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# A high glucose concentration during early stages of in vitro equine embryo development alters expression of genes involved in glucose metabolism

María J. Sánchez-Calabuig[1,2] | Raúl Fernández-González[2] | Meriem Hamdi[2] | Katrien Smits[3] | Angela P. López-Cardona[2,4] | Consuelo Serres[1] | Beatriz Macías-García[5,6] | Alfonso Gutiérrez-Adán[2]

[1]Department of Animal Medicine and Surgery, Faculty of Veterinary Science, University Complutense of Madrid, Madrid, Spain

[2]Department of Animal Reproduction, INIA, Madrid, Spain

[3]Department of Obstetrics, Reproduction and Herd Health, Ghent University, Ghent, Belgium

[4]Grupo de Investigación (GI) – Biogénesis, Universidad de Antioquia, Medellín, Colombia

[5]Animal Medicine Department, Faculty of Veterinary Sciences, University of Extremadura, Cáceres, Spain

[6]Research Group of Intracellular Signalling and Technology of Reproduction (SINTREP), Research Institute of Biotechnology in Livestock and Cynegetic (INBIO G+C), University of Extremadura, Cáceres, Spain

**Correspondence**
Alfonso Gutiérrez-Adán, Department of Animal Reproduction, INIA, Madrid, Spain.
Email: agutierr@inia.es

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## Abstract

**Background:** Equine embryos exhibit an unusual pattern of glucose tolerance in vitro and are currently cultured in hyperglycaemic conditions.

**Objective:** Our main objective was to analyse the effect of different glucose concentrations on in vitro produced equine embryo development and quality.

**Study design:** Experiments comparing in vitro and in vivo produced embryos.

**Methods:** Oocytes (n = 641) were collected from post-mortem ovaries, matured in vitro and fertilised by intracytoplasmic sperm injection (ICSI). Embryo culture was divided from Day 0 to Day 4 and from Day 4 to Day 9 in three groups: 5-10 (5 and 10 mmol/L glucose respectively; n = 87); 5-17 (5 and 17.5 mmol/L; n = 66); and 10-17 (10 and 17.5 mmol/L; n = 117). A control group of 20 in vivo produced blastocysts was included. Cleavage and blastocyst rates were evaluated and embryos were snap-frozen for analysis of the relative mRNA expression of genes related to mitochondrial function, DNA methylation, apoptosis, glucose transport and metabolism.

**Results:** No differences were observed in the cleavage or blastocyst rates among in vitro groups. Under high glucose conditions in vitro (10-17 group), *BAX*/*BCL2* was higher, and *PFKP*, *LDHA* and *COX2* were overexpressed compared to all other groups. The two groups with 5 mmol/L glucose concentration during the first culture stage (5-10 and 5-17) displayed similar patterns which differed to the 10-17 group.

**Main limitations:** Conclusions related to embryo quality are based on gene expression patterns. Transfer of in vitro produced embryos would reveal whether the observed differences improve embryo developmental competence.

**Conclusions:** Five mM glucose during the first days of culture seems to be preferable to avoid over-activation of embryonic glycolytic pathways. Further studies are necessary to determine whether this improves embryo developmental competence.

KEYWORDS

horse, embryo culture, glucose metabolism, gene expression

Beatriz Macías-García and Alfonso Gutiérrez-Adán contributed equally to this study and should be regarded as co-last authors.

## 1 | INTRODUCTION

Equine embryos appear to benefit from high glucose concentrations during in vitro embryo development.[1-3] This is in contrast with the low glucose requirements described in other species, and with the in vivo glucose concentration present in the oviductal fluid of the mare, which has been previously described to range from 0.15 to 0.3 mmol/L.[4] Our group has recently reported this value to be below 1 mmol/L.[5] However, equine embryos are often cultured in DMEM/F12 medium that contains high levels of glucose (17.5 mmol/L), which differs from normal serum glucose concentration in horses (4.2-7.3 mmol/L).[6] Moreover, in cows, sows and humans, the glucose concentrations vary between ~0.05 mmol/L in the oviduct and ~4.5 mmol/L in the uterus.[7,8]

In other species, during early pre-implantation development, embryo metabolism preferentially depends on pyruvate and lactate which are used for adenosine triphosphate (ATP) production by oxidative metabolism, and it is known that high glucose concentrations retard in vitro embryo growth in humans and mice, blocking development at the 2-cell stage or increasing the incidence of apoptosis.[9] Once in the uterus, the embryo switches its metabolism from low to medium glucose use via glycolysis and pentose phosphate pathways. For this reason, many in vitro culture (IVC) systems in different mammalian species are divided into two stages for sequential embryo culture: a low glucose medium to support early embryo development (<1 mmol/L, that mimics the oviduct conditions), and moderate glucose-containing medium to support blastocyst development (~5 mmol/L, mimicking the uterine environment).[10]

While high glucose concentrations have been described to exert a deleterious effect on embryo development and quality in mice, rat and cattle,[11-13] and a glucose concentration of 10 mmol/L appears to be lethal to mice and bovine embryos during IVC,[12,14] a significantly higher number of equine embryos are produced at high glucose concentrations during early development.[15,16] However, IVC of equine embryos during Days 0-5 at a 5 mmol/L followed by 20 mmol/L reduces total blastomere and trophectoderm cell numbers, as well as affecting blastocyst cell allocation to the epiblast or presumptive primitive endoderm when compared to early culture at 0 mmol/L followed by 17 mmol/L.[2] Although metabolic studies have shown high glucose uptake and metabolism in in vivo recovered equine blastocysts,[17,18] there is little information on the effects of excessive glucose during in vitro equine embryo development at the level of gene expression.

Thus, the objective of this work was to assess the effect of glucose concentration in a two-stage IVC system on equine embryo development and the expression of candidate genes related to glucose transport, glucose metabolism, mitochondrial function, DNA methylation and apoptosis in the produced blastocysts. Gene expression data were compared with in vivo equine blastocysts.

## 2 | MATERIALS AND METHODS

Unless stated otherwise, all chemicals were purchased from Sigma Aldrich Quimica.

### 2.1 | Oocyte collection and IVM

Ovaries were collected at a slaughterhouse located 3 hours away from our laboratory. Transport was performed at 15°C within 8 hours of slaughter. Immature cumulus-oocyte complexes (COCs) were obtained by follicular scraping as previously described[19] using TCM199 supplemented with HEPES, 1 mg/mL BSA, 10 μg/mL heparin and 2.2 g/L bicarbonate. The oocytes (n = 641) were collected and maintained in holding medium (HM, TCM199 supplemented with HEPES + 20% fetal calf serum (FCS) + 0.5% gentamycin) for a maximum of 12 hours at room temperature in the dark. Then, oocytes presenting at least three layers of cumulus cells were selected and cultured in groups of 50 COCs in DMEM-F12 supplemented with 17 mmol/L glucose with 10% (v/v) FCS, 0.1 mg/mL epidermal growth factor (EGF), 8 mU/mL FSH and 2.5 μL/mL LH in 100 μL drops (10 oocytes per drop) covered with mineral oil for 26-28 hours at 38.5°C under an atmosphere of 5% $CO_2$ in air with maximum humidity, for in vitro maturation.

### 2.2 | Sperm preparation and ICSI

Frozen semen straws (0.5 mL) from a proven stallion were used. Briefly, a straw was thawed in a water bath at 42°C for 40 seconds. Then, the sample was centrifuged at 300 ***g*** for 5 minutes, the supernatant was discarded and the pellet was resuspended in HM. Sperm were then resuspended in PVP:HM (5:1, v/v); a 20 μL droplet of this suspension was added to the manipulating dish. In the meanwhile, oocytes were denuded by repeated pipetting in hyaluronidase at a concentration of 300 IU/mL using a narrow Pasteur pipette. Intracytoplasmic sperm injection (ICSI) was performed in a 90 mm Petri dish, with the main drop of 50 μL of HM in which micromanipulation took place; when needed, new drops of sperm in PVP:HM were added every 30 minutes. Injections were performed with a PMM-150 FU piezo-impact unit (Prime Tech) using a blunt-ended pipette (inner diameter, 6–7 μm), filled with 1 μL Fluorinert FC-40 (F9755). Individual motile spermatozoa were mechanically immobilised with a pulse of the piezo unit in the middle piece of the flagellum and injected into the oocytes. Oocytes were injected in groups of five, washed twice in HM and twice in culture medium before culture.

### 2.3 | In vitro culture

Presumptive zygotes (n = 270) were randomly distributed to three culture media (DMEM/F12 + 10% FCS) supplemented with different glucose concentrations: 5-10 group (n = 87): 5 mmol/L (D1-D4) and 10 mmol/L (D5-D9); 5-17 group (n = 66): 5 mmol/L (D1-D4) and 17 mmol/L (D5-D9); and 10-17 group (n = 117): 10 mmol/L (D1-D4) and 17 mmol/L (D5-D9) and maintained until Day 9 at 38.5°C under an atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at maximum humidity. Expanded blastocysts (Day 9 after ICSI)

measuring 157-195 μm were snap-frozen in liquid nitrogen and stored at −80°C until use.

### 2.4 | In vivo blastocyst collection

In vivo equine embryos (n = 20) were collected 7 days after insemination by conventional uterine flushing as previously described[20] from mares housed at Ghent University, CENSYRA, Veterinary Faculty of Madrid (Universidad Complutense of Madrid) and Centro de Formación del Medio Rural de Navalmoral de la Mata. Embryos were snap-frozen in liquid nitrogen until use.

### 2.5 | Gene expression analysis

Pools of three blastocysts per experimental group (three replicates per experimental group) were used for RNA extraction. Poly (A) RNA was extracted using the Dynabeads® mRNA DIRECT™ Micro Kit (Ambion®, Thermo Fisher Scientific Inc.) following the manufacturer's instructions.[22] Immediately after RNA extraction, reverse transcription (RT) reaction was carried out using a Moloney murine leukaemia virus (MMLV) Reverse Transcriptase First Strand cDNA Synthesis Kit following the manufacturer's instructions (Epicentre Technologies Corp.) in a total volume of 40 μL, to prime the RT reaction and produce cDNA. Tubes were heated to 70°C for 5 minutes to denature the secondary RNA structure and then the RT mix was completed with the addition of 50 units of reverse transcriptase. Next, the tubes were incubated at 25°C for 10 minutes to promote the annealing of random primers, followed by 60 minutes at 37°C to allow the RT of RNA and finally an incubation for 5 minutes at 85°C to denature the enzyme.

All mRNA transcripts were quantified using quantitative polymerase chain reaction (qPCR) and were run in the following conditions: 95°C for 3 minutes, 35 cycles of 94°C for 15 seconds, 56°C for 30 seconds and 72°C for 15 seconds, followed by a final extension step for 10 seconds and carried out in duplicate for all genes of interest in the Rotor-Gene 6000 Real-Time Cycler TM (Corbett Research, Sydney, Australia) by adding a 2-ml aliquot of each sample (~60 μg/μL) to the PCR mix (GoTaq qPCR Master Mix, Promega Corporation) containing the specific primers selected to amplify the selected genes. Primer sequences and the approximate sizes of the amplified fragments of all transcripts are given in Table S1. The comparative cycle threshold (CT) method was used to quantify expression levels. All primers were designed using Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to span exon-exon boundaries when possible. Primers were previously validated for adequate efficiency, and the specificity of their PCR products was confirmed by electrophoresis on a 2% agarose gel. The PCR conditions were tested to achieve efficiencies close to 1. Each RT-qPCR reaction was performed in a final volume of 20 μL, containing 0.25 mmol/L of forward and reverse primers, 10 μL of GoTaq RT-qPCR Master Mix (Promega) and 2 μL of each cDNA sample using a Rotor-Gene 6000 Real-Time Cycler (Corbett Research) and SYBR Green as double-stranded DNA-specific fluorescent dye. All mRNA transcripts were quantified in duplicate. The comparative quantification cycle (Cq) method was used to quantify expression levels.[22]

Values were normalised to two endogenous controls (*H2AFZ* and *ACTB*) previously tested for stability[23] and efficiency. The expression stabilities were evaluated using the geNorm software for Microsoft, ranking the genes based on the internal control gene stability parameter M. A dilution series with pooled cDNA from all groups of embryos was included for each gene to acquire PCR efficiencies. According to the comparative CT method, the ΔCT value was determined by subtracting the housekeeping mean CT value for each sample from each gene CT value of the sample. The calculation of ΔΔCT involved using the highest treatment ΔCT value, which is the treatment with the lowest target expression, as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in the relative gene expression of the target were determined using the formula $2^{-\Delta\Delta CT}$.

### 2.6 | Data analysis

All statistical tests were performed using SigmaStat (Jandel Scientific). To check normality of distribution, a Shapiro-Wilk test was used and to test equality of variances, a Levene's test was performed. Data for cleavage rates and blastocyst yield showed normal distribution and homogeneous variances, and they were compared using a one-way analysis of variance (ANOVA) followed by a Holm-Sidak post hoc test. Data for relative mRNA abundance of *BAX*, *BCL2*, *LDHA*, *PFKP*, *COX2* and *G6PD* followed normal distribution and had homogeneous variances; they were similarly compared using one-way ANOVA followed by Holm-Sidak post hoc test or Dunn's method. Concerning the *BAX/BCL2* ratio and relative mRNA abundance of *PDK3*, *SCLA3*, *GADPH*, *UQC2 and SCLA1*, the data followed normal distributions but failed the equal variance test, therefore they were compared using the nonparametric Kruskal-Wallis test. P-values were not corrected for multiple comparisons. Values were considered significantly different when the *P*-value was lower than .05.

## 3 | RESULTS

### 3.1 | Effect of glucose during in vitro culture on early embryo development

No differences ($P = .6$) were observed in the cleavage rate between in vitro groups (46.9 ± 14.2, 40 ± 3.5 and 45.8 ± 11.3 in 5-10, 5-17 and 10-17 groups respectively). Similarly, no differences ($P = .9$) were found in the blastocyst rate between groups (20.5 ± 4.1, 24.7 ± 7.9 and 19.8 ± 7.8 in 5-10, 5-17 and 10-17 groups respectively) (Table 1).

**TABLE 1** Effect of glucose supplementation during in vitro culture on embryo development, cleavage and blastocyst yield (n = 631), 5-10: 5 and 10 mmol/L during Days 1-4 and 4-9 respectively; 5-17: 5 and 17.5 mmol/L during Days 1-4 and 4-9 respectively; 10-17: 10 and 17.5 mmol/L during Days 1-4 and 4-9 respectively

| | Oocytes collected n | Viable oocytes n | MII oocytes n (%) | Cleavage n (%) | Blastocyst n (%) |
|---|---|---|---|---|---|
| 5-10 | 219 | 101 | 87 (86.0 ± 12.0) | 47 (46.9 ± 14.2) | 21 (20.5 ± 4.1) |
| 5-17 | 124 | 67 | 66 (99.0 ± 2.0) | 27 (40 ± 3.5) | 17 (24.7 ± 7.9) |
| 10-17 | 298 | 136 | 117 (86 ± 14.0) | 61 (45.8 ± 11.3) | 27 (19.8 ± 7.8) |

## 3.2 | Effect of culture conditions on relative mRNA expression of equine blastocysts

The relative mRNA expression of genes related to apoptosis, mitochondrial function and DNA methylation of in vivo and in vitro produced embryos is shown in Figure 1. *ACTB* gene did not pass the stability and efficiency test thus, *H2AFZ* was the reference gene used for the experiment. Concerning genes related to apoptosis, we observed significantly higher transcript levels of the proapoptotic gene *BAX* in the 5-10 and 10-17 in vitro groups ($P = .001$). Whereas no differences were found in the expression of the anti-apoptotic *BCL2* gene ($P = .2$), we found a higher *BAX/BCL2* ratio in the 10-17 in vitro group ($P = .02$).

The expression of two mitochondria-encoded genes, *COX2* (cytochrome c oxidase subunit II), and *UQC2* (ubiquinol-cytochrome c reductase complex core protein 2), and one nuclear gene related to mitochondrial function *PINK1* (PTEN-induced putative kinase 1) were studied. The expression of *COX2* was lower in in vivo produced embryos compared to the in vitro counterparts, and an increase in the expression of this gene was observed in the 10-17 group ($P < .05$).

The expression of *UQC2* was also downregulated in in vivo produced embryos compared to the rest of the groups ($P = .02$). Interestingly, *PINK1*, a gene involved in the Parkin pathway which is responsible for maintaining mitochondrial integrity and function, was only expressed in in vivo derived blastocysts.

No differences were observed in the expression of mRNA encoding for the methylation gene selected, *DNMT3*, between groups ($P = .09$).

The relative mRNA expression of genes related to glucose transport and glucose metabolism is shown in Figures 2 and 3. Regarding glucose transport, the relative mRNA abundance of *SCL2A1* and *SLC2A3*, which encode for glucose transporters 1 and 3, was similar between groups. A lower expression of mRNA for both transporters was observed in the in vivo group compared to their in vitro counterparts ($P = .02$ and $P = .04$ for *SCL2A1* and *SLC2A3* respectively).

When mRNA expression of the anaerobic glycolytic pathway was studied, a consistent overexpression of *LDHA* was observed in the 10-17 in vitro group compared to the rest of the experimental groups ($P = .01$), while no significant differences were found between the 5-10 group and the in vivo retrieved embryos ($P = .06$). Regarding the aerobic glycolytic pathway, the expression of mRNA

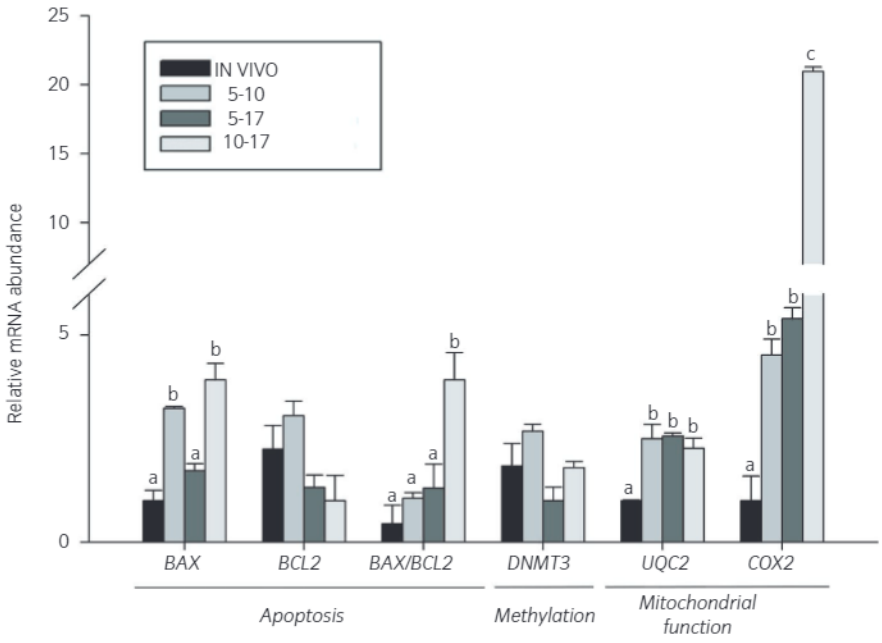


**FIGURE 1** Relative mRNA expression of genes related to apoptosis, methylation and mitochondrial function assessed in Day 7 in vivo embryos and Day 9 in vitro produced blastocysts subjected to different glucose concentrations. Bars represent the relative abundance of the transcripts analysed normalised to the housekeeping gene *H2AFZ*. Results are expressed as means ± SEM. (A) vs (B) indicate significant differences between groups for a given gene ($P < .05$)

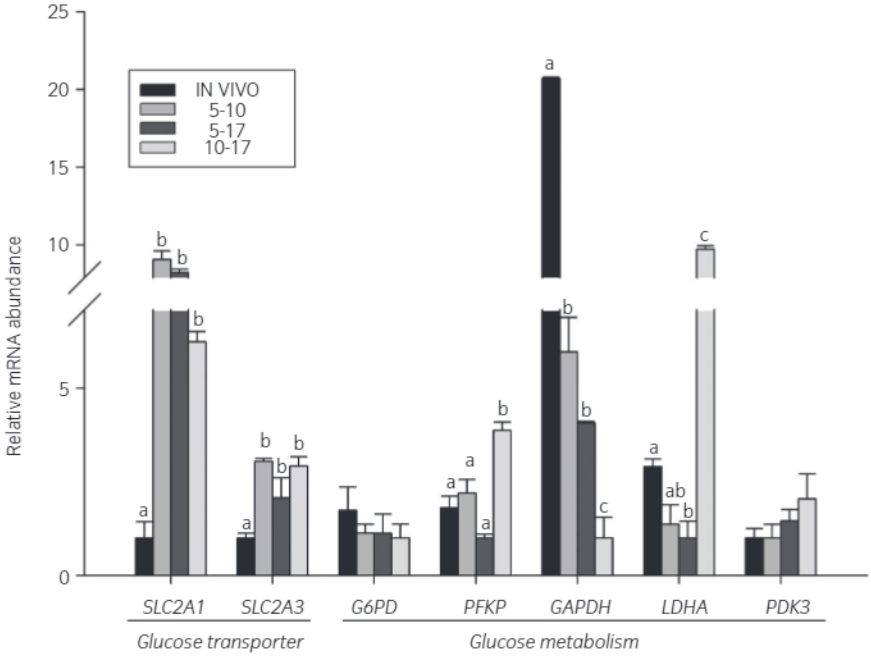


**FIGURE 2** Relative mRNA expression of genes related to glucose transporters and metabolism assessed in Day 7 in vivo embryos and Day 9 in vitro produced blastocysts subjected to different glucose concentrations. Bars represent the relative abundance of the transcripts analysed and normalised to the housekeeping gene *H2AFZ*. Results are expressed as means ± SEM. (A) vs (B) indicate significant differences between groups for a given gene ($P < .05$)

encoding for *PFKP* and *GADPH* was studied. *PFKP* expression was consistently higher in the 10-17 group compared to the rest of the groups studied ($P = .001$), and *GADPH* was consistently overexpressed in the in vivo group compared to the rest ($P = .02$), while its expression decreased in embryos exposed to higher glucose concentrations. The conversion of pyruvate to acetyl-CoA is triggered by pyruvate dehydrogenase lipoamide kinase isozyme 3 (encoded by *PDK3*), which is the link between aerobic glycolysis and the Krebs cycle. The mRNA expression for *PDK3* was no different between groups ($P > .05$).

Finally, the expression of *G6PD*, the first enzyme of the pentose phosphate pathway, was no different between groups ($P = .4$).

## 4 | DISCUSSION

The current study evaluates the effect of three different sequential glucose regimes for IVC on gene expression in equine embryos. It aimed to establish if different glucose concentrations used from Days 0 to 4 and 5 to 9 of embryo culture influenced the likelihood of development to the blastocyst stage; however, no differences were observed in blastocyst yield between groups. Moreover, the results showed that IVC from Day 0 to Day 4 at 5 mmol/L glucose (which was included in both 5-10 and 5-17 groups) reduces the expression of genes related to glucose metabolism (*LDHA* and *PFKP*) and apoptosis (*BAX*) in the 5-17 group compared to the 10-17 group. Moreover, we demonstrated that the 5-10 and 5-17 groups had a more similar expression pattern to in vivo derived Day 7 blastocysts. The gene expression pattern of the 10-17 group is suggestive of increased glycolysis which could be compared to rapidly dividing cancer cells, which take up high amounts of glucose, shifting their metabolism to glycolysis rather than through the tricarboxylic acid cycle, this metabolism being consistent with the Warburg effect.[24]

The second part of the experiment focused on the expression of candidate genes of in vitro produced D9 embryos cultured at three different sequential glucose concentrations compared to D7 in vivo embryos. As expected, different expression patterns were observed when comparing in vivo and in vitro produced blastocysts. It has been described that in vitro produced embryos behave differently to in vivo derived ones, with the development of the former being retarded.[25,26] Accordingly, Pomar[27] compared *D7* in vivo derived embryos with D9 in vitro produced embryos; the blastocyst stage was reached in both cases, although the in vitro blastocysts were smaller. However, there is no evidence of how their metabolism varies in vivo or in vitro depending on their developmental stage. It has previously been reported that IVC affected mitochondrial number suggesting that the lower mtDNA number may be a consequence of developmental retardation;[28] however, no correlation between embryonic arrest and lower mtDNA copy numbers was found.[29] Similarities in cell count and an embryonic diameter between D10 in vitro produced blastocysts and the smallest in vivo-derived blastocysts (Day 7) were reported previously.[1,3,33] However, the exact differences are still under study.

No differences were observed in blastocyst production between groups; interestingly, using the 5 mmol/L glucose concentration during the first stage (D1-D4) of in vitro embryo culture (groups 5-10 and 5-17) showed that IVP embryos followed a similar gene expression pattern irrespective of the glucose concentration used afterwards. Indeed, these patterns were more similar to those of in vivo produced embryos than the 10-17 group. In fact, when embryos were exposed to higher glucose concentrations (10 mmol/L) during the first stage of embryo culture, genes

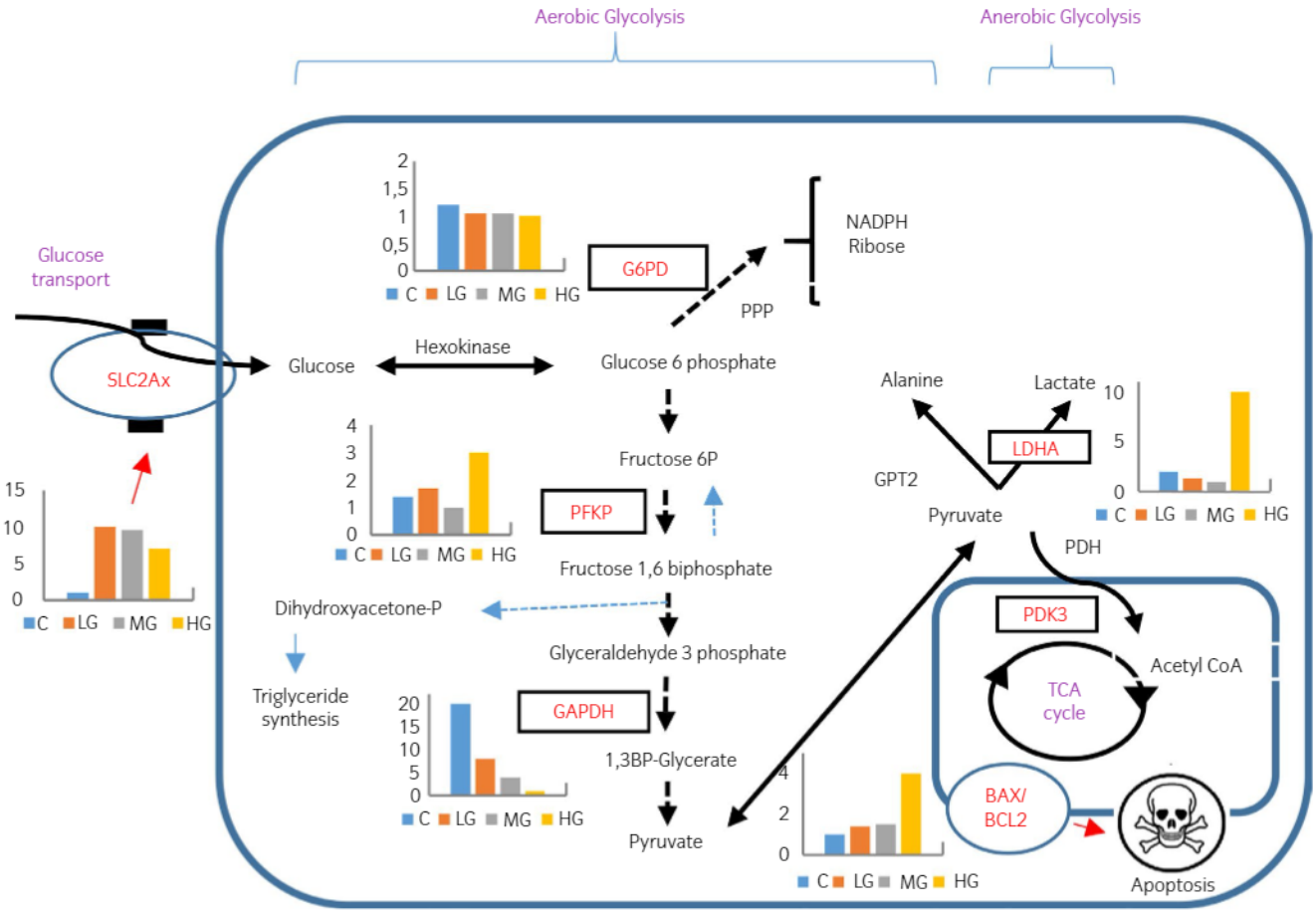


**FIGURE 3** Overview of the genes analysed in this study and their role in the regulation of glucose metabolism. SLC2A1 and SLC2A3 are the two principal glucose transporters during pre-implantation development. G6PD is either shunted through the pentose phosphate pathway (PPP) to form NADPH, ribose and glycolysis intermediates, or to generate phosphoenolpyruvate. PFKP catalyses the irreversible conversion of fructose 6-phosphate to fructose 1,6-bisphosphate and is a key regulatory enzyme in glycolysis. GAPDH serves to break down glucose for energy and carbon molecules. In addition to this long-established metabolic function, GAPDH has recently been implicated in several nonmetabolic processes, including transcription activation and initiation of apoptosis by oxidative stress. PDK3 is part of the pyruvate dehydrogenase complex (PDH) that catalyses the overall conversion of pyruvate to acetyl-CoA and $CO_2$, providing the primary link between glycolysis and the tricarboxylic acid (TCA). Any build-up of pyruvate can feedback through glycolysis and the PPP, being converted by lactate dehydrogenases LDHA to lactic acid, or via alanine aminotransferase to alanine

involved in aerobic and anaerobic glycolysis pathways were overexpressed. In a preliminary experiment (data not shown), we tried 0 mmol/L glucose during D1-D4 of IVC based on a previous study.[2] However, in our experiment, and in contrast to the above, embryos were arrested at the morula stage and no blastocysts were obtained.

Concerning expression of genes related to apoptosis, a higher expression of the proapoptotic gene *BAX* was found in both the 5-10 and 10-17 groups, which could imply an initial activation of apoptotic mechanisms when the glucose concentration is too high. Indeed, a significantly higher *BAX*/*BCL2* ratio in the 10-17 group was found. This effect was previously demonstrated in hyperglycaemic conditions in mouse, rat and bovine embryos where high glucose concentrations during IVC led to a poorer embryo quality.[12-14,34]

Regarding glucose transporters, *SLC2A1* and *SLC2A3* appeared to be overexpressed in all in vitro groups. We have recently reported that glucose concentration in the equine oviduct is lower than in other mammals,[5,32] suggesting a specific control of oviductal glucose concentration. However, the horse is the species where the highest concentration of glucose is routinely used for IVC. It has been described in the mouse that a hyperglycaemic environment in vitro is paradoxically associated with downregulation of glucose transporters 1, 2 and 3 in the blastocyst, reduced glycolytic activity and a relative drop in intra-embryonic free glucose.[31] However, this does not seem to be the case in equine embryos, where the three culture conditions similarly increased the expression of *SLC2A1* and *SLC2A3*, regardless of the glucose concentration used. Moreover, high glucose levels during gastrulation in mice due to maternal hyperglycaemia have been associated with a 150-fold higher rate of ATP production from glycolysis than from oxidative phosphorylation, but with a total reduction of ATP, implying reduced energy production in hyperglycaemic embryos.

We also found an increase in *PFKP* and *LDHA* mRNA expression levels in the 10-17 group, genes typically upregulated during blastocyst development when glycolysis begins to increase.[33] The rate-limiting enzyme PFK in glycolysis is upregulated in parallel with the increased glucose consumption in human embryos,[33] suggesting that embryos of the 10-17 group, with the higher *PFKP* expression, may display higher glucose consumption than the other groups. Moreover, fructose 1,6-bisphosphate is the product of PFK that can function as a signalling molecule to activate pyruvate kinase, inhibit mitochondrial oxidative phosphorylation and regulate reactive oxygen species levels.[34] Besides, we observed that the expression of glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) was reduced in all the in vitro experimental groups, with the 10-17 group having the lowest expression. *GAPDH* has long been recognised as an important enzyme for energy metabolism, and the production of ATP and pyruvate through anaerobic glycolysis in the cytoplasm. In addition to serving as a critical checkpoint in glycolysis, GAPDH also works as a moonlighting protein based on its ability to perform mechanistically distinct functions.[38,39] GAPDH is considered to provide a common link between hyperglycaemia-induced oxidative stress and activation of some of the major pathways associated with the pathogenesis of diabetic complications.[37] In agreement with our results, it has been reported that in bovine retinal cells a high glucose concentration decreases *GAPDH* expression.[38]

Another glycolysis-related gene, *LDHA*, is upregulated in the 10-17 group, while the 5-17 group exhibits a lower expression compared to the in vivo obtained embryos. LDHA is a gene coding for the main enzyme of anaerobic conversion of pyruvate into lactate, which produces NAD+ and stimulates glycolysis. Similar to our results, it has been reported that hyperglycaemia in bovine embryos increases *LDHA* expression,[14] suggesting that enhanced expression of metabolic genes results in an 'un-quiet' embryo that is less viable.[13]

The mitochondrial constitution seems to be critical to ensure proper developmental competence, as in vitro embryo production negatively affects mitochondrial number and function in murine and bovine embryos.[39,40] It has been reported in bovine embryos that mitochondrial gene expression levels were higher in blastocysts compared to oocytes (up to 20 times),[39] while microenvironment changes, maternal supplementation or cellular energy demand have likewise been associated with alterations in bovine blastocyst mitochondrial gene expression.[40] Accordingly, in the current study, a lower expression of the genes related to mitochondrial function (*COX2* and *UQC2*) was found in in vivo produced embryos compared to the in vitro groups, while increased expression of *COX2* was observed when 10 mmol/L glucose was added during early development (10-17 group). *COX2*, one of the three mtDNA-encoded subunits of cytochrome c oxidase, is considered a critical enzyme in the respiratory chain. Three times more *COX2* expression has also been observed in monkey blastocysts cultured in medium with glucose (HECM9) than without glucose (G1/G2).[41] It has been suggested that the upregulation of mitochondrial genes might be a compensatory response due to the generation of free radicals and the loss of function occurring in the electron transport chain.[42] Ubiquinol-cytochrome c reductase is the third complex of the electron transport chain intervening in cellular respiration and the biochemical generation of ATP by oxidative phosphorylation. An increase of expression of *UQC2* is observed in the three in vitro groups and could be due to a nonadaptation of the embryo to the external high glucose concentrations. Finally, *PINK1*, which encodes for a mitochondrial kinase involved in mitochondrial quality control and cell survival, has been shown to protect against neuronal cell death induced by oxidative stress and its deficiency has been linked to mitochondrial dysfunction and increased oxidative cellular stress.[43,44] Interestingly, in our study, we did not find the expression of this gene in in vitro produced embryos, so lower ROS scavenger capacity is expected in in vitro produced embryos compared to the in vivo retrieved ones in which this gene was clearly expressed. The analysis of cultured monkey blastocysts compared with flushed embryos showed a reduced RNA expression of several mitochondrial genes, indicating that culture may compromise mitochondrial metabolism.[41]

In summary, we provide evidence that under high glucose conditions, glycolysis and anaerobic glycolysis pathways seem to be more active in equine in vitro produced embryos, and in an attempt to regulate this hyperactivation, glucose could reduce *GAPDH* activity.[45] Indeed, the use of 10 mmol/L glucose in early culture appears to be deleterious for the embryo when compared to 5 mmol/L glucose, increasing the ratio of *BAX/BCL2* which is associated with apoptosis and reduced embryo viability.[46] In conclusion, glucose concentration during the early stages of IVC seems to regulate the activation of embryonic glycolytic pathway. Five mM glucose during this period seems to be more physiological based on similar expression patterns to in vivo blastocysts. However, further studies of the metabolic aspects of equine embryo development in both in vivo and in vitro conditions should provide a deeper knowledge that could be employed to improve in vitro conditions.

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## CONFLICT OF INTERESTS

No competing interests have been declared.

## AUTHOR CONTRIBUTIONS

R. Fernández-González, A.P. López-Cardona and M. Hamdi executed the study and contributed to the analysis and interpretation of the data. C. Serres contributed to the study execution. K. Smits

contributed to the study design and study execution and corrected the manuscript. A. Gutiérrez-Adán and B. Macías-García designed the study and corrected the manuscript. M.J. Sanchez Calabuig contributed to the study design, executed the study, analysed the data and prepared the manuscript.

## ETHICAL ANIMAL RESEARCH

The in vivo blastocyst collection procedures used were approved by the ethics committee of the Faculty of Veterinary Medicine of Ghent University (reference number EC 2016/80).

## OWNER INFORMED CONSENT

Not applicable.

## DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## PEER REVIEW

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## ORCID

*María J. Sánchez-Calabuig* https://orcid.org/0000-0002-4280-992X
*Beatriz Macías-García* https://orcid.org/0000-0001-5331-9793

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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