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Hyperglycemia Influences Apoptosis and Autophagy in Porcine Parthenotes Developing *In Vitro*

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ABSTRACT

The objective of this study was to examine the effects of high concentrations of glucose on porcine parthenotes developing *in vitro*. Addition of 55 mM glucose to the culture medium of embryos at the four-cell-stage significantly inhibited blastocyst formation, resulting in fewer cells in blastocyst-stage embryos and increased levels of apoptosis and autophagy compared to control. Quantitative reverse transcriptase (RT) PCR analysis revealed that the expression of pro-apoptotic genes (*Caspase 3*, *Bax* and *Bak*) and autophagy genes (*Atg6* and *Atg8*/*Lc3*) were increased significantly by the addition of 55 mM glucose to the culture medium compared to control. MitoTracker Green fluorescence revealed a decrease in the overall mitochondrial mass compared to control. However, the addition of 55 mM glucose had no effect on mRNA expression of the nuclear DNA-encoded mitochondrial-related genes, cytochrome oxidase (*Cox*) *5a*, *Cox5b* and *Cox6b1*. These results suggest that hyperglycemia reduced the mitochondrial content of porcine embryos developing *in vitro* and that this may hinder embryonic development to the blastocyst stage and embryo quality by increasing apoptosis and autophagy in these embryos.

(Key words : Pig embryo, Mitochondria, Apoptosis; Autophagy)

INTRODUCTION

Glucose is an energy source required by all cells and organs. The important role played by glucose as a major energy substrate during preimplantation embryo development *in vitro* has been demonstrated in many species. *In vivo,* glucose utilization in porcine embryos increases during early development and continues to increase until compaction and blastulation (Flood and Wiebold; 1988). In contrast, *in vitro* development of porcine embryos at the earliest pre-implantation stages is supported by pyruvate and lactate (Gandhi *et al*., 2001; Kikuchi *et al*., 2002). In blastocyst-stage embryos, the oxidation of pyruvate via the tricarboxylic acid cycle is replaced by the use of glucose as the main substrate of glycolysis. However, high concentrations of exogenous glucose (20 to 55 mM) inhibit blastocyst development and cell proliferation and differentiation, as well as increase apoptosis and affect intra-embryonic metabolite levels (Zusman *et al*., 1985; Diamond *et al*., 1989; Pampfer *et al*., 1990; De hertogh *et al*., 1991; Moley *et al*., 1996; Moley, 2001; Fraser *et al*., 2007).

Type I programmed cell death, or apoptosis, is crucially involved in the development and differentiation of embryos. Blastocysts recovered from diabetic mice show higher levels of mRNA and protein expression for the pro-apoptotic effector, Bax (Pampfer *et al*., 1997). Increased expression of apoptotic markers is also observed when high levels of glucose are added to the culture medium of blastocysts from normal mice or rats, suggesting that glucose enhances the induction of apoptosis (Pampfer *et al*., 1997; Moley *et al*., 1998). Recent studies have demonstrated expression of different pro-apoptotic and anti-apoptotic effectors during high glucose-induced apoptosis in rodent blastocysts and also showed an increase in reactive oxygen species (ROS) in blastocysts exposed to high concentrations of D-glucose (Leunda-Casi *et al*., 2002). Apoptosis results from changes in mitochondrial integrity caused by various effectors, such as Ca^{2+}, ROS, or production of Bax, which lead to the release of cytochrome C and activation of the

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caspase cascade (Nutt *et al*., 2005). During embryonic development, poor quality bovine embryos express more Bax and less Bcl compared with good quality embryos (Yang *et al*., 2002).

Hyperglycemia may also be involved in the induction of autophagy (Adastra *et al*., 2011). Autophagic cell death is characterized by the presence of abundant autophagic vacuoles, which engulf bulk cytoplasm and cytosolic organelles, such as mitochondria and the endoplasmic reticulum, for subsequent degradation by the cells' own lysosomal system (Gozuacik and Kimchi, 2004). The proteins encoded by autophagy-related genes (ATG) are required for the formation of autophagic vesicles (Yu *et al*., 2004; Pattingre *et al*., 2005; Suzuki and Ohsumi, 2007; Xie and Klionsky, 2007). ATG6/Beclin-1 functions in cooperation with the class III PI3-kinase signaling complex to positively control the formation of autophagic vacuoles (Petiot *et al*., 2000; Kihara *et al*., 2001). Microtubule-associated protein light chain 3 (LC3/ATG-8) is converted to a membrane-conjugated form by ATG4, while ATG9 is an integral membrane protein required for the formation of double membrane vesicles (Baehrecke, 2005; Kirisako *et al*., 2000; Noda *et al*., 2000).

Although there have been several reports on the mechanisms of diabetes and the irreversible apoptotic effects on preimplantation embryo development induced by hyperglycemia, there is little data on the effects of hyperglycemia with respect to mitochondria, apoptosis- and autophagy-related genes in porcine embryos developing *in vitro*. Therefore, the present study used an *in vitro* porcine model to investigate the effects of exposure to high concentrations of glucose on embryo viability and mitochondrial content, in addition to the expression of apoptotic, autophagic and nuclear-encoded mitochondria-related genes.

MATERIALS AND METHODS

Oocyte Collection and *In Vitro* Maturation

Prepubertal porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 25℃ in Dulbecco's phosphate-buffered saline (dPBS) supplemented with 75 mg/l penicillin G and 50 mg/l streptomycin sulfate. Cumulus-oocyte complexes (COCs) were aspirated from the follicles (2~8 mm diameter) using an 18-gauge needle and a disposable 10 ml syringe. COCs were washed three times with Hepes-buffered Tyrode's medium containing 0.1% (w/v) polyvinyl alcohol (Hepes-TL-PVA). Each group of 50 COCs was matured in 500 μl tissue culture medium (TCM)-199 with Earle's salts (Gibco, Grand Island, NY, USA) supplemented with 0.57 mM cysteine (Sigma, St. Louis, MO, USA), 10 ng/ml EGF (Sigma), 10 IU/ml PMSG (Sigma) and 10 IU/ml hCG (Sigma) under paraffin oil at 39℃ for 44 h.

Embryo Culture

Following maturation, cumulus cells were removed by pipetting in the presence of 1 mg/ml hyaluronidase for 2~3 min. Oocytes were activated for parthenogenesis with 5 mM Ca^{2+} ionophore (Sigma) for 5 min. After 3 h of culture in PZM3 (Im *et al*., 2004) medium containing 7.5 mg/ml cytochalasin B (Sigma) and 0.4% BSA, embryos were washed three times in PZM3 medium containing 0.4% (w/v) BSA and cultured in the same medium for 48 h at 39℃ in an atmosphere of 5% CO_2 and 95% air.

After 48 h of culture in PZM3 medium containing 0.4% BSA, presumptive diploid 4-cell parthenote embryos were collected and washed three times in PZM3 medium with 0.4% (w/v) BSA and then allocated and cultured in PZM3 (control), Glc 0 (pyruvate/lactate-free PZM3) Glc 5.5 or Glc 55 (pyruvate/lactate-free PZM3 supplemented with 5.5 mM or 55 mM glucose and 1.7 mM $CaCl_2{\cdot}7H_2O$) medium. Any increase in osmolarity caused by the addition of higher concentration of glucose to the culture medium was compensated for by adjusting the concentration of sodium chloride accordingly (Fraser *et al*., 2007; Funahashi *et al*., 1996). The embryos were cultured for 5 days (Day 7 post-parthenogenic activation) at 38.5℃ and 5% CO_2; embryos that developed to the blastocyst stage were analyzed.

Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Assay

Approximately 10 blastocysts were washed three times in dPBS (pH 7.4) containing 1 mg/ml PVA (dPBS/PVA) and then fixed in 3.7% paraformaldehyde in dPBS/PVA for 1 h at room temperature. After fixation, embryos were washed in dPBS/PVA and permeabilized by incubation in 0.5% Triton X-100 for 1 h at room temperature. They were then washed twice in dPBS/PVA and incubated with fluorescein-conjugated dUTP and the terminal deoxynucleotidyl transferase enzyme (*In Situ* Cell Death Detection Kit, Roche, Mannheim, Germany) in the dark for 1 h at 37℃. Nuclei were counterstained with 50 mg/ml RNase A in 40 mg/ml propidium iodide (PI) for 1 h at 37℃, after which the embryos were washed in PBS/PVA, mounted with slight coverslip compression, and examined by confocal microscopy.

For the other experiments, embryos were fixed in 3.7 % paraformaldehyde in PBS for 1 h at RT and stained with 40 mg/ml PI for 1 h at 37℃ to label the nuclei. Total cell numbers were counted under a fluorescence

microscope (Olympus, Tokyo, Japan).

Mitochondrial Staining and Image Analysis

To identify mitochondria, porcine blastocysts were fixed with 3.7% formaldehyde in dPBS/PVA for 1 h, washed twice with dPBS/PVA, and incubated with MitoTracker Green FM (M-7514, Molecular Probes, Inc.) for 20 min. Nuclei were stained with Hoechst 33342. Phase-contrast images of individual samples were digitized using laser scanning confocal microscopy (Carl Zeiss, Germany). The intensity of MitoTracker Green FM fluorescence was measured with ZEN software (Carl Zeiss). At least 10 embryos were examined, and at least 10 areas were examined in each.

Immunofluorescence Staining

To determine the expression of the LC3 autophagy protein, approximately 10 blastocysts were washed in dPBS containing polyvinyl alcohol (PVA, 1 mg/ml), fixed for 20 min in 3.7% paraformaldehyde in dPBS and permeabilized with 0.2% Triton X-100 in dPBS for 30 min at room temperature. The fixed embryos were then incubated with rabbit polyclonal LC3 antibody (Abcam, Cambridge, UK) for 1 h and then with a FITC-labeled secondary antibody (Sigma). Hoechst 33342 was used to stain the nuclei. Slides were examined under a laser-scanning confocal microscope (Carl Zeiss). The intensity of LC3 was measured with ZEN software (Carl Zeiss). At least 10 embryos were examined, and at least 10 areas were examined in each.

Real Time Reverse Transcription Polymerase Chain Reaction (Real Time RT-PCR)

Approximately 10 blastocysts were washed in Ca^{2+}- and Mg^{2+}-free PBS, snap-frozen in liquid nitrogen and stored at −70℃. mRNA was extracted using the Dynabeads mRNA Direct Kit (Dynal Biotech Asa, Oslo, Norway) according to the manufacturer's instructions. Synthesis of cDNA was achieved by reverse transcription of the RNA using Oligo (dT)12–18 primers and the superscript reverse transcriptase enzyme (Invitrogen Corporation Grand Island, NY, USA). The mRNAs for *Bcl-xL*, *Bak*, *Bax*, *caspase3* (*Casp3*), *Cox5a*, *Cox5b*, *Cox6b1*, *Atg6*, microtubule-associated protein 1 light chain 3 beta (*Atg8/Lc3*) and *β-actin* were detected by real-time RT-PCR using specific primer pairs (Table 1). PCR reactions were performed according to the instructions supplied with the real-time PCR machine (DNA Engine

Table 1. List of primers used for quantitative real-time RT-PCR

Genes	GenBank Accession No.	Primer sequence	Annealing temp (C)	Product size
β-actin	U07786	F: ACTACCTCATGAAGATCCTC R: ATCTCCTTCTGCATCCTGTC	60	391
Bcl-xL	AF216205	F: ACTGAATCAGAAGCGGAAAC R: AAAGCTCTGATACGCTGTCC	60	249
Bak	AJ001204	F: CTAGAACCTAGCAGCACCAT R: CGATCTTGGTGAAGTACTC	60	151
Bax	AJ606301	F: GCCGAAATGTTTGCTGACGG R: CGAAGGAAGTCCAGCGTCCA	60	152
Casp3	NM_214131	F: GAGGCAGACTTCTTGTATGC R: CATGGACACAATACATGGAA	55	236
Cox5a	XM_001926181	F: GGAATTGCGTAAAGGGATGA R: CATTTTGTCAAGGCCCAGTT	55	247
Cox5b	NM_001007517	F: CTATGGCATCTGGAGGTGGT R: ACAGATGCAGCCCACTATCC	55	191
Cox6b1	NM_001097497	F: CTGAGAGCCCTACCAGCATC R: GTCACCCCCTTTAGCAGTCA	55	199
Atg6	NM_001044530	F: AGGAGCTGCCGTTGTACTGT R: CACTGCCTCCTGTGTCTTCA	60	189
Atg8/LC3	NM_001190290	F: CCGAACCTTCGAACAGAGAG R: AGGCTTGGTTAGCATTGAGC	60	206

(F: forward. R: reverse)

Opticon 3 fluorescence detection system, MJ Research, Waltham, MA, USA). The threshold cycle (Ct) value represents the cycle number at which the sample fluorescence rises significantly above that of the background. The reactions were conducted according to the protocol supplied with the DyNAmo SYBR green qPCR kit containing modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM $MgCl_2$ and dNTP mix including dUTP (Finnzymes). PCR conditions were: denaturation at 95℃ for 10 min, followed by 40 cycles of amplification and quantification at 95℃ for 10 sec, 55 or 60℃ for 30 sec and 72℃ for 30 sec with a single fluorescence measurement, a melting curve program set at 65~95℃ with a heating rate of 0.2℃/sec and continuous fluorescence measurement, and a final cooling step to 12℃.

Fluorescence data were acquired after the extension step in PCR reactions containing SYBR Green. Thereafter, PCR products were analyzed by generating a melting curve. Since melting curves are sequence-specific, nonspecific PCR products could be distinguished from specific ones. The crossing point (CP) was determined for each transcript to generate the mathematical model. The CP is defined as the point at which the fluorescence rises appreciably above the background fluorescence. The relative quantification of gene expression was analyzed using the 2-ddCt method (Livak and Schmittgen, 2001). In all experiments, β-*actin* mRNA was used as an internal standard.

Statistical Analysis

The general linear models (GLM) procedure embedded within the statistical analysis system (SAS User's Guide) was used to analyze the data from all experiments. Significant differences were determined using Tukey's Multiple Range Test with $p<0.05$ considered significant. A paired Student's *t*-test was used to compare relative gene expression.

RESULTS

Effect of Different Concentrations of Glucose on Porcine Embryos Developing *In Vitro*

Different concentrations of glucose (5.5 mM and 55 mM) in pyruvate/lactate-free PZM3 medium were added to 4-cell to blastocysts developing *in vitro*. The developmental rates to the blastocyst stage of porcine 4-cell parthernotes cultured for 5 days was similar in the PZM3 and Glc 5.5 groups (56.7±2.44% *vs.* 52.8± 2.44%), but was significantly decreased in the Glc 55 group (37.1±2.44%; *$p<0.05$; Fig. 1). As expected, no embryos developed to the blastocyst stage in the Glc 0 group.

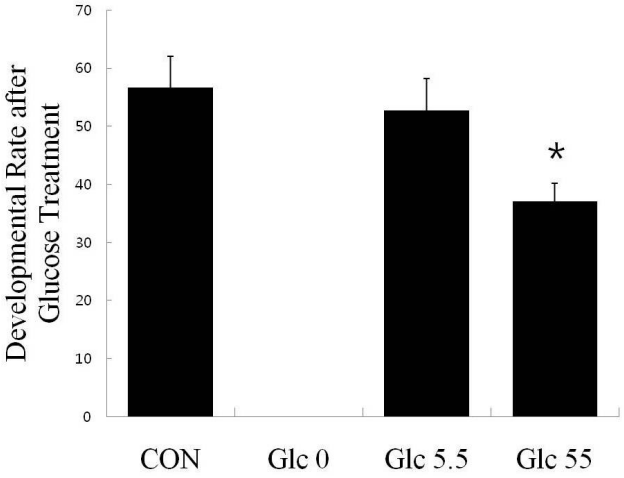

Fig. 1. The effect of glucose concentration on embryonic development from the 4-cell to the blastocyst stage. Statistically significant differences are indicated by asterisks (*$p<0.05$). Values represent the mean ± SEM of four separate experiments.

Glucose Concentration Affects Cell Number and Apoptosis in The Blastocyst Stage

Porcine blastocysts were stained with PI to label all nuclei and by TUNEL to label apoptotic nuclei. They were then analyzed by epifluorescent and confocal microscopy. At Day 7, the mean cell number within the blastocysts derived from the Glc 55 group was significantly lower than that in blastocysts derived from the PZM3 (control) and Glc 5.5 groups (38±1.9 *vs.* 51±1.7 and 53±1.5; *$p<0.05$; Fig. 2A). DNA fragmentation resulting from apoptotic nicking of genomic DNA in individual embryos was measured using the TUNEL assay. The apoptotic index (fragmented cell number/total cell number) in blastocysts derived from the Glc 55 group was significantly higher than that in blastocysts derived from the control and Glc 5.5 groups (5.7±0.44% *vs.* 4±0.39% and 3±0.35%; *$p<0.05$; Fig. 2B and C).

To investigate whether hyperglycemia modulates the mRNA expression of apoptosis-related genes in porcine parthenotes developing *in vitro*, the relative amounts of *Casp3*, *Bak*, *Bax* and *Bcl-xL* mRNA expression in blastocysts cultured in PZM3 and Glc 55 medium were measured using real-time RT-PCR. Porcine *β-actin* was used as an internal standard. Each treatment group consisted of 5~10 embryos and each experiment was repeated four times with three replicates. The addition of high levels of glucose to the medium increased the mRNA expression level of pro-apoptosis-related genes and decreased the mRNA expression of anti-apoptosis-related

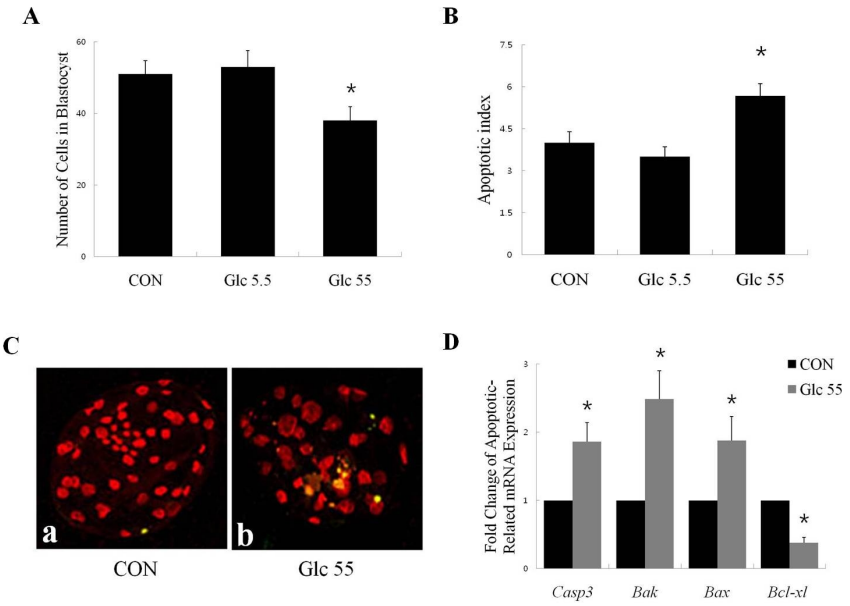

Fig. 2. Number of cells per blastocyst (A) and the apoptotic index (B; apoptotic cell no/total cell number) in blastocysts after 5 days of culture in PZM3, Glc 5.5 or Glc 55 medium. Laser scanning confocal microscopy images of total nuclei (red) and fragmented DNA (green) in porcine blastocysts (C; 400×). a, control; b, Glc 55. Relative expression of apoptosis-related genes analyzed by real-time RT-PCR (D). Statistically significant differences are indicated by asterisks (*p<0.05). Values represent the mean ± SEM of four separate experiments.

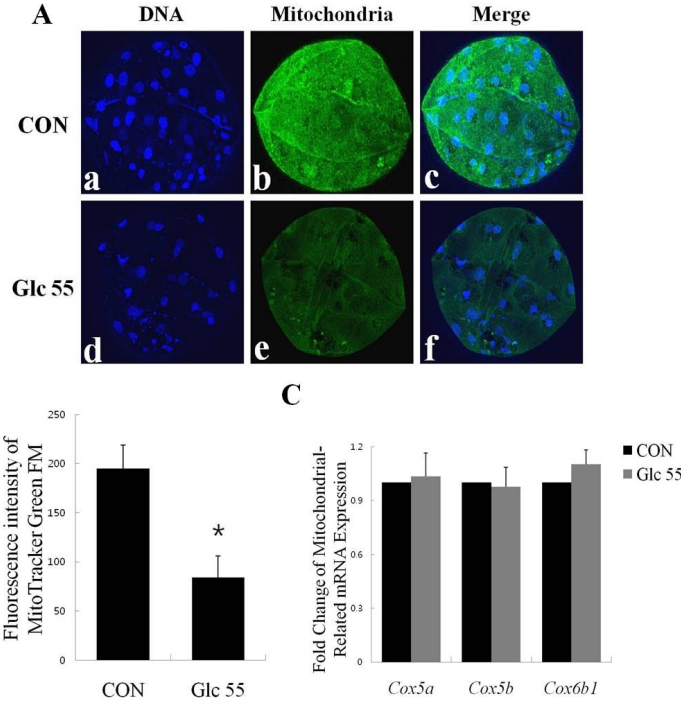

Fig. 3. Laser scanning confocal microscopy images (A, 400×) of mitochondria in blastocyst stage embryos. Blue, chromatin (Aa and Ad); green, mitochondria stained by MitoTracker (Ab and Ae); merge (Ac and Af). The intensity of MitoTracker Green FM was measured with ZEN software from Carl Zeiss (B). The relative abundance of nuclear DNA encoded mitochondria-related genes was analyzed by real-time RT-PCR (C). Statistically significant differences are indicated by asterisks (*p<0.05). Values represent the mean ± SEM of four separate experiments.

genes (*p<0.05; Fig. 2D).

Hyperglycemia Affects Mitochondrial Distribution but not Nuclear DNA-Encoded Mitochondrial Gene Expression in Blastocysts

To investigate the effect of hyperglycemia on mitochondrial distribution, blastocysts were stained with Hoechst 33342 to label the nuclei and MitoTracker to label mitochondria and then analyzed by epifluorescent and confocal microscopy. Embryos exposed to Glc 55 medium showed a significant decrease in MitoTracker Green fluorescence at the blastocyst stage compared with those in the control group (195±24 *vs*. 84±22; *p<0.05; Fig. 3Ab and Ae).

To investigate the effect of hyperglycemia on mitochondrial gene expression, the mRNA expression level of the mitochondria-related genes *Cox5a*, *Cox5b* and *Cox6b1*, encoded by nuclear DNA, was measured by real-time RT-PCR. No significant difference was detected in the mRNA expression levels of *Cox5a*, *Cox5b* and *Cox6b1* between the control and Glc 55 groups (Fig. 3C).

Effect of Hyperglycemia on Autophagy and Autophagy-Related Gene Expression in Blastocysts

To determine the effect of hyperglycemia on autophagy, we examined LC3 expression in porcine blastocysts derived from the control and Glc 55 groups. Embryos exposed to Glc 55 medium showed a significant increase in the amount of LC3 protein compared with the control group (36±9.4 *vs*. 98±11.2; *p<0.05; Fig. 4Ab and Ae).

To determine whether hyperglycemia modulates the expression of autophagy-related genes in porcine parthenotes developing *in vitro*, the relative amounts of *Atg6* and *Atg8/Lc3* mRNA expression in blastocysts cultured in PZM3 and Glc 55 medium were measured by

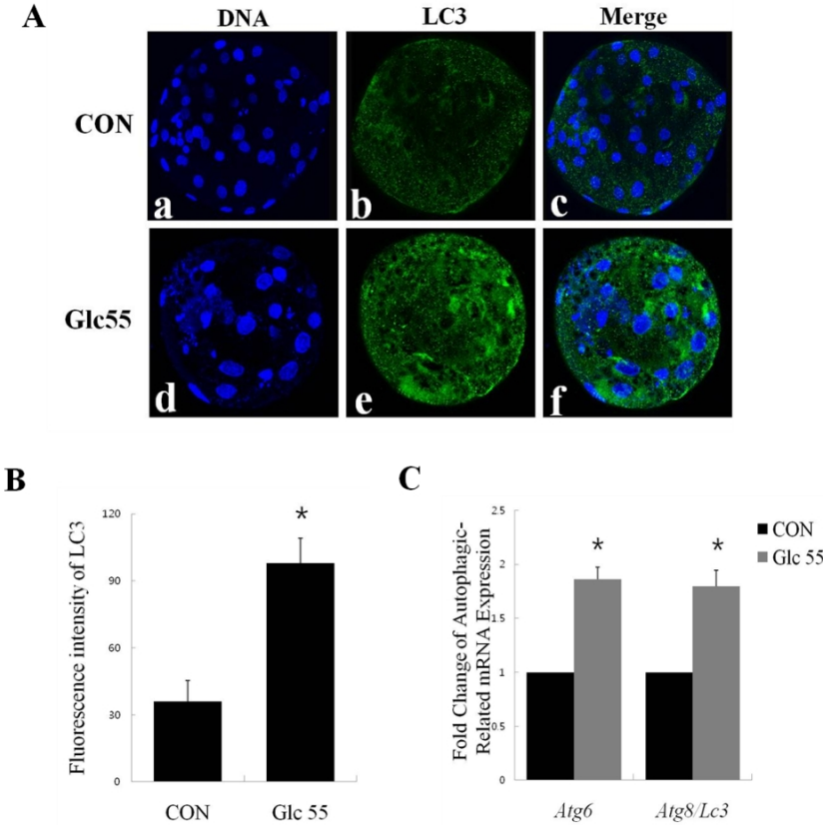

Fig. 4. Images of total nuclei (blue) and autophagosomes (LC3; green) in porcine blastocysts obtained from *in vitro* culture of 4-cell parthenotes for 5 days (A, 400×). Embryos were stained with Hoechst 33342 (Aa and Ad) and a FITC-conjugated secondary antibody for anti-LC3 (Ab and Ae). The merged images are presented in green and blue for FITC-conjugated and Hoechst 33342 (Ac and Af), respectively. LC3 intensity was measured using ZEN software from Carl Zeiss (B). The relative abundance of autophagy-related genes was analyzed by real-time RT-PCR (C). Statistically significant differences are indicated by asterisks (*p<0.05). Values re the present the mean ± SEM of four separate experiments.

real-time RT-PCR. Embryos exposed to Glc 55 medium showed significantly increased expression of mRNA for *Atg6* and *Atg8/Lc3* (*p<0.05; Fig. 4C).

DISCUSSION

Previous analysis of murine and rat models of diabetes and hyperglycemia suggests that increased rates of apoptosis lead to increased rates of malformation and absorption of blastocyst-stage embryos (Funahashi *et al*., 1996; Moley *et al*., 1998). Similarly, our results showed that when pyruvate/lactate-free PZM3 medium is supplemented with 55 mM glucose, the development rate significantly decreased (p<0.05) compared to that in the control (PZM3) and low glucose (Glc 5.5) groups. In addition, the mean cell number per blastocyst significantly decreased in embryos exposed to 55 mM glucose, and supplementation with a high concentration of glucose significantly increased the percentage of apoptotic TUNEL-labeled nuclei. The propensity of a cell to undergo apoptosis is continuously counterbalanced by factors that stimulate cell survival and proliferation. For example, during embryogenesis, the *Bcl-2* gene family controls developmental programmed cell death. In contrast, other conserved genes are positive regulators of apoptosis. These include the Bak proteins, which are involved in the caspase cascade in the pig. In the present study, the addition of high concentrations of glucose to pyruvate/lactate-free PZM3 medium decreased Bcl-xl mRNA expression and increased the expression of Bax, Bak and caspase 3 mRNA, indicating that high concentrations of glucose induce apoptosis-related gene expression.

Apoptosis results from changes in mitochondrial integrity caused by effectors such as Ca^{2+}, ROS, or production of Bak, which induce the release of cytochrome C and activation of the caspase cascade (Nutt *et al*., 2005). Cytochrome C oxidase is an important energy-generating enzyme that is critical for the proper functioning of most cells. In mammalian tissues, *Cox5a*, *Cox5b* and *Cox6b1* are encoded in the nucleus and synthesized in the cytoplasm (Cui *et al*., 2006). In the present study, no significant differences in the mRNA expression of *Cox5a*, *Cox5b* or *Cox6b1* were detected between the PZM3 and Glc 55 groups. However, we showed that embryos treated with a high concentration of glucose showed a significant decrease in mitochondrial distribution within blastocysts. Yu *et al* (2006) reported that hyperglycemia causes an increase in the generation of ROS and mitochondrial malfunction. Taken together with the results of our study, this suggests that hyperglycemia leads to the release of cytochrome C and changes in the mitochondrial transmembrane potential in porcine embryos developing *in vitro*, which than induces apoptosis.

Recently, hyperglycemia was shown to be involved in the induction of autophagy in mouse embryos (Adastra *et al*., 2011). Autophagy, a process by which eukaryotic cells degrade and recycle macromolecules and organelles, plays an important role in cellular responses to hyperglycemia. Moreover, several studies have indicated that ROS are involved in the induction of autophagy in many cell types (Zhang *et al*., 2009; Lee *et al*., 2009; Chen and Gibson, 2008). In the present study, we observed autophagy in porcine embryos subjected to hyperglycemic conditions. Our results showed that embryos cultured in Glc 55 medium had significantly enhanced expression of LC3, which is a marker of autophagosomes. In addition, embryos exposed to hyperglycemia also showed enhanced expression of the autophagy-related genes *Atg6* and *Atg8/Lc3*. Both previous observations and the results of the present study suggest that hyperglycemia leads to increased autophagy in porcine preimplantation embryos.

In conclusion, the present study shows that hyperglycemia induces apoptosis and autophagy in porcine preimplantation embryos. We found increased expression levels of pro-apoptotic and autophagy-related genes, but not of nuclear DNA-encoded mitochondria-related genes. Hyperglycemia decreased the mitochondrial content in porcine embryos developing *in vitro,* resulting in slowed embryonic development to the blastocyst stage and decreased embryo quality, as well as increased apoptosis and autophagy in these embryos.

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