% triton X-100) and protease inhibitor cocktail (Complete Mini, Roche Diagnostics, IN, U.S.A.). Homogenates were kept on ice for 30 min, and then centrifuged at 15,000 × g for 10 min at 4°C and the supernatants were collected. Total protein concentrations were measured by the Lowry method using RC DC protein Assay Kit (BioRad Laboratories, Hercules, CA, U.S.A).

Total protein was diluted with Laemmli sample buffer (2% SDS, 62.5 mM Tris-HCL, pH6.8, 25% glycerol and 0.01% bromophenol blue). Equal amounts of protein from the samples were subjected to polyacrylamide gel electrophoresis and transferred to a polyvinylidene diflouride membrane (Millipore, Bedford, MA, U.S.A.) by semi-dry blotting. The membranes were then treated with 5% nonfat dried milk overnight at 4°C and immunoblotted for 1 hr with goat polyclonal GLUT1 (C-20, Santa Cruz Biotechnology, Santa Cruz , CA, U.S.A.). Membranes were washed with 0.1% tween 20-contained PBS (PBS-T), then incubated with biotin-conjugated anti-goat IgG (Vector Lab., Burlingame, CA, U.S.A.). Followed by incubation with VECTASTAIN ABC reagent (Vector Lab), immunoreactive blots were identified using chemiluminescence detection ECL plus kit (GE healthcare, Buckinghamshire, UK) and LAS 3000 lumino-image analyzer (Fujifilm, Tokyo, Japan). As a reference, GAPDH was monitored using mouse monoclonal anti-GAPDH antibody (Chemicon, Temecula, CA, U.S.A.) and peroxidase-linked anti-mouse IgG (Vector Lab). The band intensity was analyzed by using NIH image analysis software (NIH, Bethesda, ML, U.S.A.).

Immunohistochemistry: Paraffin sections prepared from rabbit placenta fixed with Zamboni's solution were used. Sections were deparaffinized in xylene, rehydrated through a graded series of alcohol and washed with PBS. After treatment with 0.1% trypsin solution for 20 min, sections were incubated with 3% H_2O_2 and then with 5% non–fat dry milk for blocking. The primary antibody polyclonal anti-GLUT1 raised in goat (Santa Cruz) or monoclonal anti-GLUT3 raised in mouse (R&D systems, Minneapolis, MN, U.S.A.),

applied to section overnight at 4°C. After several washes, the slides were incubated with biotin anti-goat or anti-mouse IgG antibody (Vector Lab). A Vectastain Elite ABC kit (Vector Lab) was used to detect the secondary antibodies, and then diaminobenzidine was applied for visualization. Sections were briefly counterstained with Mayer's hematoxylin.

Statistical analysis: Results were reported as mean ± standard deviation (SD). The concentration of glucose, relative mRNA expression, and protein levels were evaluated using one-way ANOVA. A P value <0.05 was considered statistically significant.

RESULTS

Maternal and fetal glucose concentration: Our result showed the significant decrease of the glucose concentration in maternal blood serum in comparison with that of non-pregnancy (Table 1). On the other hand, the maternal and fetal glucose levels were elevated accompanied with pregnancy progress. In addition, the fetal glucose concentrations were significantly lowered compared with maternal concentration.

Expression of GLUT1 and GLUT3 in rabbit placentas: Real time RT-PCR showed that expression of both glucose transporters was observed in the developing or matured rabbit placentas (Fig. 1). GLUT1 mRNA was detectable at day 13 (early placentation period) and significantly increased to day 18 (placentation period), and then continued till the end of pregnancy (day 28). At day 13, mRNA expression of GLUT3 was significantly increased as compared with non-pregnancy. Significant decrease was observed from day 13 to 18 and low level of GLUT3 expression was remained till the end of pregnancy.

Regarding GLUT1 that showed obvious expression in the rabbit placentas, western blot experiments were additionally conducted (Fig. 2). The expected size of the rabbit GLUT1 was 55 kDa. Our result showed that GLUT1 protein level did not change significantly during the course of pregnancy, although increasing tendency was observed at early period (day 13) and advanced period (day 25) of placentation in comparison with non-pregnant uteri.

Localization of GLUT1 and GLUT3: Immunohistochemical studies on the both glucose transporters were conducted especially at day 25 of pregnancy (Fig. 3), corresponding to a period for GLUT1-abundant and rabbit placental completion. In the mature rabbit placentas, GLUT1 was detected at the labyrinth region and GLUT3 was observed in the limited labyrinth region close to the junctional zone. In detail, GLUT1 seemed to be abundant at the outer trophoblasts that form blood sinuses filled with maternal nutrient blood. GLUT3-positive reaction was observed specifically at the inner trophoblast layer surrounding fetal blood vessels.

DISCUSSION

To the best of our knowledge, it is the first report to evaluate glucose transporters GLUT1 and GLUT3 in the rabbit placenta during successful pregnancy. Our present study clarified elevation of placental GLUT1 from early placentation to placentation period and maintenance to term. These results are consistent with the previous finding in the hemochorial placenta from human and mouse. In the human placenta, GLUT1 is a dominant glucose transporter increasing expression level during gestation maximal at delivery [28]. In mice, the level of GLUT1 mRNA increases as gestation proceeds, whereas the amount of GLUT3 mRNA is unchanged throughout the gestation [46]. GLUT1 and GLUT3 in the rabbit placenta were not in agreement with pervious study on the rat and sheep placenta. In rat, placental GLUT1 mRNA decreased throughout pregnancy and GLUT3 levels do not change significantly during the course of gestation [47]. In sheep, placental GLUT3 is increased in mRNA and protein levels as gestation advances, whereas GLUT1 abundance is unaffected and/or decreased toward term [17].

The relationship between GLUT1 mRNA and protein

Table 1. Maternal and fetal glucose concentrations[a)]

Gestation days	Maternal glucose (mg/d*l*)	Fetal glucose (mg/d*l*)
0 (non-pregnant)	123 ± 0.66 (3)	N.A
13	104 ± 1.19* (3)	N.A
18	113 ± 0.92* (4)	76.7 ± 0.23* (3)
25	120 ± 0.20* (3)	82.7 ± 0.79* (3)
28	122 ± 0.17* (4)	84.8 ± 0.35* (4)

a) Maternal serum, fetal liver (day18) and fetal serum (day 25 and 28) were used for assay. Values are mean ± SD. *: P<0.05 vs. non-pregnant. N.A=not applicable. Parentheses= number of dams or fetuses tested.

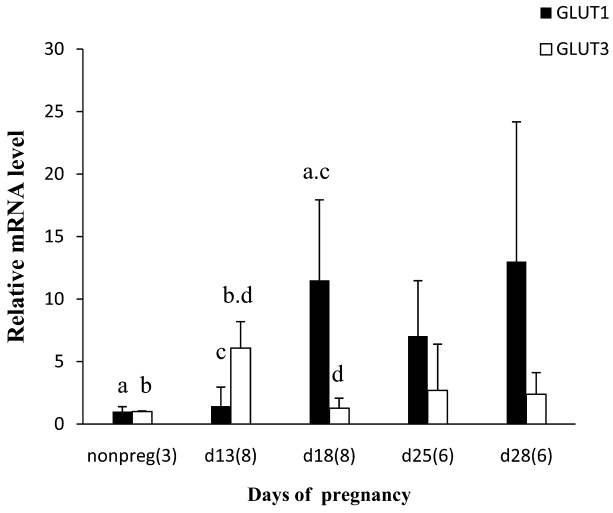

Fig. 1. Quantitative mRNA expression of GLUT1 and GLUT3 in the rabbit non-pregnant uteri and placentas. a-d: There are statistical differences among the same characters. Each bar represents values of mean and SD. The number of used specimens is shown in the parentheses. nonpreg = non-pregnant uteri.

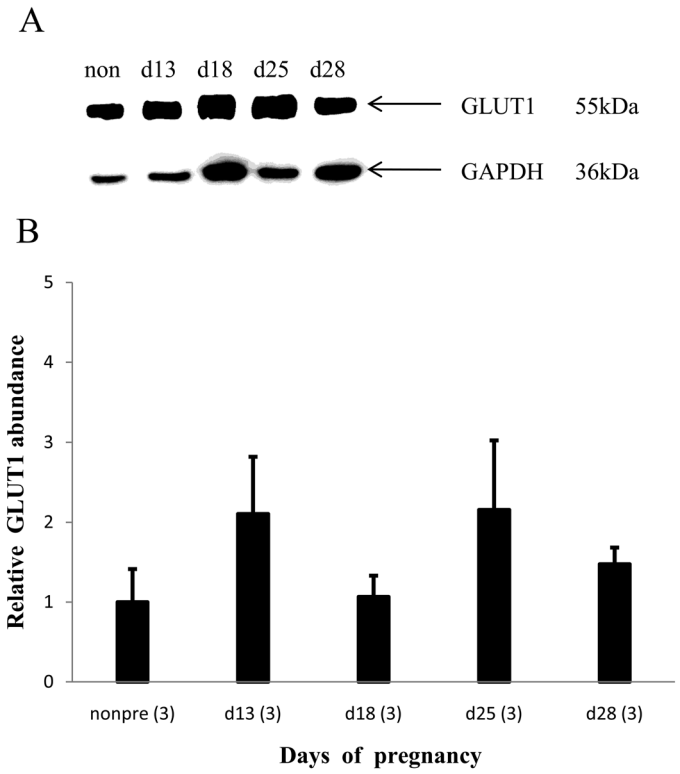

Fig. 2. Western blot analysis of GLUT1 in the rabbit placenta. A) Representative data of western blot at non-pregnant uteri and placentas. B) Quantitative comparison of GLUT1 protein levels. Each bar represents values of mean and SD. The number of used specimens is shown in the parentheses. non-preg=non-pregnant uteri.

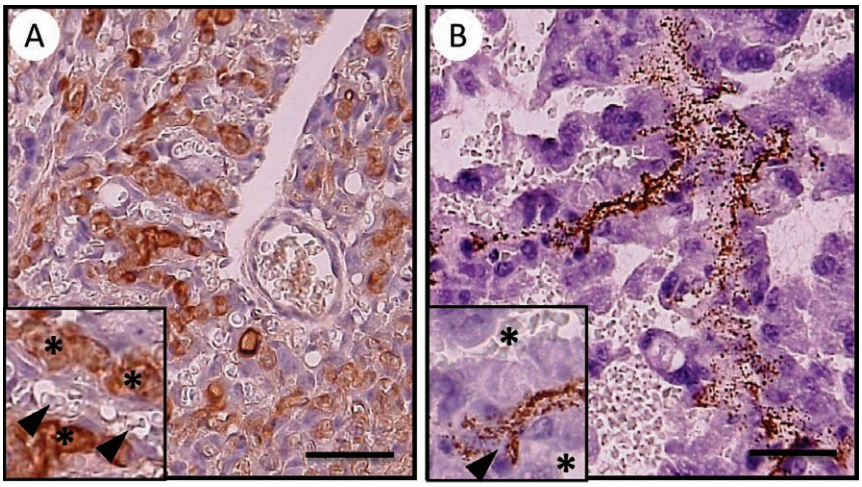

Fig. 3. Immunohistochemical localization of GLUT1 (A) and GLUT3 (B) in the rabbit placenta at day 25. Inset: High magnification of the placental labyrinth regions, containing maternal blood sinuses (asterisks) and fetal blood vessels (arrowheads). A) Specific immune-reaction for GLUT1 is seen around the maternal blood sinuses, which are formed by the outer trophoblasts. B) GLUT3 reactivity is seen at the inner trophoblast layer around the fetal blood vessels. Bar scale=50 μm.

levels at early placentation (day13) was inconsistent in our present finding, implying that the mRNA level could not be taken as an index of the GLUT1 protein concentration. Resemble tendencies were observed in the rat brain and mouse fetal heart [9, 38]. The activity of glucose transport in the heart of mouse embryo increased as acute response to hypoglycemia with increase expression of GLUT1 protein, whereas the alteration of GLUT1 mRNA was not clearly detected. It was suggested that delayed increase of GLUT1 protein may be causable following the processing of protein maturation such as glycosylation, vesicular trafficking and insertion to the lipid raft, i.e., post-transcriptional controls. Alteration of maternal blood glucose in pregnant rabbits, which was first hypoglycemic, may be capable to induce prompt action of GLUT1 replenishment via post-transcriptional maturation. Still further study is needed to elucidate this functional hypothesis in placenta.

In present study, the maternal blood glucose was significantly decreased during gestation in comparison with that of the non-pregnant blood serum, which is almost in accordance with those most recently reported in pregnant New Zealand white and Japanese white rabbits [22, 29]. Change of maternal glucose concentration during gestation may reflect glucose supply or replenishment according to nutritional demand for the rabbit development [43]. In addition, our results about the glucose analysis in maternal blood serum and the fetus confirmed the previous report in rabbits that glucose concentration in offspring was significantly decreased compared to that of the mother [28]. Furthermore glucose analysis in the rabbit fetus further attached to the hypothesis that the blood glucose level in the fetus is about two-thirds of that in the mother [12, 32].

Immunohistochemical study clearly showed the localization of the GLUT1 in the outer layer of rabbit trophoblasts. In the human full term placenta, GLUT1 is found in abundance at the microvillus membrane and the basal membrane of the syncytiotrophoblast. Furthermore, GLUT3 expression restricted to the fetal capillary endothelium [23]. In the hemochorial placenta of rat and mouse, GLUT1 protein is localized both in the junctional and labyrinthine regions [15, 47]. Especially, GLUT1 in the rat placenta is present in the syncytial cell layers [36]. Immunohistochemical analysis in the rat trichorial placenta revealed the localization of GLUT1 at the plasma membranes of the maternal blood side of syncytiotrophoblast layer I and the fetal blood side of syncytiotrophoblast layer II [39]. GLUT3 resides on the maternal facing surface of syncytiotrophoblast layer I lining the labyrinthine region that is responsible for feto-maternal glucose exchange [36].

Essential nutrient and adequate oxygen supply is required for proper development and growth of fetus. Glucose transport, directed from maternal to fetal circulation under physiological conditions, is facilitated by the placental ability and important for development of the healthy fetus [5]. Our present study about localization of GLUT1 and GLUT3 partitioned in the diverse types of trophoblasts indicates importance of both glucose transporters for fetus development in the rabbit placenta, as deficiency of either GLUT1 or GLUT3 may adversely affect the glucose supply to the growing fetus resulting in IUGR [19, 42]. This etiology seems to be multifactorial and complicate, but a disorder of placental glucose transfer is a fatal risk and essential pathway for pregnancy success. Our findings further support the recent hypothesis that neither GLUT 1 nor GLUT3 alone is sufficient for the well being of the developing fetus [19]. Thus, there appears to be interdependency of GLUT1 and GLUT3 in the pregnant rabbit mediating the glucose transport necessary for fetal development and growth. A related collaboration between GLUT1 and GLUT3 guarantees an adequate glucose supply to the developing conceptus as previously reported in the epitheliochorial placenta of sheep [13, 14].

Currently, reproductive roles of GLUT1 and GLUT3 have been explored by the researches of transgenic mice. The embryos obtained from the homozygous mating of GLUT1-deficient mice revealed severe failure of organogenesis, growth retardation and other malformations reminiscent of the human infants from hyperglycemic diabetic mothers [24]. In the heterozygous mice, nitric oxide-dependent endothelial relaxation was reduced [34], showing a possible effect for GLUT1-dependent glucose metabolism involving in the regulation of placental blood flow. Emphasis is also placed on the transgenic GLUT3 null mutant mice. In the homozygous null mutation of GLUT3, despite of the intact presence of GLUT1, a decrease in transplacental fetal glucose is observed. These data showed an importance of coordinated functionality of GLUT1 and GLUT3 in the transplacental glucose transport [19]. Recently, transgenic techniques have been developed in the laboratory rabbits as a useful tool for the molecular researches and production of therapeutic proteins. Transgenic research may be useful to establish a reproductive role of glucose transporters in the rabbit placenta.

In conclusion, this study demonstrates the distinct histological pattern of glucose transporter, GLUT1 and GLUT3 in the rabbit placenta. Due to presence of the GLUT1 mainly to surface of the outer trophoblast, while that of GLUT3 to the basal site of the inner trophoblast, suggesting that GLUT1 is responsible for glucose supply as a use of placental fuel and further transportation to the fetal circulation through GLUT3 as in mice [47].

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