Theriogenology 121 (2018) 82–90

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Placental and uterine expression of GLUT3, but not GLUT1, is related with serum progesterone levels during the first stages of pregnancy in queens

Lluís Ferré-Dolcet [a], Marc Yeste [b], Meritxell Vendrell [a], Teresa Rigau [a], Joan Enric Rodríguez-Gil [a], Maria Montserrat Rivera del Álamo [a, *]

[a] Department of Animal Medicine and Surgery, Faculty of Veterinary Medicine, Autonomous University of Barcelona, Bellaterra (Cerdanyola del Vallès), E-08193, Spain
[b] Department of Biology, Faculty of Sciences, University of Girona, Girona, E-17071, Spain

ARTICLE INFO

Article history:
Received 20 March 2018
Received in revised form
1 August 2018
Accepted 2 August 2018
Available online 7 August 2018

Keywords:
Glucose transport
Queen
Endometrium
Placenta
Trophoblast
GLUT1
GLUT3

ABSTRACT

The present study investigated the expression of GLUT1 and GLUT3 in the uterus and placental transference zone of non-pregnant and pregnant queens throughout different pregnancy ages, using immunohistochemistry and immunoblotting techniques. Both GLUT1 and GLUT3 were expressed in both uterine glandular and luminal epithelia and myometrium in pregnant and non-pregnant queens. While endometrial endothelia showed expression of GLUT1 in both pregnant and non-pregnant queens, GLUT3 was only expressed in the pregnant counterparts. Regarding placental structures, GLUT3 was present in cytotrophoblasts, syncytiotrophoblasts and chorionic endothelia and GLUT1 showed a similar location but was absent in cytotrophoblasts. The presence of GLUT1 (55 kDa) and GLUT3 (60 kDa) was confirmed in both uterine and placental tissues through immunoblotting. When the expression of both GLUT1 and GLUT3 were analysed as a whole in the total of the pregnancy period, no significant differences in the relative content of both GLUTs were observed between pregnant and non-pregnant queens. However, when GLUTs expression was analysed in a time-period basis and related with progesterone levels, results were different. Thus, whereas the relative content of GLUT1 showed no correlation with serum progesterone levels, a significant ($P < 0.05$) and negative correlation was found between the relative GLUT3-content in the uterus on days 30 and 40 of pregnancy as well as in the placental transference zone on day 30 and serum progesterone levels. In summary, our results indicate that whereas GLUT1 could be considered as a basal, constant sugar intake system for the whole of pregnancy in queens, GLUT3 is specially required for optimizing glucose uptake during the first half of pregnancy in this species through a progesterone-related mechanism.

1. Introduction

Glucose metabolism is vital to prepare uterine epithelium and stroma for embryo implantation and for the differentiation of the *functionalis* layer to support the developing conceptus [1–4]. Due to its polar nature and hydrophilic condition, glucose is not able to pass through the lipid bilayer and has to be transported via specific integral proteins to reach the cytoplasm [5]. These carrier proteins are known as glucose transporters and are divided into two groups. The first group is formed by the Na^+/Glucose co-transporters protein family (SGLTs) and the second group consist of the members of GLUT proteins family, which act through facilitated diffusion [5].

Focusing on GLUT proteins (GLUTs) family, they are molecules composed by about 500 aminoacidic residues and are classified according to the transported hexose [6]. Thus far, a total of 14 GLUTs have been described in the literature. However, only few have been determined to be present in the female reproductive tract; namely GLUT1, 3, 4 and 8 [1–3,7–11]; and placental structures; namely GLUT1, 3, 4, 8 and 12 [12–19]. To date, GLUT1 and 3 are the most described facilitative glucose transporters in both male and female reproductive tracts of several species.

GLUT1 is the most ubiquitous facilitating glucose transporter

* Corresponding author.
E-mail address: mariamontserrat.rivera@uab.cat (M.M. Rivera del Álamo).

https://doi.org/10.1016/j.theriogenology.2018.08.002
0093-691X/

[20] and is responsible for the glucose basal uptake and storage in all eukaryotic cells [21]. It has been described to be present in rat and human endometrium [1,22] and placenta in humans, marmoset monkeys, rats [12], cats, minks and dogs [18]. On the other hand, GLUT3 shows a high affinity for glucose and a much lower affinity for other monosaccharides, such as mannose, xylose or galactose [23]. The GLUT3 transporter is very abundant in tissues that present an intensive glucose metabolism, such as brain, testis and placenta [24–27].

Previous studies have demonstrated that steroid hormones are involved in the regulation of glucose metabolism via modulating the expression of GLUTs. In effect, progesterone up-regulates the expression of GLUT1 in human and murine endometrium [1,4], and down-regulates that of GLUT3 in rat uterus and mouse placenta and decidua [7,22]. This relationship between serum progesterone levels and two of the most important hexose transporters highlights the importance that a fine regulation of glucose uptake and, hence utilization, has for the proper maintenance of pregnancy. Nonetheless, pregnancy presents many differences between species in aspects as relevant as length, number of foetuses and type of placentation [28]. These differences could underlie species-specific adaptations in mechanisms such as the aforementioned progesterone-related control of hexoses during pregnancy. In this regard, it is reasonable to hypothesise that the described relationship between progesterone levels and GLUT1 and GLUT3 expression during pregnancy in mice and humans [1,4,7,22] could not be the same in other species with very different pregnancy conditions.

Taking into account the possibility of the existence for species-specific differences in the relationship of serum progesterone levels and GLUT1/GLUT3 expression during pregnancy, the main aim of this work was to study that link in queens. Two reasons explain the election of this species. The first reason is the practically total absence of data regarding hexose utilization control during pregnancy in this species. The second reason is the specific features of queen gestation, which strongly differ from those of species in which the relationship progesterone/GLUT1-GLUT3 has been established. Therefore, the results obtained in this study are likely to provide new insights into the species-related differences in the involvement and control of both GLUT1 and GLUT3 during mammalian pregnancy and foetal development.

2. Materials and methods

2.1. Samples collection and processing

Seventy-five queens belonging to a program of sterilization of stray-cats at the Universitat Autònoma de Barcelona (Spain) were initially included in the present study. For inclusion, negative tests to feline leukemia (FeLV) and feline immunodeficiency (FIV), and no sign of illness were mandatory parameters. Sexual stage of the queens was not considered relevant for the inclusion or exclusion of the females in the study.

First of all, queens were pre-medicated with ketamine (5 mg/kg, Imalgene 1000, Laboratorios Merial, Barcelona, Spain), buprehorphine (20 μg/kg, Buprecare; Divasa-Farmavic S.A, Gurb, Spain) and midazolam (0.2 mg/kg, Midazolam Normon; Laboratorios Normon S.A., Madrid, Spain) IM. Anaesthesia was performed with IV propofol (4 mg/kg, Vetofol; Laboratorios Esteve, Barcelona, Spain) and anaesthetic status was maintained with 1.5–2% isoflorane (Isoflo; Laboratorios Esteve, Barcelona, Spain). When sedated, a blood sample from the jugular vein was obtained from each queen to determine serum progesterone concentration.

Routine ovariohysterectomy was performed by midline laparotomy. Once removed, uteri were macroscopically examined and classified as macroscopically pregnant (n = 27) and non-macroscopically non-pregnant (n = 48). When macroscopically pregnant, queens were divided according to their gestational age [29,30] into 30 (D_{30}), 40 (D_{40}), 50 (D_{50}) and 60 (D_{60}) days of pregnancy. According to the diameter of embryo vesicles, nine queens were on D_{30} of pregnancy, but one was excluded from the study because serum progesterone concentration was of 1.68 ng/ml. Progesterone values on days 25–50 of pregnancy range from 15 to 30 ng/mL [31]. Thus, such a low concentration of progesterone may indicate a forthcoming foetal loss and the queen was then consequently removed from the study. Five queens were on D_{40} of pregnancy and four were on D_{50}. Finally, although nine cat females were on D_{60} of pregnancy, the blood from one queen and the uterine sample from another were lost. Consequently, these two queens were excluded from the study.

Regarding to non-macroscopically pregnant queens, serum progesterone was determined to discriminate between non-pregnant queens and those that could be in early pregnancy. Thus, queens with serum progesterone levels ≥1 ng/ml were excluded from the study, following the criteria established by Tsutsui and Stabenfeldt [31]. Moreover, post-partum queens were also removed from the study. Therefore, the final study included a total of 31 queens: 7 non-pregnant queens, 8 queens at D_{30} of pregnancy, 5 queens at D_{40} of pregnancy, 4 queens at D_{50} of pregnancy and 7 queens at D_{60} of pregnancy.

Uterine full-thickness biopsies were obtained from each queen. From pregnant queens, biopsies of the placental transference zone were also collected. Biopsies were split into two samples. One was kept frozen at −80 °C for Western blotting analyses, whereas the other was fixed in 10% paraformaldehyde during 48 h for immunohistochemistry evaluations. For Western blotting analyses, only the endometrium was used. With this purpose, endometrium was removed from the myometrium with a scalpel and kept frozen until analyses were performed.

The present study only included non-pregnant queens with evidence of lack of ovulation and animals in which pregnancy was positively determined by macroscopic observation of pregnancy inside the uterus. This rationale, which implies dismissing cat females that showed ovulation but did not exhibit macroscopic signs of pregnancy, was based upon the specific characteristics of the feline sexual cycle, which differs from others that have been commonly utilised for placenta studies such as rodents or humans.

Sampling collection was performed under the guidelines of the Ethical Committee Animal Care and Research, Autonomous University of Barcelona (CEEAH, code 2939).

2.2. Progesterone concentration

Blood samples from the jugular vein were allowed to clot at room temperature in glass tubes and centrifuged at 2500 × *g* for 5 min. Serum was then collected and kept frozen in Eppendorf tubes at −20 °C until progesterone analysis was performed (Immulite® 1000; Siemens Healthcare Diagnostics, Cornellà del Llobregat, Spain).

2.3. Immunohistochemistry

Biopsy samples were embedded in paraffin blocks prior to 4 μm-thick sections were obtained. For dewaxing, sections were immersed two times in xylene for 5 min. After that, sections were hydrated by immersing twice for 5 min in a descending concentration of alcohols (100%, 96% and 70% respectively) and washed twice in PBS (1x) for 5 min with slight agitation. This process was performed at room temperature.

For antigen retrieval, sections were plunged into citrate buffer (10 mM sodium citrate, 0.05% Tween20, pH = 6.0) for 20 min at

96 °C and rinsed twice in PBS for 5 min. Sections were subsequently immersed for 1 h in 2% BSA at room temperature for blocking unspecific antibody reactions. After blocking, samples were incubated with the corresponding primary antibody overnight at 4 °C at a dilution factor of 1:100 (v/v). Afterwards, sections were washed six times of 10 min each in PBS and then incubated with a secondary anti-mouse Alexa Fluor 568-linked antibody at 4 °C for 3 h at 1:50 dilution (v/v). Nuclear staining was performed with Hoescht-33342 (Thermo Fisher scientific Inc. Waltham, MA, USA) at room temperature for 20 min at 1:1000 dilution (v/v). Biopsies of mice liver were used as a positive control for both GLUT1 and GLUT3. On the other side, for negative controls, sections of both queen uterus and placental transference zone were only incubated with the secondary antibody in order to detect non-specific bindings. Tissue samples were mounted using Vectashield antifading mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA) and sealed with nail polish. A confocal laser scanning microscope (Leica TCS SP5) equipped with a HyD detector (Leica Microsystems GmbH, Mannheim, Germany) was used to evaluate the proteins of interest (Alexa Fluor® 568; excitation 568 nm) and nucleus (Hoechst; excitation 405 nm).

2.4. Western blotting

Samples were homogenized in 1 mL of cold protein extraction buffer (50 mM TRIS HCl, 1 mM EDTA, 10 mM EGTA, 25 mM DTT, 1.5% Triton-X-100, 1 mM PMSF, 10 μg/mL Leupeptine, 1 mM Ortovanadate and 1 mM Benzamidine; pH = 7.4) with an Ultra-Turmax T25 basic homogenizer (IKA-WERKE, Staufen, Germany). Supernatants were collected after centrifuging the samples at 4 °C and 12,000 × *g* for 10 min and total protein concentration was determined by the Bradford technique [32].

Separation of proteins was performed by SDS-PAGE electrophoresis in 10% (w/v) acrylamide separating gels [33]. After that, proteins were transferred into nitrocellulose membranes and Ponceau S dye at 0.1% (w/v) was used to confirm that the transference had been successful. To avoid unspecific unions, nitrocellulose membranes were blocked in 2% BSA (w:v) for 1 h.

Membranes were incubated with primary antibodies at 4 °C overnight. Following this, membranes were washed in a TBST solution (Tris buffered saline, 0.1% Tween20) three times for 5 min to remove the excess of primary antibody. After washing, membranes were exposed to an anti-rabbit secondary antibody (Santa Cruz Biotechnology Inc., 10410 Finnell Street, Dallas, Texas, USA) for 45 min at room temperature and at 1:1000 (v:v). Membranes were washed again six times of 10 min each with TBST and then incubated with Inmobilon TM Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA, USA) for 5 min. Membranes were subsequently exposed to radiograph films to reveal GLUT1 and GLUT3 bands (50–60 KDa).

After evaluating the expression of GLUT1 and GLUT3, membranes were immersed in stripping buffer (10 g glycine, 1 g SDS, 10 ml Tween 20, pH 2.2, in 1 L of pure water) at room temperature for removal of specific GLUT-markings. Membranes were then reprobed with an anti-mouse α-tubulin antibody at 1:1000 dilution (ABR Affinity BioReagents, CO, USA) to verify that the same amount of protein for each sample was loaded.

Specific GLUT1, GLUT3 and α-tubulin bands densities were analysed with a computer-assisted image analysis system (Multi Gauge v 3.0 software system, Fujifilm, Tokyo, Japan) and ratios between densities of each GLUT band and its corresponding α-tubulin band were calculated.

2.5. Antibodies

For GLUT1 and GLUT3 detection, anti-GLUT1 and anti-GLUT3 polyclonal primary anti-rabbit antibodies (Sigma-Aldrich, Saint Louis, MO, USA) at 1:1000 (v:v) dilution were used.

2.6. Statistical analysis

Analysis of results was conducted with a statistical package (SPSS for Windows, Ver. 21.0; Chicago, IL, USA). Data were checked through normality (Shapiro-Wilk test) and homogeneity of variances (Levene test). As data were not distributed normally, they were transformed through arcsin $\sqrt{x}$. However, this transformation did not correct the distribution and non-parametric tests were used as an alternative to ANOVA. Therefore, Kruskal-Wallis test was used as a non-parametric ANOVA and this was followed by Mann-Whitney test for pair-wise comparisons. Dependent variables were the relative abundances (i.e. normalised against α-tubulin) of GLUT1 and GLUT3.

Spearman correlations were calculated between P4 levels and relative abundances of GLUT1 and GLUT3. The level of significance was set at $P < 0.05$.

3. Results

3.1. Inmunohistochemistry analyses

Immunohistochemistry localizations for GLUT1 in the uterus and placental transference zone are summarised in Table 1, whereas those of GLUT3 analyses are summarised in Table 2.

Focusing on GLUT1 localisation, this protein was detected in the plasma membrane and cytoplasm of luminal and glandular uterine epithelia, myometrium and endometrial endothelia (Fig. 1C). No changes in the uterine localisation of GLUT1 were observed between pregnant and non-pregnant queens, nor between gestational phases. Regarding to placental transference zone, GLUT1 was present in syncytiotrophoblasts and endothelium in all the evaluated gestational phases (Fig. 1D), but was absent from cytotrophoblasts.

In a similar fashion to GLUT1, GLUT3 was found in the plasma membrane and cytoplasm of luminal and glandular uterine epithelia, as well as in the myometrium of both non-pregnant and pregnant queens in all gestational phases (Figs. 1 and 2

Table 1
Expression of GLUT1 in uterine and placental transference zone samples. **NP**: non-pregnant queens; **D_{30}**: day 30 of pregnancy; **D_{40}**: day 40 of pregnancy; **D_{50}**: day 50 of pregnancy; **D_{60}**: day 60 of pregnancy. Presence or absence of expression in the different evaluated structures is indicated with + or − respectively.

	Uterus			Placental Transference zone		
	Epithelium	Myometrium	Endothelium	Syncytiotrophoblasts	Cytotrophoblasts	Endothelia
NP	+	+	+			
D_{30}	+	+	+	+	−	+
D_{40}	+	+	+	+	−	+
D_{50}	+	+	+	+	−	+
D_{60}	+	+	+	+	−	+

Table 2
Expression of GLUT3 in uterine and placental transference zone samples. **NP**: non-pregnant queens; **D_{30}**: day 30 of pregnancy; **D_{40}**: day 40 of pregnancy; **D_{50}**: day 50 of pregnancy; **D_{60}**: day 60 of pregnancy. Presence or absence of expression in the different evaluated structures is indicated with + or − respectively.

	Uterus			Placental Transference zone		
	Epithelium	Myometrium	Endothelium	Syncytiotrophoblasts	Cytotrophoblasts	Endothelia
NP	+	+	−			
D_{30}	+	+	+	+	+	+
D_{40}	+	+	+	+	+	+
D_{50}	+	+	+	+	+	+
D_{60}	+	+	+	+	+	+

respectively). By contrast, expression of GLUT3 in the endometrial endothelium was restricted to pregnant queens, regardless of their gestational age (Fig. 2C). Finally, as far as placental transference zone is concerned, GLUT3 was present in syncytiotrophoblasts, cytotrophoblasts and endothelium (Fig. 2E).

3.2. Determination of serum progesterone levels and relative expression of GLUT1 and GLUT3

Mean serum progesterone concentration for pregnant queens was 7.81 ± 5.36, 8.61 ± 3.88, 6.61 ± 4.37 and 5.10 ± 2.66 ng/ml

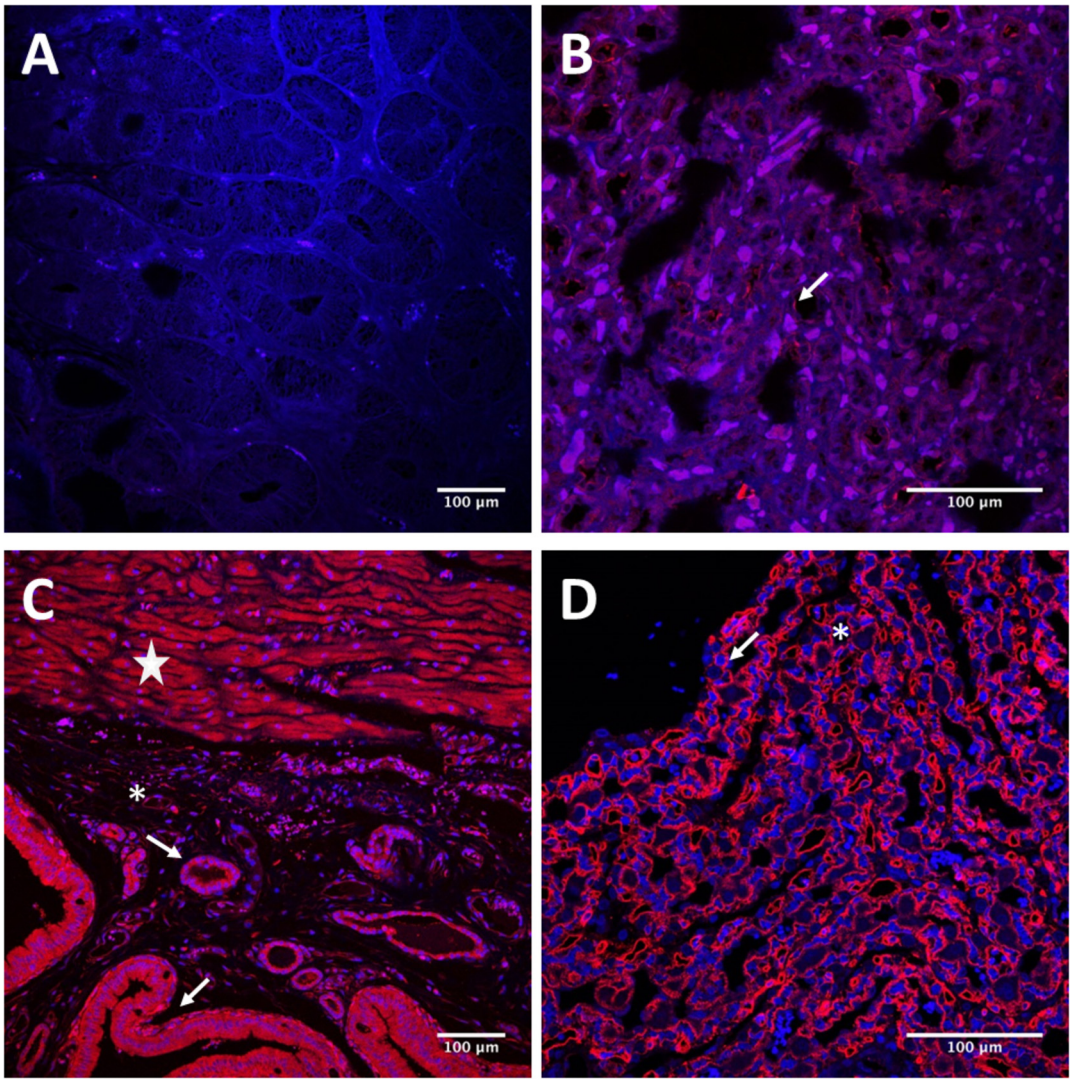

Fig. 1. Immunohistochemistry images for GLUT1 expression. Cellular nuclei are blue-stained with DAPI dye, while GLUT1 is red-stained. **A**: negative control in a kidney sample from a rat; **B**: positive control in a kidney from a rat. Note the red mark for GLUT1 around the collecting ducts membrane of the kidney (white arrow); **C**: uterine sample. Note the red mark in the cellular membrane and cytoplasm in cells from luminal and glandular epithelia (arrows), myometrium (big asterisk) and endometrial endothelia (small asterisk); **D**: sample from transference zone. Note the red mark in the apical membrane of the syncytiotrophoblasts (arrow) and endothelia (asterisk).

(mean ± standard deviation, SD for D_{30}, D_{40}, D_{50} and D_{60}, respectively (Table 3). As expected, non-pregnant queens included in the study showed a serum concentration for progesterone below 1 ng/ml (Table 3).

Regarding the relative expression of both GLUT1 and GLUT3 evaluated through immunoblotting, samples from both uterus and placental transference zone showed specific bands of 55 kDa and 60 kDa, corresponding to GLUT1 (Fig. 3-A) and GLUT3 respectively (Fig. 3-B). When the relative content of GLUT1 and GLUT3 (i.e. normalised against α-tubulin) was compared, no significant differences between pregnant and non-pregnant queens were observed either when the gestation day or when all the gestational time as a whole were considered (Fig. 3-C and 3-D).

3.3. Correlations between serum progesterone levels and relative GLUT1 and GLUT3 content

There was no correlation between serum progesterone levels and the relative content of GLUT1 in any of the evaluated experimental points (Table 4). In contrast, a negative, significant ($P < 0.05$) correlation was observed between serum progesterone levels and relative content of GLUT3 in both transference zone and uterus samples from pregnant queens, with r values of −0.557 and −0.521, respectively (Table 4).

When pregnancy day was set as the reference point for correlation analyses, there was no correlation between serum progesterone levels and relative GLUT1 content on any pregnancy day, either in uterus or in placental transference zone (Table 5). On the contrary, there was a significant ($P < 0.05$), negative correlation between the relative GLUT3 content and serum progesterone levels in uterine samples on D_{30} and D_{40} and in samples from placental transference zone on D_{30} (Table 6).

4. Discussion

The results shown in this manuscript are the first reference about the concomitant presence of both GLUT1 and GLUT3 hexose transporters in both uterine and placental-transference tissues of queens during pregnancy, since GLUT1, but not GLUT3, has been already described in cats [18]. More importantly, our results also indicate the existence of a clear relationship between serum progesterone levels and GLUT3 expression, but not GLUT1, in both uterus and placental-transference area during the first half of pregnancy. These results match with previous reports showing the presence of GLUT1 in the uterus and placental transference zone in other mammalian species, such as rodents, human, minks, dog and sheep [16,18,22,34]. Likewise, a relationship between maternal serum progesterone levels and the utero-placental expression of GLUT1 and GLUT3 has also been found in rodents and humans [4,7,16,22]. However, there are striking differences between our study and these previous reports in other species. Centring on uterine samples, our results indicate that while the presence of GLUT3 in the endothelium, but not that of GLUT1, is related with the start of pregnancy, the expression of both GLUTs in other uterine locations does not rely upon pregnancy onset. Although GLUT3 is more specific for glucose transport than GLUT1, the affinity of both transporters for glucose is practically the same [34,35]. Thus, the modulation of the precise rhythm of glucose transport in the utero-placental system during early pregnancy would not require the presence of two glucose transporters with similar efficiency. This would imply that GLUT3 could play other roles than being just a reinforcement for glucose transport during the early stages of pregnancy in queens. Placental function and foetal development depend on maternal endometrial vascular remodelling [36,37]. Thus, the implementation of an optimal endometrial vascularization plays an important role in embryo implantation [38]. At this respect, the pregnancy-related presence of GLUT3 in the endometrial endothelium suggests that this hexose transporter might be involved in this remodelling process. Meanwhile, there are other putative roles for feline uterine/placental GLUT3 in the early stages of pregnancy than acting on remodelling of endometrial vascularization. On this respect, a relationship between GLUT3 expression in human trophoblast and the activity of mTORC1 protein has been described [39]. This is important, since mTORC1 is involved in a transduction signalling pathway sensing nutrients in the placenta and regulating protein synthesis in embryo and foetus [40]. Thus, a progesterone-related modulation of GLUT3 in the early phases of pregnancy in queens could be also related with the control of embryo/early foetus through a GLUT3/mTORC1-regulated pathway. Therefore, the progesterone-linked regulation of GLUT3 endometrial expression in the early phases of gestation would suggest that this specific hexose transporter is also involved in the implantation of feline conceptus.

In this sense, feline females are coitus-induced ovulators and regardless they get pregnant or not, serum progesterone levels increase over 1.5 ng/ml once ovulation has occurred [42]. On the other hand, queen embryos reach the uterus on days 6–8.5 after coitus [29]. Due to their small size, embryos are difficult to detect at naked-eye at that stage, meaning that queens with serum progesterone concentrations higher than 1.5 ng/ml could be either pregnant or pseudopregnant. For this reason, only queens with apparent pregnancy and non-pregnant queens with serum progesterone concentration below 1 ng/ml were included in the current study.

The lack of correlation between the relative content of GLUT1 with serum progesterone concentration are in disagreement with those observed in other species, such as rats [7] and humans [2]. Indeed, those studies in rats and humans observed a significant increase in GLUT1 expression during foetal development and a positive correlation with serum progesterone levels [2,7]. A possible explanation for those differences could be that while mice and humans present a haemochorial placenta, queens, as indicated above, present an endotheliochorial placenta type. The most important difference between both placenta types is that haemochorial placenta has greater invasiveness and permeability [28]. This would imply that the diffusion of molecules, especially those with low molecular weight such as glucose, would need stricter control to avoid unlimited diffusion through the utero-placental wall. In this way, stricter control of trans-placental diffusion of glucose would be exerted by a closer regulation of the main hexose transporter as GLUT1 is. Thus, the presence of different types of placenta with different degrees of invasiveness could explain the different mechanisms of regulation of hexose transport during pregnancy. In fact, the same hypothesis can be raised to explain other discrepancies between the results of our study and the other previous ones. For instance, it has been described that GLUT3 is present in placental layers of mice and humans only at the first stages of pregnancy [15,19,23,43]; however, our study found that GLUT3 was present in the queen's placenta throughout the whole pregnancy. In addition, non-pregnant queens with low levels of progesterone and pregnant queens at D_{60} express more GLUT3 in both endometrium and placental transference zone, which, again, is in contrast with the results reported in the human and mouse [15,19,23,43]. The greater permeability of rodent and human placentas when comparing with that of the queen would be again of high relevance to explain our results, as transport of solutes from maternal circulation to the foetus takes place until delivery in the case of endotheliochorial placenta. These differences, of course, do not dismiss the existence of other species-specific factors that could contribute to explain the species-linked differences in the utero-

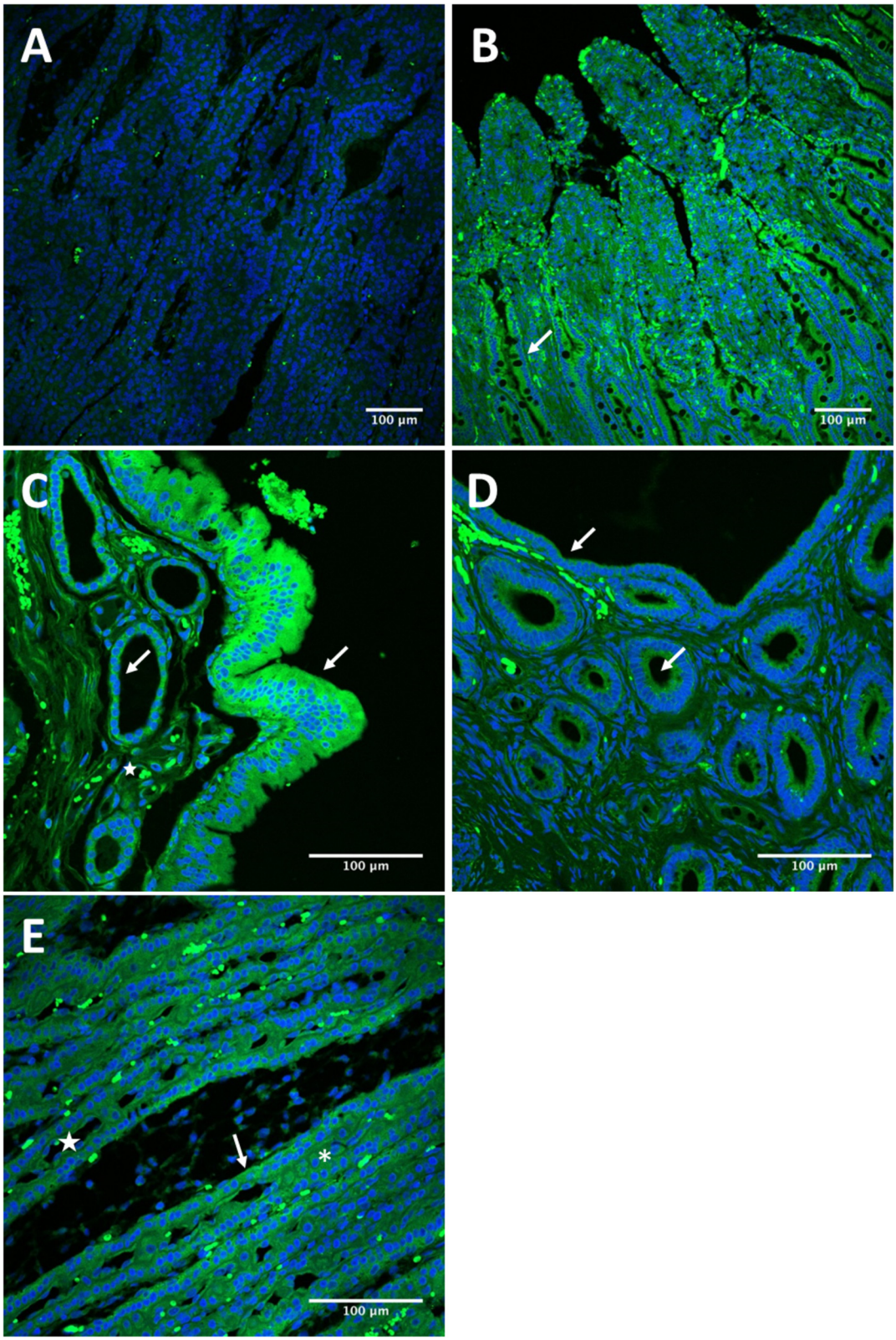

Fig. 2. Immunohistochemistry images for GLUT3 expression. Cellular nuclei are blue-stained with DAPI dye, while GLUT3 is green-stained. **A**: negative control in a placenta sample; **B**: positive control in a small intestine sample from a rat. Note the green mark for GLUT3 around the collecting ducts membrane of the kidney (white arrow); **C**: uterine sample from a pregnant queen. Note the green mark in the cellular membrane and cytoplasm in cells from luminal and glandular epithelia (arrows) and endometrial endothelia (asterisk); **D**: uterine sample from a non-pregnant queen. Note the green mark in the cellular membrane and cytoplasm in cells from luminal and glandular epithelia (arrows). **E**: sample from transference zone. Note the red mark in the apical membrane of syncytiotrophoblasts (arrow), cytotrophoblasts (small asterisk) and endothelia (big asterisk).

Table 3
Serum progesterone concentration and relative abundance of GLUT1 and GLUT3 calculated by means of the Multi Gauge v3.0 software system in the uterus and the transference zone of queens at different stages of the sexual cycle and different gestational ages. Statistically significant differences ($P < 0.05$) among the different stages are expressed with different superscripts. Values are expressed as mean ± standard error of the mean (SEM). **NP**: non-pregnant queens (n = 7); **D_{30}**: pregnant queens on day 30 of pregnancy (n = 8); **D_{40}**: pregnant queens on day 40 of pregnancy (n = 5); **D_{50}**: pregnant queens on day 50 of pregnancy (n = 7); **D_{60}**: pregnant queens on day 60 of pregnancy (n = 7). Statistically significant differences at different sexual and pregnancy stages for each AQP are indicated with different superscripts.

Stage	Progesterone (ng/ml)	GLUT1 (arbitrary units)		GLUT3 (arbitray units)	
		Uterus	Transference zone	Uterus	Transference zone
NP	0.26 ± 0.05^{a}	0.98 ± 0.17	–	0.74 ± 0.07	–
D_{30}	7.81 ± 5.36^{b}	1.24 ± 0.11	1.23 ± 0.10	0.57 ± 0.16	0.55 ± 0.16
D_{40}	8.61 ± 3.88^{b}	1.22 ± 0.11	0.90 ± 0.23	0.81 ± 0.10	0.89 ± 0.06
D_{50}	6.61 ± 4.37^{b}	1.10 ± 0.06	1.09 ± 0.06	0.77 ± 0.16	0.71 ± 0.14
D_{60}	5.10 ± 2.66^{b}	1.23 ± 0.12	1.12 ± 0.06	0.73 ± 0.14	0.68 ± 0.15

placental GLUTs expression during pregnancy. Thus, further research is warranted to better elucidate this point.

Centring on the precise location of both GLUT1 and GLUT3 in the chorionic structures, both transporters have previously been described to be present in the haemochorial-type placenta [12,25–27]. In the present study, GLUT1 was expressed in syncytiotrophoblasts and chorionic endothelium, whereas GLUT3 was present in syncytiotrophoblasts, cytotrophoblasts and chorionic endothelium, the current study being the first one to report the expression of GLUT3 in a carnivore placenta. These results are partially in disagreement with previous studies that showed the presence of GLUT1, but not that of GLUT3, in the carnivore placenta [18]. This discrepancy could, however, have a technical origin. Thus, whereas the anti-GLUT3 antibody utilised by Wooding et al. [18] was not specifically directed against carnivore GLUT3, the antibody utilised in our study did. Thus, a difference in the sensitivity of the utilised antibodies in both studies could be one of the factors involved in the existence of the observed discrepancies.

Assuming thus a positive signal for both GLUT1 and GLUT3 in our conditions, a more detailed explanation of the results observed through immunocytochemistry is needed. In this way, it must be reminded that syncytiotrophoblasts compose the outer layer of the trophoblast, are in direct contact with maternal endometrium and are responsible for the biomolecular interactions between the mother and the foetus [44]. The presence of both GLUT1 and GLUT3 in these specific trophoblastic cells strongly suggests that these hexose transporters are involved in the implantation of queen embryos. On the other hand, cytotrophoblasts are stem cells that compose the inner layer of the trophoblast and that replicate and differentiate into syncytiotrophoblasts [44]. Besides this, our results also showed that vascular endothelia only expressed GLUT3 in the chorionic membrane of pregnant queens but not in the endometria of pregnant and non-pregnant queens. These results agree with those of Brown et al. [19], who described that GLUT3 was present in the vascular endothelia of human placenta from the first trimester of pregnancy on. Thus, the presence of GLUT3 in both syncythiotrophoblasts and chorionic cells would reinforce the above formulated hypothesis focused in the existence of some relationship between GLUT3 and the previously described mTORC1-related pathways involving embryo development and early foetus growth during at least the cytotrophoblastic-linked embryonic and early foetal development [40]. Furthermore, the

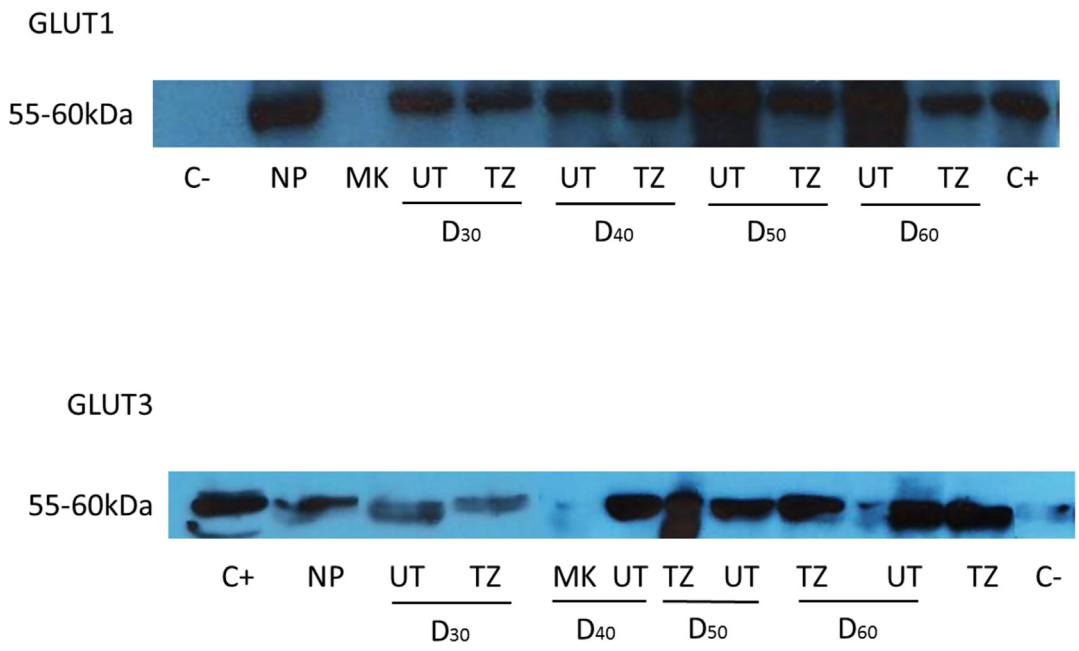

Fig. 3. A-B Western blotting expression for GLUT1 (A) and GLUT3 (B). Radiograph films show 55–60 kDa bands compatible with GLUTs. Note that the expression is present in all the evaluated groups. **NP**: non-pregnant queens; **UT**: uterus; **TZ**: transference zone; **D_{30}**: 30 days of pregnancy; **D_{40}**: 40 days of pregnancy; **D_{50}**: 50 days of pregnancy; **D_{60}**: 60 days of pregnancy. **C-D** Relative expression of GLUT1 and GLUT3 (immunoblotting) in the uterus (C) and the transference zone (D) of pregnant queens throughout different gestational ages. **UT**: uterus; **TZ**: transference zone; **D_{30}**: 30 days of pregnancy (n = 8); **D_{40}**: 40 days of pregnancy (n = 5); **D_{50}**: 50 days of pregnancy (n = 4); **D_{60}**: 60 days of pregnancy (n = 7). Values are expressed as mean ± standard error of the mean (SEM).

Table 4
Overall correlations of P_4 levels with relative (i.e. normalised against α-tubulin) content of GLUT1 and GLUT3. UT-NP: uterine samples from non-pregnant queens; **UT-P**: uterine samples from pregnant queens; **TZ**: samples from placental transference zone. Statistically significant correlations between P_4 and the corresponding GLUT are marked with an "*".

	Relative GLUT1-content			Relative GLUT3-content		
	UT-NP	UT-P	TZ	UT-NP	UT-P	TZ
Spearman Correlation coefficient	0.201	−0.020	−0.290	−0.020	−0.521*	−0.557*
P-value	0.702	0.935	0.905	0.965	0.032	0.020

Table 5
Correlations of P_4 levels with relative (i.e. normalised against α-tubulin) content of GLUT1 throughout pregnancy. **UT-P**: uterine samples from pregnant queens; **TZ**: samples from placental transference zone. Statistically significant correlations between P_4 and the corresponding GLUT are marked with an "*".

Relative GLUT-1 content								
	UT-P				TZ			
	D_{30}	D_{40}	D_{50}	D_{60}	D_{30}	D_{40}	D_{50}	D_{60}
Spearman Corr coeff	−0.068	−0.079	0.382	0.007	−0.174	−0.543	0.248	0.093
P-value	0.913	0.921	0.750	0.987	0.779	0.634	0.840	0.827

Table 6
Correlations of P_4 levels with relative (i.e. normalised against α-tubulin) content of GLUT3 throughout pregnancy. **UT-P**: uterine samples from pregnant queens; **TZ**: samples from placental transference zone. Statistically significant correlations between P_4 and the corresponding GLUT are marked with an "*".

Relative GLUT-3 content								
	UT-P				TZ			
	D_{30}	D_{40}	D_{50}	D_{60}	D_{30}	D_{40}	D_{50}	D_{60}
Spearman Corr coeff	−0.981*	−0.963*	−0.840	−0.202	−0.977*	−0.174	−0.667	−0.565
P-value	0.019	0.037	0.365	0.701	0.023	0.826	0.535	0.243

establishment of a feasible foetal-maternal circulation is mandatory for foetal development [45]. In this sense, cytotrophoblasts play a vital role in the establishment of foetal-maternal circulation [46]. Taking this into account, the presence of a complete array of glucose transporters with similar affinity characteristics in the chorionic vascularization related with both syncythiotrophoblast and chorionic cells suggests the existence of a strict regulation of the hexoses transport during the whole process of queen placenta development.

In conclusion, the present results suggest that, whereas GLUT1 could play a structural role in the control of hexoses uptake in both uterus ad placenta, GLUT3 could play a more dynamic role. That role would not only be restricted to the role of hexose transport and would depend on the exact physiological situation of the uterine and/or placental tissues before and during pregnancy. At this respect, it is interesting to remind that while GLUT1 is a ubiquitous protein without practically no specificity for any hexose, GLUT3, instead, has a strong tissue-dependent expression and an almost total specificity for glucose, dismissing any other monosaccharide [23]. These differences could be in the basis of the observed separate evolution of the expression pattern of both GLUT1, with no changes during all of the analysed period, and GLUT3, with a progesterone-related dynamics. Related to this, further research is needed to stablish the actual role of these hexose transporters during the sexual cycle and pregnancy.

References

[1] Von Wolff M, Ursel S, Hahn U, Steldinger R, Strowitzki T. Glucose transporter proteins (GLUT) in human endometrium: expression, regulation, and function throughout the menstrual cycle and in early pregnancy. J Clin Endocrinol Metab 2003;88:3885–92.

[2] Frolova A, Flessner L, Chi M, Kim ST, Foyouzi-Yousefi N, Moley KH. Facilitative glucose transporter type 1 is differentially regulated by progesterone and estrogen in murine and human endometrial stromal cells. Endocrinology 2009;150:1512–20.

[3] Kim ST, Moley KH. Regulation of facilitative glucose transporters and AKT/MAPK/PRKAA signaling via estradiol and progesterone in the mouse uterine epithelium. Biol Reprod 2009;81:188–98.

[4] Frolova AI, Moley KH. Glucose transporters in the uterus: an analysis of tissue distribution and proposed physiological roles. Reproduction 2011;142:211–20.

[5] Bell GI, Kayano T, Buse JB, Burant CF, Takeda J, Lin D, Fukumoto H, Seino S. Molecular biology of mammalian glucose transporters. Diabetes Care 1990;13:198–208.

[6] Joost HG, Thorens B. The extended GLUT-family of sugar/polyol transport facilitators: nomenclature, sequence characteristics, and potential function of its novel members (review). Mol Membr Biol n.d.;18:247–256.

[7] Yamaguchi M, Sakata M, Ogura K, Miyake A. Gestational changes of glucose transporter gene expression in the mouse placenta and decidua. J Endocrinol Invest 1996;19:567–9.

[8] Welch RD, Gorski J. Regulation of glucose transporters by estradiol in the immature rat uterus. Endocrinology 1999;140:3602–8.

[9] Mozzanega B, Miori R, Granzotto M, Chiarelli S, Xamin N, Zuliani L, Sicolo N, Marchesoni D, Vettor R. Obesity reduces the expression of GLUT4 in the endometrium of normoinsulinemic women affected by the polycystic ovary syndrome. Ann N Y Acad Sci 2004;1034:364–74.

[10] Korgun ET, Celik-Ozenci C, Seval Y, Desoye G, Demir R. Do glucose transporters have other roles in addition to placental glucose transport during early pregnancy? Histochem Cell Biol 2005;123:621–9.

[11] Kohan K, Carvajal R, Gabler F, Vantman D, Romero C, Vega M. Role of the transcriptional factors FOXO1 and PPARG on gene expression of SLC2A4 in endometrial tissue from women with polycystic ovary syndrome. Reproduction 2010;140:123–31.

[12] Hahn T, Desoye G. Ontogeny of glucose transport systems in the placenta and its progenitor tissues. In: Early Pregnancy, 2; 1996. p. 168–82.

[13] Sciullo E, Cardellini G, Baroni M, Torresi P, Mazziotti F, Pozzilli P, Fallucca F. Glucose transporters (GLUT 1, GLUT 3) mRNA in human placenta of diabetic and non-diabetic pregnancies. Ann Ist Super Sanita 1997;33:361–5.

[14] Xing AY, Challier JC, Lepercq J, Caüzac M, Charron MJ, Girard J, Hauguel-de Mouzon S. Unexpected expression of glucose transporter 4 in villous stromal cells of human placenta. J Clin Endocrinol Metab 1998;83:4097–101.

[15] Hahn D, Blaschitz A, Korgun ET, Lang I, Desoye G, Skofitsch G, Dohr G. From maternal glucose to fetal glycogen: expression of key regulators in the human placenta. Mol Hum Reprod 2001;7:1173–8.

[16] von Wolff M, Ursel S, Hahn U, Steldinger R, Strowitzki T. Glucose transporter proteins (GLUT) in human endometrium: expression, regulation, and function throughout the menstrual cycle and in early pregnancy. J Clin Endocrinol

Metab 2003;88:3885–92.
[17] Gude NM, Stevenson JL, Rogers S, Best JD, Kalionis B, Huisman MA, Erwich JJ, Timmer A, King RG. GLUT12 expression in human placenta in first trimester and term. Placenta 2003;24:566–70.
[18] Wooding FBP, Dantzer VB, Klisch K, Jones CJ, Forhead AJ. Glucose transporter 1 localisation throughout pregnancy in the carnivore placenta: light and electron microscope studies. Placenta 2007;28:453–64. https://doi.org/10.1016/j.placenta.2006.07.006.
[19] Brown K, Heller DS, Zamudio S, Illsley NP. Glucose transporter 3 (GLUT3) protein expression in human placenta across gestation. Placenta 2011;32:1041–9. https://doi.org/10.1016/j.placenta.2011.09.014.
[20] Palfreyman RW, Clark AE, Denton RM, Holman GD, Kozka IJ. Kinetic resolution of the separate GLUT1 and GLUT4 glucose transport activities in 3T3-L1 cells. Biochem J 1992;284(Pt 1):275–82.
[21] Mueckler M. Family of glucose-transporter genes. Implications for glucose homeostasis and diabetes. Diabetes 1990;39:6–11.
[22] Korgun E, Demir R, Hammer A, Dohr G, Desoye G. Glucose Transporter Expression in Rat Embryo and Uterus During Decidualization, 1370; 2001. p. 1364–70.
[23] Simpson IA, Dwyer D, Malide D, Moley KH, Travis A, Vannucci SJ. The facilitative glucose transporter GLUT3: 20 years of distinction. Am J Physiol Endocrinol Metab 2008;295:E242–53.
[24] Maher F, Vannucci S, Takeda J, Simpson IA. Expression of mouse-GLUT3 and human-GLUT3 glucose transporter proteins in brain. Biochem Biophys Res Commun 1992;182:703–11.
[25] Burant CF, Davidson NO. GLUT3 glucose transporter isoform in rat testis: localization, effect of diabetes mellitus, and comparison to human testis. Am J Physiol 1994;267:R1488–95.
[26] Boileau P, Mrejen C, Girard J, Hauguel-de Mouzon S. Overexpression of GLUT3 placental glucose transporter in diabetic rats. J Clin Invest 1995;96:309–17.
[27] Hauguel-de Mouzon S, Challier JC, Kacemi A, Caüzac M, Malek A, Girard J. The GLUT3 glucose transporter isoform is differentially expressed within human placental cell types. J Clin Endocrinol Metab 1997;82:2689–94.
[28] Senger P. Pathways to pregnancy and parturition. Psychiatr Rehabil J 2005;35:381.
[29] Knospe C. Periods and stages of the prenatal development of the domestic cat. Anat Histol Embryol 2002;31:37–51.
[30] Fresno L, Rodriguez-Gil JE, Rigau T, Pastor J, Rivera del Alamo MM. Modulation of the biochemical composition of amniotic and allantoic fluids as a control mechanism of feline foetal development. Placenta 2012;33:522–7. https://doi.org/10.1016/j.placenta.2012.03.002.
[31] Tsutsui T, Stabenfeldt GH. Biology of ovarian cycles, pregnancy and pseudopregnancy in the domestic cat. J Reprod Fertil Suppl 1993;47:29–35.
[32] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[33] Sirois J, Doré M. The late induction of prostaglandin G/H synthase-2 in equine preovulatory follicles supports its role as a determinant of the ovulatory process. Endocrinology 1997;138:4427–34.
[34] Dandrea J, Wilson V, Gopalakrishnan G, Heasman L, Budge H, Symonds ME. Maternal nutritional manipulation of placental growth and glucose transporter 1 (GLUT-1) abundance in sheep. Reproduction 2001;122:793–800.
[35] Carruthers A. Facilitated diffusion of glucose. Physiol Rev 1990;70:1135–76.
[36] Maher F, Vannucci SJ, Simpson IA. Glucose transporter proteins in brain. Faseb J 1994;8:1003–11.
[37] Biberoglu E, Kirbas A, Daglar K, Biberoglu K, Timur H, Demirtas C, et al. Serum angiogenic profile in abnormal placentation. J Matern Neonatal Med 2016;29:3193–7.
[38] Lacko LA, Hurtado R, Hinds S, Poulos MG, Butler JM, Stuhlmann H. Altered feto-placental vascularization, feto-placental malperfusion and fetal growth restriction in mice with Egfl7 loss of function. Development 2017;144:2469–79.
[39] Plaisier M. Decidualisation and angiogenesis. Best Pract Res Clin Obstet Gynaecol 2011;25:259–71.
[40] Xu J, Lu C, Wang J, Zhang R, Qian X, Zhu H. Regulation of human trophoblast GLUT3 glucose transporter by mammalian target of rapamycin signaling. Int J Mol Sci 2015;16:13815–28.
[42] Schmidt PM, Chakraborty PK, Wildt DE. Ovarian activity, circulating hormones and sexual behavior in the cat. II. Relationships during pregnancy, parturition, lactation and the postpartum estrus. Biol Reprod 1983;28:657–71.
[43] Esterman A, Greco MA, Mitani Y, Finlay TH, Ismail-Beigi F, Dancis J. The effect of hypoxia on human trophoblast in culture: morphology, glucose transport and metabolism. Placenta n.d.;18:129–136.
[44] Wang Y, Zhao S. Vascular Biology of the Placenta 2010;2.
[45] Kaufmann P, Mayhew TM, Charnock-Jones DS. Aspects of human fetoplacental vasculogenesis and angiogenesis. II. Changes during normal pregnancy. Placenta 2004;25:114–26. https://doi.org/10.1016/j.placenta.2003.10.009.
[46] Garnier V, Traboulsi W, Salomon A, Brouillet S, Fournier T, Winkler C, et al. PPARγ controls pregnancy outcome through activation of EG-VEGF: new insights into the mechanism of placental development. Am J Physiol Endocrinol Metab 2015;309:E357–69. https://doi.org/10.1152/ajpendo.00093.2015.