Full Length Research Paper

Effect of glucose, lactate and pyruvate concentrations on *in vitro* growth of goat granulosa cell

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Carbohydrates are among the most influential of the numerous components of culture medium that affect metabolism and developmental potential. Glucose, lactate and pyruvate are required for the growth of oocytes and other follicular cells *in vitro*. The aim of this study was to determine the effects of different concentrations of glucose, lactate and pyruvate on promoting DNA synthesis of granulosa cells in a serum-free medium. Effects of glucose (0.75, 1.5 or 3 mM), pyruvate (0.1 or 0.33 mM) and L-lactate (3, 6 or 12 mM) concentrations in the maturation medium on the relative granulosa cell growth during metaphase II (MII) were examined in a 3 × 2 × 3 factorial design. The greatest relative granulosa cell growth response (p<0.05) was observed in the presence of 1.5 mM glucose and 0.33 mM pyruvate or in 6 mM lactate and 0.33 mM pyruvate. Increasing pyruvate concentrations from 0.1 to 0.33 mM resulted in an increase in DNA synthesis in granulosa cells. In conclusion, the results of this study showed that increasing glucose and pyruvate concentrations in the maturation medium increased the growth of goat granulosa cells.

Key word: Energy substrate, granulosa cell growth, methyl-^{3}H-thymidine, goat.

INTRODUCTION

Metabolism plays a crucial role in oocyte quality because glycolytic activity in mature oocytes is correlated with increased embryonic development (Krisher, 2004). Carbohydrates are among the most influential of the numerous components of the culture medium that affect metabolism and developmental potential (Herrick et al., 2006). Glucose, lactate and pyruvate are essential for the growth of oocytes and other follicular cells *in vitro* (Nandi et al., 2008).

High glucose concentration (28 mM) in bovine IVM medium decreased glutathione concentration in the cytoplasm of oocytes and increased intracellular oxygen free radicals (Hashimato et al., 2000). On the other hand, insufficient concentration of glucose led to incomplete nuclear maturation and cumulus expansion. Sutton-McDowall et al. (2003) demonstrated that glucose was a major metabolite used in bovine cumulus oocyte complex in maturation medium. McGowan and Bucher (1983) showed that pyruvate promoted DNA synthesis in serum free primary cultures of rat hepatocytes. It has been shown that pyruvate but not glucose is the main energy used directly by the oocyte (Biggers et al., 1967). It is also known that cumulus cells must be present for resumption of meiosis when glucose is the only energy source present (Eppig, 2005). A study by Nandi et al. (2008) revealed that oocyte maturation was higher in medium containing supraphysiological concentrations of either glucose (5 mM) or pyruvate (10 mM) alone, or physiological concentrations of glucose and lactate and pyruvate in combination. They suggested that pyruvate

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acted both as an energy source and antioxidant for oocytes in the culture medium. Recently, emerging evidence points to the existence of an oocyte-granulosa cell regulatory loop by which complementary signaling and metabolic pathways drive the development and function of both the oocytes and follicular somatic compartments (Su et al., 2008). On the other hand, oocyte regulates folliculogenesis by modulating a broad range of ganulosa cell and cumulus cell functions associated with somatic cell growth and differentiation, primarily achieved through the secretion of soluble growth factors acting locally on these cells (Eppig, 2001; Gilchrist et al., 2004). However, few studies have examined the effects of changing the energy substrates of the maturation medium on follicular cell proliferations (Nandi et al., 2008).

Therefore, the aim of the present study was to determine the effects of different concentrations of glucose, lactate and pyruvate on promoting DNA synthesis of goat ganulosa cell in a serum-free medium.

MATERIALS AND METHODS

Collection of ovaries and preparation of culture medium

Goat ovaries were collected from a local abattoir and transported to the laboratory at 30-35°C in physiological saline (0.9% NaCl) containing 100 µg/ml streptomycin and 100 IU/ml penicillin within 2 to 3 h after collection. Ovaries were washed five times with warm fresh physiological saline. Follicles between 2 and 6 mm in diameter were dissected under sterile conditions using a surgical blade in plastic Petri dishes containing warm (37°C) HEPES-buffered synthetic oviduct fluid (SOF) supplemented with 1.5 mM glucose, 0.33 mM pyruvate, 1.0 mM glutamine, 3.0 mM L-lactate, 1x nonessential minimal essential medium (MEM) amino acids, 1x essential MEM amino acids, and 1.0 mg/ml BSA (Herrick et al., 2006). High-quality cumulus-oocyte complexes (COCs) with multilayered compact cumulus cells and evenly granulated cytoplasm were rinsed 4 to 5 times in SOF-HEPES washing solution. After preparation, the washing and maturation media were filtered through sterilized Sartorius filter with a 0.22-mm pore membrane. The media were adjusted to 280 mOsm.

Modified SOF maturation medium was used as the basal medium in which glucose, L-lactate and pyruvate concentrations were manipulated. Defined maturation medium contained 1.0 mM alanyl-glutamine, 0.1 mM taurine, 1× NEAA, 0.5× EAA, 1× MEM vitamins, insulin-transferrin-selenium (5 µg/ml I, 5 µg /ml T, and 5 ng/ml S), 0.1 mM cysteamine, 50 mg/ml gentamicin, 50 ng/ml EGF (Roche Applied Science), 0.5 mM citrate, 4 mg/ml BSA and 0.01 IU/ml each of ovine LH and ovine FSH. The maturation medium was equilibrated with CO_2 by placing in a CO_2-incubator (Memmert, Germany) for 2 h before the oocytes were transferred. The COCs together with granulosa cells were matured in a CO_2-incubator containing 5% CO_2 in air and 96% humidified environment.

Preparation and culture of granulosa cells

Large antral follicles in intact ovaries were punctured with an 18-gauge needle and mural granulosa cells were gently harvested from follicles. Cells were washed twice in HEPES-buffered SOF and twice in culture media. Mural ganulosa cells together with 16

cumulus-oocyte complex were co-cultured in Nunc 96-well tissue culture plates at a density of 2×10^5 in culture volume of 100 µl. After 18 h of culture, 1 µCi/ml methyl-^{3}H-thymidine (ICN) was added to each well and the culture continued for an additional 6 h after which the cells were harvested with a cell harvester onto a thin filter mat. Filter mats were then saturated with scintillation fluid, and counted with a Wallac microbeta scintillation counter.

Experimental design and data analysis

Effects of glucose (0.75, 1.5 and 3 mM), pyruvate (0.1 and 0.33 mM) and L-lactate (3, 6 and 12 mM) concentrations in the maturation medium on the relative granulosa cell growth during metaphase II (MII) were examined in a $3 \times 2 \times 3$ factorial design. Statistical analysis was carried out using SAS software (SAS Version 9.1, SAS Institute Inc., Cary, NC) by using the general linear model (GLM) procedure, followed by Tukey's test for comparison of means ($P < 0.05$). Data were expressed as mean ± SEM.

RESULTS AND DISCUSSION

The first-order significant interactions between glucose, lactate and pyruvate were included in the data analysis because the second-order interactions were not significant. There was a significant interaction ($p<0.05$) between the energy substrates on the relative granulosa cell growth during metaphase II (Tables 1 and 3). The interaction effect of glucose and lactate concentrations on relative granulosa cell growth was not significant (Table 2). The greatest relative granulosa cell growth responses ($p<0.05$) were observed following maturation in 1.5 mM glucose and 0.33 mM pyruvate (Table 1) and 6 mM lactate and 0.33 mM pyruvate (Table 3). Increasing pyruvate concentrations from 0.1 to 0.33 mM promoted increases in DNA synthesis in the granulosa cells (Tables 1 and 3).

In this study, we used a defined medium to determine the effect of different glucose, lactate and pyruvate concentrations on the relative granulosa cell growth responses. A chemically defined medium is useful for analyzing the physical action of substances such as inorganic compounds, energy substrates (glucose, lactate and pyruvate), hormones, cytokines and vitamins on oocyte maturation and the development of pre-implantation embryos, because it eliminates undefined factors present in serum or serum albumin (Bavister, 1995; Herrick et al., 2006). Although a role for energy substrates has been stated for oocyte competence and embryo development (Thompson, 2006), little is known about the effects of primary nutrients (glucose, lactate and pyruvate) on cell growth response.

It was shown that the presence of 1.5 mM glucose and 0.33 mM pyruvate in the maturation medium resulted in the highest mural granulosa cell [3H] counts than in other treatments. Both low and high glucose concentrations had detrimental effect on follicular cell growth and oocyte maturation. Inadequate glucose during IVM leads to impaired completion of nuclear maturation (Hashimato et

Table 1. Effects of glucose and pyruvate concentrations on relative granulosa cell growth (cpm/2×10⁵) during *in vitro* maturation (mean ± SEM).

Pyruvate (mM)	Glucose (mM)			Main effect of pyruvate
	0.75	1.50	3.00	
0.10	1.01 ± 0.02^{b}	0.98 ± 0.02^{b}	1.00 ± 0.04^{b}	0.99 ± 0.02
0.33	1.00 ± 0.03^{b}	1.15 ± 0.05^{a}	1.11 ± 0.06^{a}	1.09 ± 0.03
Main effect of glucose	1.00 ± 0.02	1.07 ± 0.03	1.05 ± 0.03	

Different superscripts indicate significant ($P<0.05$) difference amongst glucose or pyruvate interactions (means comparison by Tukey's test).

Table 2. Effects of different glucose and lactate concentrations on relative granulosa cell growth (cpm/2×10⁵) during *in vitro* maturation (mean ± SEM).

Lacate (mM)	Glucose (mM)			Main effect of lactate
	0.75	1.50	3.00	
3.00	0.97 ± 0.02	1.00 ± 0.05	1.12 ± 0.09	1.03 ± 0.03
6.00	0.99 ± 0.04	1.10 ± 0.05	1.07 ± 0.06	1.06 ± 0.03
12.00	1.04 ± 0.03	1.09 ± 0.08	0.98 ± 0.03	1.03 ± 0.03
Main effect of glucose	1.00 ± 0.02	1.07 ± 0.03	1.05 ± 0.03	

Different superscripts indicate significant ($P<0.05$) difference amongst glucose or lactate interactions (means comparison by Tukey's test).

Table 3. Effects of different pyruvate and lactate concentrations on relative granulosa cell growth (cpm/2×10⁵) during *in vitro* maturation (mean ± SEM).

Pyruvate (mM)	Lactate (mM)			Main effect of pyruvate
	3.00	6.00	12.00	
0.10	0.99 ± 0.03^{b}	1.02 ± 0.04^{b}	0.98 ± 0.02^{b}	0.99 ± 0.02
0.33	1.07 ± 0.06^{a}	1.10 ± 0.04^{a}	1.09 ± 0.05^{a}	1.09 ± 0.03
Main effect of glucose	1.03 ± 0.03	1.06 ± 0.03	1.03 ± 0.03	

Different superscripts indicate significant ($P<0.05$) difference amongst lactate or pyruvate interactions (means comparison by Tukey's test).

al., 2000) and cumulus expansion, probably due to insufficient substrates for hyaluronic acid synthesis (Sutton-McDowall, 2005).

Oocytes cultured in high glucose concentrations (10 to 28 mM glucose) had decreased ooplasmic glutathione concentrations and increased intracellular reactive oxygen species (Hashimato et al., 2000). Also, maternal diabetes adversely affected oocyte maturation and follicular somatic cell growth (Chang et al., 2005). Hyperglycemia inhibits thymidine incorporation and cell proliferation in human umbilical vein endothelial cells, and this is associated in increased PKC and eNOS activity, cGMP and cAMP levels and p42/44mapk phosphorylation (Rojas et al., 2003).

Pyruvate deficiency induces cell death in both primary rat calvarial cells and in the clonal murine osteoblastic cell line MC3T3-E1 at the proliferative stage (Hinoi et al., 2002). In this study, the growth of follicular somatic cells in the medium containing 0.33 mM pyruvate and 6 mM

lactate were significantly higher that than in the other treatments. Increasing the pyruvate concentration from 0.1 to 0.33 mM in the maturation medium significantly ($p<0.05$) amplified DNA incorporation into the granulosa cells. This agrees with Nandi et al. (2008) who showed that pyruvate had a bi-functional role both as an energy substrate and as an antioxidant. Also, Downs and Hudson (2000) reported that the medium containing pyruvate as the sole metabolite decreased the maturation rate of oocytes.

In this study, the interaction effect of glucose and lactate concentrations on the relative granulosa cell growth was not significant. Our results are in agreement with that of Nandi et al. (2008) who reported that glucose or pyruvate supply energy to the follicular cells, and those follicular cells did not particularly rely on lactate as a source of energy. However, lactate may have additional, non-metabolic roles in the acidification of the local environment (Leese and Lenton, 1990). Funahashi et

(2008) showed that high concentrations of pyruvate (5.55 mM) in IVM medium significantly increased the intracellular GSH content of the porcine oocyte. They concluded that both glucose and pyruvate play a critical role in the release of oocyte from arrest at the germinal vesicle (GV) stage, probably through the PPP pathway, whereas supplementation with pyruvate improved the cytoplasmic maturation as determined by oocyte glutathione level.

Oocytes are powerful stimulator of bovine mural granulosa cells DNA synthesis (Li et al., 2000; Gilchrist, et al., 2003). Gilchrist et al. (2003) showed that oocyte secreted growth factors (TGF-b1 and TGF-b2) and denuded oocytes significantly increased ^{3}H-thymedine incurporation in mural granulosa cells. Most likely, oocyte growth factors directly or indirectly impact on the growth of granulosa cells.

In conclusion, our finding showed that increasing medium glucose and pyruvate concentrations, but not lactate, affected the relative growth of granulosa cells, implying that these cells use glucose and pyruvate as their preferred energy substrates.

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