

Effects of Glucosamine on the Development and Related Gene Expression of Buffalo (*Bubalus bubalis*) Embryos

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Abstract: O-linked β -N-Acetylglucosamine Glycosylation (O-GlcNAc) is one of the main types of glycosylation in mammalian cells while Glucosamine (GlcN) is an O-GlcNAc substrate. Thus, effects of GlcN on the embryonic development, level of O-GlcNAc and related gene expression of buffalo embryos were examined in this study. Buffalo zygotes derived from *In Vitro* Fertilization (IVF) were randomly allocated into culture in the medium supplemented with different concentration of GlcN (0, 1, 2 and 4 mM) during the different culture period (0-72, 72-172 and 0-172 h). When GlcN was added to the medium in the culture period of 0-72 h after IVF, addition of 2 mM GlcN resulted in more zygotes developing to blastocysts (26.1%) in comparison with control (14.3%), 1 mM (13.6%) and 4 mM (11.3%) groups ($p < 0.05$). However, the blastocyst yield decreased gradually when GlcN was added to the medium during 72-172 h of culture and decreased significantly when the concentration of GlcN was arrived at 4 mM (3.1 vs. 14.2%, $p < 0.05$). When GlcN was added to the medium in the whole culture period (0-172 h) there were no significant difference in either cleavage rate or blastocyst yield among the four groups ($p > 0.05$). Immunofluorescence analysis revealed that addition of 2 mM GlcN to medium from 0-72 h after IVF resulted in a significant increase ($p < 0.05$) in the O-GlcNAc level of embryos at 2, 4, 8 cells and morula stage with the exception of blastocysts. QRT-PCR revealed that culture of zygotes with 2 mM GlcN in the culture period of 0-72 h after IVF resulted in a significant increase ($p < 0.05$) in the expression of O-GlcNAc transferase gene in the embryos at the 2, 4, 8 cells and morula stage and did not affect the expression of O-GlcNAc-selective N-acetyl β -D-glucosaminidase gene. These results indicate that appropriate concentration of GlcN can improve the development of buffalo embryos and this action is stage dependent and mediated by O-GlcNAc transferase gene.

Key words: O-linked β -N-acetylglucosamine glycosylation, glucosamine, embryos, gene expression, buffalo

INTRODUCTION

Protein glycosylation is an important Post-Translational Modification (PTM) (Hart, 1992; Seitz, 2000) and more than half of all proteins are glycosylated according to the SWISS-PROT database (Apweiler *et al.*, 1999). O-linked β -N-acetylglucosamine glycosylation (O-GlcNAc) was discovered in 20 years ago by Torres and Hart (1984) and many glycoproteins have been found to have variety of physiological functions (Hart *et al.*, 2007) including nuclear, mitochondrial and cytoplasmic proteins.

Glucosamine (GlcN) is metabolized to UDP-N-acetyl Glucosamine (UDP-GlcNAc) via the Hexosamine Biosynthesis Pathway (HSP) (Wells *et al.*, 2001). The enzymes involved in HSP include the O-linked N-acetyl Glucosaminyl Transferase (OGT) and the β -selective N-acetyl glucosaminidase (OGA) (McClain *et al.*, 2002). The HSP end product is UDP-GlcNAc (McKnight *et al.*,

1992). Levels of UDP-GlcNAc directly respond to modulate OGT affinity for different peptides and regulate O-GlcNAc (Kreppel and Hart, 1999). Fluctuating UDP-GlcNAc levels and differentially targeted OGT isoforms could then lead to discrete modifications of various cellular targets, potentially causing a change in the target's function and may also serve to regulate OGT directly (Kreppel *et al.*, 1997; Lubas *et al.*, 1997; Lubas and Hanover, 2000). Three features suggest that O-GlcNAc performs a regulatory role: O-GlcNAc occurs at sites on the protein backbone that are similar or identical to those modified by protein kinases O-GlcNAc competes with phosphorylation on some well studied proteins such as RNA Polymerase II (RNA Pol II) O-GlcNAc is highly dynamic with rapid cycling in response to cellular signals such as cellular stress (Hart *et al.*, 2007). Changes in O-GlcNAc levels have been shown to alter the behavior of proteins such as enzyme activity (Du *et al.*, 2001; Federici *et al.*, 2002; Parker *et al.*, 2003) protein protein

interactions (Roos *et al.*, 1997; Han *et al.*, 1998; Yang *et al.*, 2001) DNA binding ability (Gao *et al.*, 2003) subcellular localization (Bachmann *et al.*, 1989; Duverger *et al.*, 1993; Monsigny *et al.*, 1999; Juang *et al.*, 2002) the half life and proteolytic process of proteins (Datta *et al.*, 1989; Ray *et al.*, 1992; Han and Kudlow, 1997; Cheng *et al.*, 2000). O-GlcNAc levels have been shown to be elevated in response to different forms of cellular state (Zachara *et al.*, 2004).

GlcN supplementation is well known to up-regulate HSP via increasing O-GlcNAc. GlcN enters the cell and can be transported by the facilitated glucose transporters and is readily phosphorylated to Glucosamine 6-Phosphate (GlcN6P) (Marshall *et al.*, 2004) then finally metabolized to UDP-GlcNAc. Thus, GlcN can provide high flux and change the terminal transfer/removal of the O-GlcNAc moiety to target proteins. Elevated extracellular GlcN concentrations lead to increase modification of intracellular proteins with O-GlcNAc. Activation of pathways leading to O-GlcNAc formation improves cell survival whereas inhibition of O-GlcNAc formation decreases cell survival (Champattanachai *et al.*, 2007). Increasing O-GlcNAc formation was associated with increased mitochondrial levels of the prosurvival Bcl-2 which can contribute to the increased tolerance to apoptosis (Champattanachai *et al.*, 2007). Meanwhile, O-GlcNAc is also necessary for cell viability in embryogenesis and development (Shafi *et al.*, 2000).

The aim of this study is to examine the effects of GlcN supplementation on the development, O-GlcNAc level and expression of OGT/OGA in buffalo preimplantation embryos and explore the function of O-GlcNAc signaling pathway in the embryogenesis of buffalos.

MATERIALS AND METHODS

Reagents and media: Unless otherwise mentioned, all reagents used were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The TCM 199 powder was purchased from Gibco BRL (Paisley, Scotland, UK) and Fetal Bovine Serum (FBS) were purchased from Hyclone Company (Logan, UT, USA). The *In Vitro* Maturation (IVM) medium was TCM-199 supplemented with 26.2 mM NaHCO₃, 5 mM HEPES, 5% estrous cow serum (OCS, self preparation), 2% Bovine Follicular Fluid (BFF, collected without regard to the stage of the reproductive cycle) and 0.1 µg mL⁻¹ FSH. The Fertilization Medium (FM) was modified Tyrode's medium supplemented with 50 µg mL⁻¹ heparin, 2.5 mM caffeine and 0.6% BSA. The embryo Culture Medium (CM) was TCM-199 supplemented with 26.2 mM NaHCO₃, 5 mM HEPES and 3% OCS. All of the media were supplemented with 60 µg mL⁻¹ penicillin G and

100 µg mL⁻¹ streptomycin sulfate then sterilized by passing through a 0.20 µm filter and stored at 4°C for up to 4 weeks.

***In vitro* maturation of oocytes:** Chinese swamp buffalo ovaries were collected from a local abattoir. Buffalo Cumulus Oocytes Complexes (COCs) from 2-6 mm follicles with multi layers of cumulus cells were selected for IVM. Then, COCs were washed twice in the IVM medium and cultured in a 30 mm glass dish containing 1.5 mL IVM medium under a humidified atmosphere of 5% CO₂ in air at 38.5°C. After 24 h of IVM, the surrounding cumulus cells of oocytes were partly removed and the oocytes were kept in CM for subsequently IVF.

***In vitro* fertilization:** Frozen semen was thawed in a waterbath at 37°C and then layered under 1.5 mL fertilization medium in a conical tube for the swim-up procedure. After incubation for 30 min at 38.5°C, the top 0.8-1.0 mL medium containing the more motile sperm was collected and then centrifuged (500×g) for 5 min to harvest sperm. The pellet of semen was resuspended in fertilization medium and adjusted to a final concentration at 5×10⁶ sperm/mL. Oocytes matured *in vitro* were placed into a droplet of 30 µL fertilization medium under sterile mineral oil (10-15 oocytes per drop). Then, 4-5 µL of semen was added to the droplet containing oocytes to give a final sperm concentration at 1-1.5×10⁶ sperm/mL and co-incubated with oocytes for 8-10 h at 38.5°C under a humidified 5% CO₂ in air atmosphere.

***In vitro* culture of embryos:** At 8-10 h after insemination, presumed zygotes were placed into co-culture with granulosa cell monolayers in a 30 µL droplet of CM overlaid with mineral oil under a humidified atmosphere of 5% CO₂ in air at 38.5°C. The granulosa cell monolayers were established at 48-72 h before introduction of embryos. After introduction of zygotes, half of the medium was replaced with fresh medium every 24 h. After 2 days of co-culture, cleavage of zygotes was checked and the number of developed blastocysts was recorded within 7 days of co-culture.

Assessment of nuclear O-GlcNAc levels by RL2 IHC: To assess the level of O-GlcNAc in buffalo embryos during the early development by RL2 IHC, embryos at different developmental stage (2, 4, 8 cells, morula and blastocyst) were recovered and fixed in 4% Paraformaldehyde (PFA) in Phosphate Buffered Saline (PBS, pH 7.2) for 30 min at Room Temperature (RT). Then, they were washed in PBS containing 0.1% Tween 20 for 1 h and permeabilized with 1% Triton X-100 in PBS for 30 min at RT. The samples

were then blocked with PBS supplemented with 1% BSA for 1 h at RT. After washing three times with Triton-PBS-BSA, the embryos were incubated with the primary antibody RL2 antiserum for overnight. The samples were mounted on slides with mounting medium containing fluorescein isothiocyanate 488 (FITC; Cell Signaling Technology, CST, America). The RL2 immunoreactivity was assessed and quantified by image analysis using the CARL ZEISS LSM 510 laser-scanning confocal microscope. For assessment of nuclear O-GlcNAc levels, the mean green scale intensity of nuclear in the same optical plane for each embryo was quantified. This was done at the same optical depth from the coverslip for each adherent embryo in each treatment experiment group to minimize any confounding impact that variable cellular depth may have fluorescence intensity.

mRNA extraction and cDNA synthesis: Five embryos of different developmental stages were, respectively collected and treated with a Cells to cDNA™ II kit (Ambion Co., Austin, TX) according to the manufacturer's instructions with a little modifications. Briefly, embryos were transferred to 8 µL ice cold lysis buffer (0.8% Igepal, 5 mM DTT, 1 IURNase OUT) and stored at 80°C until measurement. The mRNA was extracted from embryos following the manufacturer's instructions using the high capacity cDNA reverse transcription kit (Applied Biosystems, Madrid, Spain). After the denaturation of the secondary RNA structure at 80°C for 5 min, mRNAs were reverse transcribed in a total volume of 25 µL containing 1×random primers, 1×Reverse Transcription buffer, 1 IU mL⁻¹ Rnase inhibitor, 2.5 IU mL⁻¹ Multiscribe murine leukemia virus reverse transcriptase enzyme and 4 mM dNTP mix. The cDNA was generated by reverse transcription of mRNAs under the following conditions: kept at 25°C for 10 min, 37°C for 2 h and 85°C for 5 sec.

Quantification real-time PCR: The quantification of all gene transcripts was carried out by quantitative real-time reverse transcriptase PCR on a GeneAmp 7500 using SYBR Green PCR Master mix (Applied Biosystems, America). The amounts of primer and cDNA were firstly optimized by semiquantitative PCR and proved optimal by real-time PCR. The PCR reaction mixture contained 10 µL SYBR Premix EX Taq™, 1.0 µL cDNA template, 0.5 µL of primer and Diethylpyrocarbonate (DEPC) treated water. Thermal cycling conditions were 95°C for 5 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec, followed extension stage at 72°C for 30 sec. The

comparative C_T Method was used for the quantification of expression levels as earlier described (Wee *et al.*, 2006).

Experimental design: Experiment 1 was designed to examine the effect of GlcN added at different culture periods on the development of buffalo embryos. At 8-10 h after insemination, presumed zygotes derived from IVF were randomly allocated into culture in the CM supplemented with different concentrations (0, 1, 2 or 4 mM) of GlcN at different culture period (0-72, 72-172 and 0-172 h). Then, the cleavage rate and blastocyst rate were evaluated at 2 and 7 days of culture.

Experiment 2 was conducted to investigate the effect GlcN on the expression of O-GlcNAc, OGT and OGA of buffalo embryos at different developmental stages. According to the results of the experiment 1, the optimal GlcN concentration was added to the CM at different culture period. The expression of O-GlcNAc, OGT and OGA in embryos developed from different culture treatment at different developmental staged was determined by IHC and QRT-PCR.

Statistical analysis: Within each experiment, the difference between treatments in frequencies of oocytes undergoing cleavage and developing to the blastocyst stage was analysed by χ^2 -test. Relative quantification of target gene expression is presented as fold difference and the difference was analyzed using SPSS 17.0 $p < 0.05$ were considered to be significant.

RESULTS AND DISCUSSION

Effects of GlcN on the development of buffalo embryos: As illustrated in Table 1-3 when IVF presumed zygotes were cultured in the CM supplemented with different

Table 1: Effects of glucosamine added in 0-72 h of culture on the *in vitro* development of buffalo IVF embryos

Glucosamine concentration (mM)	Oocytes	Cleaved (%)	Blastocysts (%)
0	175	110 (63.1) ^{ab}	25 (14.3) ^a
1	168	93 (58.5) ^{ab}	23 (13.6) ^a
2	172	117 (68.1) ^{bc}	45 (26.1) ^b
4	166	89 (53.6) ^a	19 (11.3) ^a

Table 2: Effects of glucosamine added in 72-172 h of culture on the development *in vitro* of buffalo IVF embryos

Glucosamine concentration (mM)	Oocytes	Cleaved (%)	Blastocysts (%)
0	197	119 (60.4)	28 (14.2) ^{bc}
1	190	115 (60.5)	21 (11.1) ^c
2	188	112 (59.6)	14 (7.4) ^c
4	195	109 (55.9)	6 (3.1) ^b

*Values with different superscripts within a column are significantly different ($p < 0.05$)

concentration (0, 1, 2 and 4 mM) of GlcN at 0-72 h of culture, there was not significant difference in the cleavage rate among the four groups ($p>0.05$) but addition of 2 mM GlcN resulted in significantly more zygotes developing to blastocysts (26.1%) in comparison with

Table 3: Effects of glucosamine added in 0-172 h of culture on the development *in vitro* of buffalo IVF embryos

Glucosamine concentration (mM)	Oocytes	Cleaved (%)	Blastocysts (%)
0	249	138 (55.4)	37 (14.9)
1	242	131 (54.1)	27 (11.2)
2	253	146 (57.7)	41 (16.2)
4	258	143 (55.4)	30 (11.6)

*Values with different superscripts within a column are significantly different ($p<0.05$)

addition of 1 mM GlcN (13.6%), 4 mM GlcN (11.3%) and control (14.3%, $p<0.05$). When different concentration (0, 1, 2 and 4 mM) of GlcN was added to the CM at 72-172 h of culture, addition of 4 mM GlcN resulted in lower blastocyst yield (3.1 vs. 14.2%, $p<0.05$). When different concentration (0, 1, 2 and 4 mM) of GlcN was added to the CM in the whole culture period (0-172 h) the cleavage rate and blastocyst yield did not change significantly ($p>0.05$).

Effects of GlcN on the O-GlcNAc level of buffalo embryos: As shown in Fig. 1a and b, the O-GlcNAc was concentrated in nuclei area rather than elsewhere in the

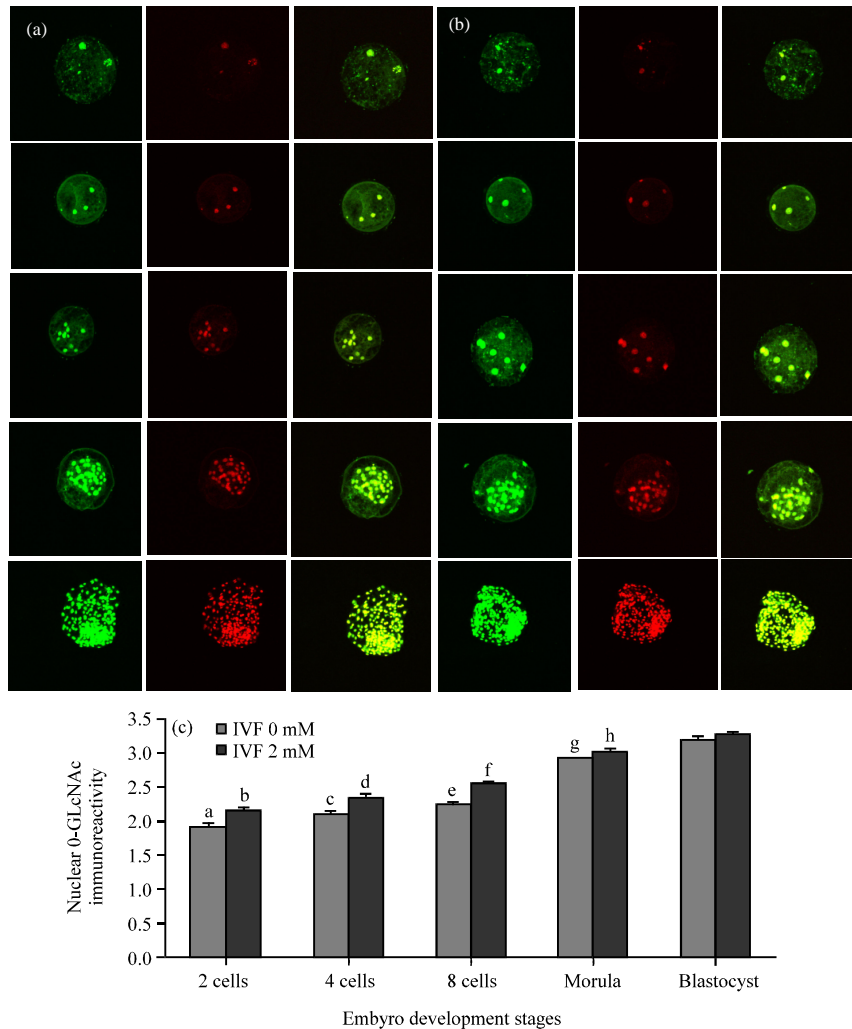


Fig. 1: Confocal images of O-GlcNAc stained with; a) FITC and nuclei stained with Prodiun Iodide (PI); b) embryos developed from the CM without GlcN; embryos developed from the CM supplemented with 2 mM GlcN in the culture period of 0-72 h; c) graph of O-GlcNAc expression levels in each developmental stage based on the fluorescence intensity of confocal images values are presented as mean±SEM and with different superscripts within a column are significantly different ($p<0.05$)

embryo and the O-GlcNAc level slightly increased from the 2 cells stage to the blastocyst stage. Profile of O-GlcNAc level in embryos at each developmental stage was related to the culture treatment. Addition of 2 mM GlcN in the culture period of 0-72 h resulted in an increase in the O-GlcNAc level of embryos at the 2, 4, 8 cells and morula stage ($p < 0.05$) but did not affect the GlcN level of blastocysts ($p > 0.05$).

Effects of GlcN on the expression of OGT and OGA: To examine the relation of O-GlcNAc with the expression of OGT and OGA, the expression levels of OGT and OGA were detected at various developmental stages using real-time PCR. As shown in Fig. 2, the OGT expression level gradually increased but the OGA expression level slightly decreased from the 2 cells stage to the blastocyst stage. Addition of 2 mM GlcN in the culture period of 0-72 h resulted in a significant increase in the OGT expression of embryos at the 2, 4, 8 cells and morula stage ($p < 0.05$) with the exception of blastocysts. However, the OGA expression level was decreased ($p < 0.05$) at all of the embryonic developmental stages when 2 mM GlcN was added to CM in the culture period of 0-72 h.

Precise control of Embryonic Genome Activation (EGA) is essential for normal embryogenesis. It is generally known that changes in nuclear structure, chromatin structure and cytoplasm are related to the EGA. These processes are orchestrated by many signals

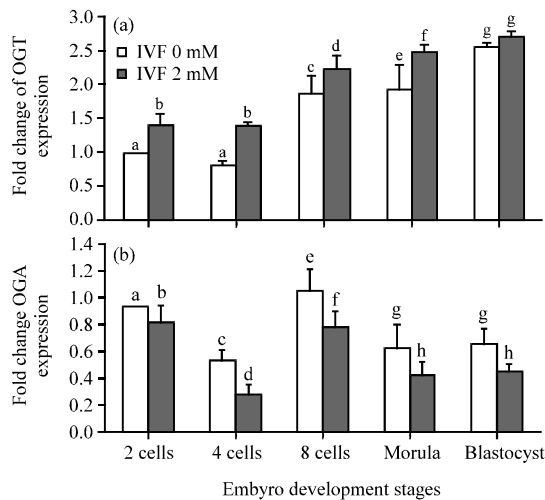


Fig. 2: Expression profiles of; a) OGT and b) OGA in the embryos developed from the culture medium supplemented with or without 2 mM GlcN in the culture period of 0-72 h. Data from three replicates and values are presented as mean±SEM. Values with different superscripts are statistically different ($p < 0.05$, Tukey's post test)

brought by PTM. O-GlcNAc is one of the important PTM regulators. The O-GlcNAc regulates the activity of nuclear and cytoplasmic proteins and is involved in a variety of biological processes including cell cycle regulation, transcriptional control, mitochondrial and peroxisomal protein transport, response to heat shock, development, extracellular stimuli and cellular stress (Zachara *et al.*, 2004). Recent proteomics studies suggested that more than half of all proteins in the cell were modified by O-GlcNAc and these proteins include cytoskeletal proteins, nuclear pore proteins, RNA Pol II, transcription factors, proto-oncogene products, hormone receptors, phosphatases and kinases (Apweiler *et al.*, 1999; Vosseller *et al.*, 2005; Nandi *et al.*, 2006; Khidekel *et al.*, 2007; Wang *et al.*, 2008). Therefore, O-GlcNAc has been shown to be crucial in many aspects of the cellular life and embryonic development.

GlcN acts as a signal via HSP to activate embryonic gene expression, differentiation and development. O-GlcNAc is the end product of the HSP which is subsequently utilized in the glycosylation of proteins. The enzymes involved in the terminal transfer of the GlcNAc moiety to and from target proteins, respectively are OGT and OGA. O'donnell *et al.* (2004) reported that knockout of the OGT resulted in stem cell and embryonic lethality.

In this study, researchers explored the effect of GlcN on the embryonic development and found that addition of 2 mM GlcN to the embryo culture medium in culture period of 0-72 h resulted in a significant increase in the proportion of zygotes developing to blastocysts. However, when GlcN was added to culture medium in the culture period of 72-172 h, the blastocyst yield decreased significantly. Moreover, addition of GlcN to CM in the whole culture period (0-172 h) did not affect the embryonic development. Kimura *et al.* (2008) reported that addition of 2.5 mM GlcN to the embryo culture medium before Maternal-Zygotic Transition (MZT) increased the blastocyst development rate but addition of GlcN was added to the maturation medium and culture medium after MZT the embryonic development was decreased. This result suggested that once the embryonic genome is activated, the expression of OGT and O-GlcNAc levels would be increased and some transcription factors and their activities could be changed.

In order to explore the relationship between OGT/OGA expression levels and O-GlcNAc levels, the O-GlcNAc levels were examined by IC and expression of OGT/OGA was detected by QRT-PCR in this study. Addition of 2 mM GlcN to the embryo culture medium in culture period of 0-72 h resulted in a significant increase in the expression of O-GlcNAc and the mRNA expression

of OGT at each developmental stage of embryos. The O-GlcNAc was observed in nuclei rather than cytoplasm and extracellular matrix which was consistent with the report by Holt and Hart (1986). Acey *et al.* (1977) demonstrated that ¹⁴C-label grains were accumulated in the nucleus of embryos when GlcN was labeled with ¹⁴C. Pantaleon *et al.* (2010) reported that increasing flux through the HSP using 0.2 mM GlcN significantly increased nuclear immunoreactive O-GlcNAc by about 70% as anticipated. This modification is mediated by the unique OGT in which unlike other protein glycosyltransferases is not found in the Golgi secretory pathway and is concentrated in the nucleoplasmic compartment (Holt and Hart, 1986).

The HSP provides the early embryo with a mechanism with which to tightly couple key cellular processes. This mechanism may explain the molecular basis of the impact of O-GlcNAc on embryonic development and related gene expression. The O-GlcNAc mechanism is not well understood. Therefore, this dynamic modification in buffalo on metabolic differentiation and blastocyst formation requires further study.

CONCLUSION

Supplementation of 2 mM GlcN into the CM in culture period of 0-72 h can improve the development of buffalo embryos, this action is stage dependent and may be mediated by promoting the expression of O-GlcNAc, up-regulating the expression of OGT and down regulating the expression of OGA.

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