Carbohydrate Uptake in Water Buffalo Cumulus-Oocyte Complexes (COCs) Supplemented with Retinoic Acid During In Vitro Maturation

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Carbohydrate uptake by water buffalo oocytes after in vitro maturation was evaluated in the present study. The concentrations of glucose and pyruvate were analysed in the spent culture media. Glucose uptake (mM) was increased significantly in the COCs that were supplemented with all-trans RA at doses of 1 (5.00), 3 (4.98), and 5 (5.20) μM compared to control (4.14) and vehicle (4.25). Pyruvate concentration (μM) in the spent culture media was very minimal indicating sufficient uptake by the COCs. However, pyruvate uptake was not significantly different between the control and vehicle compared with RA-treated groups. The data suggest that pyruvate uptake by the COCs does not require all-trans RA. Likewise this research suggests a potential role of all-trans RA in glucose uptake which coincides with its effect in enhancing the developmental competence of water buffalo oocytes.

Key Words: All-trans RA, Glucose uptake, Pyruvate uptake

INTRODUCTION

During mammalian oogenesis, the oocyte grows by more than twice of its original volume, produces large quantities of a myriad macromolecules, as it undergoes a complex series of morphological and developmental changes. In vivo the process begins with the formation of primordial germ cells (PGCs) and proceeds through a series of cellular transformations, from PGC to oogonia to oocytes then to eggs in the adult (Wassarman 1999). In both natural and artificial conditions, oocyte development encompasses a variety of cellular changes categorized as either nuclear or cytoplasmic maturation necessary for normal fertilization and successful embryonic development (Eppig 1996).

Evidently, all these developmental processes require a tremendous supply of energy. Therefore, the oocyte must meet its energy requirements during development by modulating a number of metabolic pathways that generate ATP (Preis et al. 2005).

Carbohydrates are the major source of metabolic energy for most mammalian cells and one of the components of culture medium that mostly affect the developmental competence of oocytes (review, Sutton-McDowall 2010). Hence, carbohydrate uptake and utilization are potentially valuable for assessment of oocyte development (Harris et al. 2007) and developmental competence (Preis et al. 2005) in vitro. Glucose and pyruvate stand out as the important energy substrates for oocyte maturation (Harris et al. 2007) in in vivo and in vitro maturation (IVM). In mouse, glucose is a major energy substrate for
the ovarian follicles (Boland et al. 1993; 1994), whereas pyruvate is essential for the oocyte (Biggers et al. 1967; Johnson et al. 2007). In porcine oocytes, glucose plays a key role in the control of nuclear and cytoplasmic maturation in vitro (Herrick et al. 2006). High levels of glucose and pyruvate uptake measured non-invasively in the microdroplets culture medium of human oocytes, may relate to oocyte maturation (Lesse et al. 1986; Hardy et al. 1989). Likewise, glucose and pyruvate are the principal energy sources for oocytes and follicular somatic cells in buffalo and sheep (Nandi et al. 2008).

Much earlier in vitro studies using mouse models demonstrated that during the course of oogenesis, oocytes metabolize pyruvate oxidatively through mitochondrial oxidative metabolic pathways, yielding ATP and CO2: (Brinster 1971; Zeilmaker and Verhamme 1974; Eppig, 1976). The importance of glycolytic pathway for oocyte development is exemplified by studies of various mammalian oocytes. In mice, gonadotropininduced meiosis is dependent on the presence of glucose (Fagbohun and Downs 1992; Downs and Mastropolo 1994) increased metabolism of glucose through one or more metabolic pathways also occurs simultaneously with the progression of meiosis to MII in oocytes from cats (Spindler et al. 2000) and cattle (Steeves and Gardner, 1999). In primate oocytes, glucose is necessary for cytoplasmic maturation, although nuclear maturation can occur in the absence of carbohydrates (Zheng et al. 2001). In addition, elevated glucose metabolism in mature oocytes is correlated with, and predictive of, improved embryonic development in feline and bovine animals (Krisher and Bavister 1999; Spindler and Wildt 2002).

The findings highlight the importance of glucose and pyruvate metabolism in oocyte maturation. Thus, understanding the energy substrate metabolism by oocyte during IVM may aid in optimizing maturation conditions. In addition to assisting medium optimization, carbohydrate uptake may also serve as a potential marker of oocyte viability (Lane and Gardner 1996; Gardner et al. 2000) aside from oocyte morphology which is often used as a predictor of development. This investigation determined if all-trans RA supplementation affects the glucose and pyruvate uptake of the water buffalo oocytes. Moreover, the IVM media supplemented with all-trans RA used in the present research resulted in blastocyst production rates of >39% (data not presented here), demonstrating that oocytes derived from this system were developmentally competent. Thus, the result generated in this work further validates the efficiency of all-trans RA in improving the developmental competence of water buffalo oocytes.

MATERIALS AND METHODS

Unless specified all chemicals and reagents used were obtained from Sigma Chemical Co., St. Louis, MO, USA.

Collection and culture of oocytes

The water buffalo oocytes collected from a local slaughter house were placed in a thermoflask containing 0.9% NaCl with 100 U penicillin/mL and 100 μg streptomycin/ml at 15-20°C and transported to the Reproductive Biotechnology Laboratory, Philippine Carabao Center, Muñoz, Nueva Ecija. The immature oocytes were obtained as previously described (Hufana-Duran et al. 2004). Briefly, the cumulus-oocyte complexes (COCs) were aspirated in modified phosphate buffered saline (mPBS) from 3-8 mm diameter follicles. Under the stereo microscope, selected compact COCs with 2-5 layers of cumulus cells were washed thrice and incubated in the standard culture media (SCM) containing TCM 199 (Earle’s salts with 25 mM HEPES, Gibco-BRL, Life Technologies, Inc., Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL), 0.2 mM pyruvate, 50 μg/mL follicle stimulating hormone (FSH) and gentamycin, 10 ng/mL epidermal growth factor (EGF, Invitrogen) and 1 μg/mL estradiol.

Prior to IVM, the all-trans RA was dissolved in ethanol, aliquoted, and stored in the dark at -20°C. The treatments used were: Control (SCM only); T1 (SCM+1μM RA); T2 (SCM + 3μM RA); and T3 (SCM + 5μM RA). Five (5) to ten (10) oocytes were incubated for 24 hr at 39°C in water-saturated air with 5% carbon dioxide. After in vitro maturation, oocytes were processed and prepared for in vitro embryo production (IVP) and nuclear examination (results not presented here). The spent culture media were collected from each droplet (50-100μL) in the culture dish, transferred to separate eppendorf tubes and stored at -80°C until analysis.

Measurement of Glucose

The concentration of glucose in the spent culture media was analyzed by the Hexokinase Method using the Glucose Assay Kit (Sigma Chemical Co., St. Louis, MO, USA). The depletion of glucose was measured by analyzing the difference between glucose concentration in the control and incubation drops as described previously (Gardner and Leese 1990; Hardy et al. 1995).

Measurement of Pyruvate

The amount of pyruvate in the spent culture media was assessed using Pyruvate Assay Kit (BioAssay Systems, Hayward, CA, USA). The production of pyruvate was measured by taking the difference between pyruvate concentration in the control and incubation drops as described previously (Gardner and Leese, 1990).
Statistical Analysis
ANOVA was used to analyse differences between incubation drops followed by Tukey’s honest significance difference (HSD). Data were expressed as mean ± S.E. A probability of $P < 0.05$ was considered to be significant.

RESULTS
The TCM I99 used for buffalo oocytes maturation in vitro in the present study initially had 5.6 mM glucose (Sutton 2010). As shown in Figure 1, glucose concentration in the spent culture media decreased significantly in the COCs supplemented with all-trans RA at 1 (0.596 ± 0.142), 3 (0.616 ± 0.073) and 5 (0.410 ± 0.079) μM compared to control (1.46 ± 0.160) and vehicle (1.35 ± 0.35). Consequently, an increase in glucose uptake by the oocytes was observed. A significant difference ($p< 0.05$) was noted between the control and vehicle and RA-treated groups with 4.14 ± 0.160; 4.29 ± 0.35; 5.00 ± 0.142; 4.98 ± 0.073 and 5.20 ± 0.079 mean values for control, vehicle, 1, 3 and 5 μM RA, respectively.

Figure 2 depicts the concentration of pyruvate not utilized by the water buffalo oocytes after the period of IVM. The amount of pyruvate detected from the spent culture media was very minimal and not significantly different between the control, vehicle and RA-treated groups with the following mean values expressed in μM concentration: 0.3458 ± 0.17; 0.3160 ± 0.12; 0.3560 ± 0.41; 0.3220 ± 0.025; 0.3520 ± 0.013 for control, vehicle and 1, 3, 5 μM all-trans-RA, respectively. Result suggests possible increased uptake of pyruvate by the COCs both in control and RA-treated groups during in vitro maturation.

Glucose consumption at the end of 24 h IVM period was significantly higher in the RA-treated COCs compared to control whereas; pyruvate uptake was not significantly different among all groups.

Figure 1. Glucose uptake after IVM of water buffalo oocytes supplemented with all-trans RA at 0 (control and vehicle), 1(RA1), 3 (RA2), and 5 (RA3) μM doses. Data are expressed as mean± standard error. Significant differences between control, vehicle and treatment groups are indicated by *($P < 0.05$). n=3 trials.

Figure 2. Pyruvate concentration in spent culture media after IVM in water buffalo oocytes supplemented with all-trans RA at 0 (control and vehicle), 1(RA1), 3(RA2), and 5 (RA3) μM doses. Data are expressed as means± standard error. There were no significant differences among the treatments. Initial pyruvate concentration in the media is 0.2 mM. n=5 trials.
DISCUSSION

The supply of adequate concentration of glucose and pyruvate in the IVM medium is necessary for the development and maturation of oocytes (Krisher and Bavister 1998; Rose-Hellekant et al. 1998; Nandi et al. 2008). The depletion of the glucose and pyruvate in the spent culture media indicates a possible utilization of these energy substrates by the growing COCs during the period of in vitro maturation. The minimal amount of pyruvate left in the oocyte droplets after the 24h IVM period is not surprising since this substrate is readily utilized by the oocyte to fuel its maturation-related activities. A specific action of pyruvate in enhancing oocyte development whether alone or in combination with glucose has been documented in water buffalo (Nandi et al. 2008). The direct effect that pyruvate has on bovine oocytes was demonstrated by the promotion of nuclear maturation and blastocyst development in cumulusfree oocytes in vitro matured in non-serum, pyruvate supplemented medium (Geshi et al. 2000). Previously, it was reported that pyruvate in the IVM medium can also support maturation of denuded mouse oocytes whereas, glucose can do so only when follicular cells are present in the culture medium (Biggers 1967). Furthermore, for proper completion of oogenesis, oxidative metabolism of pyruvate is essential as a vital source of energy during meiotic maturation (Johnson et al. 2007). In the present study, lactate production was not quantified but it is possible that some of the pyruvate present in the IVM media may have been converted to lactate, thus contributing to the low amount of pyruvate detected in the spent culture media.

Glucose concentration in the spent media significantly decreased in the RA-treated samples compared to control and vehicle. Suggesting increased uptake by the growing COCs. Gutnisky et al. (2007) reported that increase in the rate of glucose consumption by COCs occur during the final hour of oocyte IVM in order to generate matrix via the hexosamine biosynthetic pathway (Gutnisky et al. 2007). Roberts et al. (2004) also noted that glucose uptake in murine is highest when COCs contain metaphase II oocytes at the end of the culture. Similarly, Preis et al. (2005), demonstrated that in mouse oocytes during the final hours of maturation fertilizable COCs take up more glucose and produce more lactate than those not subsequently fertilized. These findings are relevant because a significant increase in the proportion of oocytes at M II stage (>84%) was observed in the COCs treated with all-trans RA compared to the control group in a simultaneous experiment utilising the same IVM system. Cumulus cells supply the oocyte with pyruvate by metabolizing glucose through glycolysis. Previous study confirmed that when oocytes are enclosed in cumulus cells, they can complete meiotic maturation (MII) in the absence of exogenous pyruvate, provided that glucose is present in the media (Leese and Barton 1985). Moreover, as demonstrated by Downs and co-workers (1994; 1999) using murine oocytes, it is the pentose phosphate pathway, and not glycolysis, that is the metabolic route that mediates ligand-induced resumption of meiosis.

Interestingly, one of the biological functions of retinoids in vertebrates is the regulation of glucose metabolism by inducing the expression of glucose transporter proteins. Previous studies using insulin secreting cells demonstrated that all-trans RA can induce both insulin release and expression of the glucose transporter (GLUT 2) gene (Blumentrath et al. 2001). More importantly, glucose transport in oocytes is also facilitative and mediated by glucose transporters such as solute carriers SLC2A2, protein symbol GLUT (Zheng et al. 2007; Pisani et al. 2008; Zhao and Keating 2007). Furthermore, Nishimoto et al. (2006) revealed that cumulus cells express an additional glucose transporter SLC2A4 that is insulinsensitive. The facilitative glucose transporter family (solute carriers SLC2A, protein symbol GLUT) mediates a bidirectional and energy-independent process of glucose transport in most tissues and cells (Zhao and Keating 2007) including the COCs.

In the present study, it is highly possible then, that the significant increase in glucose uptake measured from the culture droplets of COCs could be attributed to the release of glucose transporter genes induced by the supplementation of RA. In addition, AMP-dependent protein kinase (AMPK) signalling may also be implicated as a possible mechanism employed by all-trans RA in enhancing the glucose uptake of water buffalo oocytes. This was demonstrated in a previous study using skeletal muscle, retinoic acid activated AMPK, a phylogenetically conserved intracellular energy sensor, resulting to upregulation of glucose uptake in the myotubes (Lee et al. 2008).

The findings of the present research validates the efficiency of all-trans RA in improving the developmental competence of water buffalo oocytes as demonstrated in the concomitant study done using the same IVM systems and in-vitro matured oocytes that resulted to higher blastocyst yield. The non-significant difference in all the RA-treated groups suggests that only low physiological doses of all-trans RA are required during IVM.

SUMMARY AND CONCLUSION

The data presented here suggest a positive relationship between glucose uptake and developmental competence of the water buffalo oocytes. This finding supports the possibility of using glucose uptake in vitro as a
biochemical indicator of the viability and developmental competence of buffalo oocytes before in vitro fertilization. Likewise, all-trans RA has a potential in regulating glucose metabolism and its positive effect on oocyte maturation was more reinforced.

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