Development of improved procedures for in vitro maturation of oocytes collected from prepubertal goats has applications for in vitro embryo production and accompanying strategies for genetic improvement. The objective of described studies was to determine the effects of oocyte grade, in vitro maturation time, antioxidant supplementation and concentrations of estradiol in the maturation medium on in vitro maturation of oocytes harvested from 1-6 mm follicles present on the ovaries (obtained from an abattoir) of 1-6-month-old prepubertal Boer goats. Rates of progression to metaphase II were greater for Grade 1 oocytes (>3 compact layers of cumulus cells and evenly granulated cytoplasm) than Grade 2 oocytes (<3 layers cumulus cells and evenly granulated cytoplasm) and were lowest for Grade 3 oocytes (devoid of cumulus cells with abnormal cytoplasm). No significant effects of maturation for 24 vs 30 h on progression of oocytes to metaphase II were observed. The addition of 5 μM β-mercaptoethanol to maturation medium increased the proportion of oocytes progressing to metaphase II and glutathione content in such oocytes, whereas supplementation with 10 mM hypotaurine was without effect. A significant inhibitory effect of in vitro maturation in the presence of high concentrations of estradiol (10 and 100 μg/mL) on progression to metaphase II was observed, and no effect was observed in response to 1 μg/mL estradiol treatment as compared with control. Results suggest that oocyte selection and β-mercaptoethanol supplementation can positively influence progression to metaphase II of oocytes harvested from ovaries of prepubertal goats, whereas high concentrations of estradiol are inhibitory to in vitro maturation.

Introduction

Goats are a prominent food source and critical to the livestock industry in China, particularly in rural agricultural regions. Goat inventories in China are estimated at over 1x10⁹ animals nationwide (Yonghong, 2002), the vast majority of which represent indigenous breeds. Boer goats were introduced into China in 1995 and offer advantages over indigenous breeds in terms of size, meat quality, disease resistance and other production traits (Yonghong, 2002). Practical and effective strategies for distribution of Boer goat genetics in China have not been widely implemented.

Production of Boer goat embryos in vitro and transfer into recipient females of indigenous breeds in rural areas represents one potential strategy for genetic improvement. Ovaries from 1-6-month-old animals slaughtered for meat represent a readily available source of oocytes for in vitro embryo production. The use of prepubertal animals as embryo donors holds significant potential for reducing generation intervals and increasing the rate of genetic gain through embryo transfer (Duby et al., 1996). However, developmental competence of oocytes from prepubertal animals is known to be compromised in several species (Eppig and Schroeder, 1989; Salamone et al., 2001; Leoni et al., 2007).

Our long-term goal is to develop effective procedures for in vitro production and subsequent transfer of embryos derived from oocytes of prepubertal Boer goats. Successful in vitro embryo production in prepubertal animals is dependent on development of effective procedures for in vitro meiotic maturation of oocytes harvested from such animals. Overall, efficiency of in vitro maturation of goat oocytes is still far from optimal and dramatically influenced by culture conditions (Mermillod et al., 1999; Cognie et al., 2003). We hypothesize that oocyte selection, duration of in vitro maturation, and (or) supplementation of maturation medium with antioxidants or estradiol will positively influence the percentage of oocytes harvested from ovaries of prepubertal Boer goats that progress to metaphase II stage during in vitro maturation. Above variables have been previously shown to significantly impact in vitro maturation of oocytes in other species (de Matos et al., 1997; Beker et al., 2002) and (or) oocytes harvested from postpubertal goats (Han et al., 2006; Katska-Książkiewicz et al., 2007), but their impact on in vitro maturation of oocytes harvested from prepubertal Boer goats has not been investigated previously, to our knowledge.

Materials and methods

Collection of cumulus-oocyte complexes

Ovaries of 1-6-month-old Boer goats with no visible corpora lutea were harvested at an abattoir and transported to the laboratory at 20-30°C in sterile 0.9% saline containing 0.3 mg/mL gentamicin (Hua Bei Medicine Factory, Shijiazhuang City, China). Upon return to the laboratory (within 3 hr), ovaries were washed 3 times in sterile 0.9% NaCl and two times in phosphate buffered saline, (DPBS, Ca²⁺ and Mg²⁺ free) containing 1 mg/mL L-glucose, 0.1 mg/mL penicillin (Hua Bei Medicine Factory), 0.1 mg/mL streptomycin (Hua Bei Medicine Factory) and 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA USA) at 38.5°C. Oocytes were liberated from all visible 1-6 mm follicles via aspiration using a syringe and 16 gauge needle and cumulus oocyte complexes (COCs) selected for in vitro maturation (IVM) under a stereomicroscope and classified into quality grades as described below. The COCs were then washed 3

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times in DPBS and 2 times in maturation medium (TCM 199/Hepes; Invitrogen) supplemented with 2.2 mg/mL sodium bicarbonate, 0.05 mg/mL gentamicin, 10 µg/mL FSH (Ning Bo Hormone Factory, China), 10 µg/mL LH (Ning Bo Hormone Factory), 1 µg/mL estradiol (Sigma-Aldrich; St. Louis, MO USA), unless specified otherwise below, and 10% FBS prior to in vitro maturation as described below.

Effect of quality grade on in vitro maturation of prepubertal goat oocytes

To determine the effect of morphological indicators of oocyte quality on in vitro maturation of prepubertal goat oocytes, oocytes were classified and grouped into one of three quality grades derived from previously described criteria for morphological assessment of oocyte quality (Pawese et al., 1994). The Grade 1 oocytes were surrounded with at least three complete and compact layers of cumulus cells and had an evenly granulated cytoplasm. The Grade 2 oocytes were surrounded by less than three layers of cumulus cells and (or) contained a partly denuded zona pellucida but had a normal appearing cytoplasm. The Grade 3 oocytes had non-evenly granulated cytoplasm, abnormal conformation, and were surrounded by little or no cumulus cells. All remaining oocytes which did not fit clearly into one of three above categories were excluded and not utilized for experimental analysis. Groups of 8-10 oocytes from each quality grade (Grade 1, 2 and 3) were cultured separately in 50 µL microdrops of basal maturation medium (under paraffin oil) containing 0.1% polyvinyl alcohol (PVA) instead of FBS. Culture in defined media lacking serum was performed to prevent potential confounding effects of growth factors present in serum on measurement of effects of antioxidant treatments on oocyte glutathione content after in vitro maturation and associated effects on progression to metaphase II stage. Groups of 8-10 Grade 1 oocytes were cultured (under paraffin oil) in 50 µL microdrops containing PVA supplemented maturation medium (described above) in the presence or absence of 10 mM hypotaurine or 5 µM β-mercaptoethanol (n=88, 84 and 103 oocytes, respectively). Cultures were conducted at 38.5°C in a humidified atmosphere of 5% CO₂ for 27 h until determination of percent progression to metaphase II as described above. For each replicate, a subset of 10 metaphase II oocytes in each treatment group were subjected to glutathione assay according to previously published procedures (de Matos et al., 1997), to determine whether antioxidant treatments resulted in increased glutathione content in matured oocytes.

Effect of estradiol concentrations on prepubertal goat in vitro oocyte maturation

To determine the influence of estradiol concentrations on in vitro maturation, groups of 8-10 Grade 1 oocytes were cultured in 50 µL microdrops of maturation medium (under paraffin oil) containing 0, 1, 10 or 100 µg/mL estradiol. Oocytes were cultured at 38.5°C in a humidified atmosphere of 5% CO₂ for 24, 27 or 30 h (n=120, 117 and 98 oocytes per time-point, respectively). Percent of oocytes in each group that had progressed to metaphase II within denoted period of in vitro culture was determined as described above.

Effect of hypotaurine and β-mercaptoethanol supplementation on prepubertal goat oocyte maturation

To determine the effects of antioxidant supplementation on meiotic maturation of prepubertal goat oocytes, Grade 1 oocytes were cultured in 50 µL microdrops of basal maturation medium (under paraffin oil) containing 0.1% polyvinyl alcohol (PVA) instead of FBS. Culture in defined media lacking serum was performed to prevent potential confounding effects of growth factors present in serum on measurement of effects of antioxidant treatments on oocyte glutathione content after in vitro maturation and associated effects on progression to metaphase II stage. Groups of 8-10 Grade 1 oocytes were cultured (under paraffin oil) in 50 µL microdrops containing PVA supplemented maturation medium (described above) in the presence or absence of 10 mM hypotaurine or 5 µM β-mercaptoethanol (n=88, 84 and 103 oocytes, respectively). Cultures were conducted at 38.5°C in a humidified atmosphere of 5% CO₂ for 27 h until determination of percent progression to metaphase II as described above. For each replicate, a subset of 10 metaphase II oocytes in each treatment group were subjected to glutathione assay according to previously published procedures (de Matos et al., 1997), to determine whether antioxidant treatments resulted in increased glutathione content in matured oocytes.

Statistical analysis

All experiments were replicated three times using oocytes collected on different days. Effects of treatments on percent of oocytes progressing to metaphase II stage and on oocyte glutathione content were determined using one way analysis of variance using the general linear model (GLM) procedure of SAS with binomial percent data (progression to metaphase II) arcsine transformed prior to analysis. Differences between treatment means were compared using Fisher’s protected least significant differences test. Results for binomial data are depicted as the mean +/- SE of data prior to transformation.

Results

The in vitro maturation ability of oocytes of different quality grades

The relationship between quality grade and in vitro maturation of oocytes harvested from prepubertal Boer goats is depicted in Table 1. Progression to metaphase II stage was noted for 68.6% of Grade 1 oocytes subjected to in vitro maturation and a 29% reduction in maturation rate was observed for Grade 2 oocytes (P<0.05). For the lowest quality oocytes (Grade 3) progression to metaphase II was further reduced relative to Grade 1 and 2 oocytes.

Investigation of potential time dependent effects on in vitro maturation of prepubertal goat oocytes

The effect of maturation time (length of culture) on ability of Grade 1 oocytes harvested from 1-6 month-old goats to resume meiosis and progress to metaphase II stage was also investigated. As depicted in Table 2, while the percent of oocytes reaching metaphase II stage was reduced relative to Grade 1 oocytes, there was no significant difference between the Grade 2 and 3 oocytes.

Table 1. Effect of oocyte selection on progression to metaphase II (MII).

| Quality grade | Total oocytes | MII oocytes | % MII oocytes* |
|---------------|---------------|-------------|----------------|
| 1             | 245           | 168         | 68.6±6.2*      |
| 2             | 150           | 65          | 43.3±1.7*      |
| 3             | 191           | 21          | 10.9±1.8*      |

*Mean±SE. **P<0.05.
was maximal following 30 h of in vitro maturation, percent of oocytes that reached metaphase II stage following 24, 27 and 30 h was not significantly different in the current studies.

Effects of hypotaurine and \(\beta\)-mercaptoethanol supplementation on in vitro maturation of prepubertal goat oocytes

Treatment with 10 mM hypotaurine did not enhance progression of oocytes to metaphase II during in vitro maturation (Table 3), but supplementation with 5 \(\mu\)M \(\beta\)-mercaptoethanol did enhance the percentage of oocytes that progressed to metaphase II (P<0.05). Assay of oocyte glutathione content demonstrated that treatment with \(\beta\)-mercaptoethanol but not hypotaurine resulted in elevated glutathione concentrations in resulting oocytes that progressed to metaphase II (Figure 1).

Effects of different estradiol concentrations in maturation media on progression of prepubertal goat oocytes to metaphase II

As depicted in Table 4, treatment with the lowest dose of estradiol tested (1 \(\mu\)g/mL) had no effect on progression to metaphase II stage of Grade 1 oocytes harvested from 1-6 month-old animals. However, meiotic maturation was reduced in response to treatment with 10 and 100 \(\mu\)g/mL estradiol (P<0.05).

Discussion

Ability to resume meiosis is one of the early developmental milestones used to define oocyte competence (Sirard et al., 2006). Results of described studies indicate that among the variables examined, oocyte selection had the single greatest impact on successful progression to metaphase II of oocytes harvested from 1-6 month-old Boer goats. Similar rates of progression to metaphase II as observed in the present studies were previously reported for in vitro matured goat oocytes of abattoir origin, but age of animals was not specified (Rho et al., 2001). Furthermore, similar rates of in vitro maturation to metaphase II of prepubertal vs adult goat oocytes have been reported previously (Martino et al., 1995; Mogas et al., 1997a). Thus, there is an important role for oocyte selection prior to in vitro maturation of oocytes from adult animals as well. In fact, visual selection of oocytes for in vitro maturation was the most important factor determining embryo production efficiency across multiple sires, when oocytes harvested from FSH primed adult goats were utilized for in vitro embryo production (Katska-Książkiewicz et al., 2007). Oocyte selection prior to in vitro maturation for in vitro embryo production is critical because oocytes obtained from abattoir derived ovaries of adult and prepubertal animals are highly variable in their developmental competence. This heterogeneity is attributable in part to the different stages of oocyte growth and atresia within and between animals (Han et al., 2006). Given the pronounced differences in maturation rates for Grade 1 vs Grade 2 oocytes, results also indicate extent and integrity of the cumulus cell layer are major determining factors in successful in vitro maturation of prepubertal goat oocytes. The interaction between the oocyte and its surrounding cumulus cells is critical to maintenance of oocyte developmental competence (Eppig, 1991).

The effect of in vitro maturation time on proportion of prepubertal goat oocytes progressing to metaphase II stage was also examined in the current study. Increased rates of meiotic maturation of goat oocytes were observed in response to 27 vs 24 h incubation in previous studies (Martino et al., 1994; Rho et al., 2001). No significant effects of matura-
tion for 24 vs 27 vs 30 h on progression of oocytes to metaphase II (nuclear maturation) were observed in this study. The reasons for this discrepancy with previous studies are not clear. However, it will be important in future studies to also determine effects of maturation time on ability of prepubertal goat oocytes to develop to the blastocyst stage following fertilization (cytoplasmic maturation).

Estradiol is a common component of in vitro maturation media used in the goat and other species. Concentrations of 1 μg/mL are commonly added to oocyte maturation medium in the goat (Mogas et al., 1997a; Katska-Ksiazkiewicz et al., 2004) and bovine (Beker et al., 2002), presumably to mimic concentrations present in prevulatory follicles. However, to our knowledge, dose dependent effects of estradiol supplementation on in vitro maturation of oocytes collected from 1-6 month-old prepubertal goats have not been previously investigated. Treatment with 1 μg/mL estradiol did not increase proportion of oocytes progressing to metaphase II in the current study. Treatment with 1 μg/mL estradiol, in the absence of FSH, was inhibitory to in vitro maturation of bovine oocytes and did not enhance FSH induced in vitro bovine oocyte maturation (Beker et al., 2002). Furthermore, supplementation with higher concentrations of estradiol (10 and 100 μg/mL) during in vitro maturation was found to be inhibitory to progression to metaphase II, which may represent the most significant new information derived from the current study. Inhibitory effects of estradiol on nuclear maturation of in vitro matured bovine oocytes have been observed previously (Beker-van Woudenberg et al., 2004), but only a single dose of estradiol was tested in either of the aforementioned studies. Furthermore, although cause-effect relationships have not been directly established, abnormalities in oocyte maturation have been observed with superovulation procedures in the goat that yield highly superphysiological concentrations of estradiol in follicular fluid (Kumar et al., 1992). Collectively, results indicate that very high concentrations of estradiol are inhibitory to nuclear maturation of oocytes collected from 1-6 month-old prepubertal goats. While beneficial effects of supplementation with 1 μg/mL of estradiol on progression of in vitro matured oocytes to metaphase II were not observed in the present study, potential beneficial effects on cytoplasmic maturation and subsequent embryonic development following fertilization cannot be ruled out without further investigation.

Glutathione is an important scavenger of free oxygen radicals and protects cells against oxidative damage. Intracellular glutathione levels in mature oocytes help mediate sperm DNA decondensation and male pronucleus formation post fertilization in several species (Perreault et al., 1988; Yoshida et al., 1993; Sutovsky et al., 1997). The lower than desirable rates of blastocyst development reported following in vitro maturation and in vitro fertilization of prepubertal goat oocytes are associated with abnormal sperm head decondensation at fertilization (Martino et al., 1995; Mogas et al., 1997b). Furthermore, oocyte glutathione content is an indicator of cytoplasmic maturation (Funahashi et al., 1994; de Matos et al., 1997) and glutathione levels are higher in prepubertal goat oocytes that progressed to metaphase II stage during in vitro maturation than in those ones that did not reach metaphase II (Rodriguez-Gonzalez et al., 2003). To determine potential beneficial effects of antioxidant supplementation on nuclear maturation of prepubertal goat oocytes, the effects of β-mercaptoethanol and hypnotaurine supplementation (in defined media lacking serum) on progression to metaphase II and oocyte glutathione content in resulting metaphase II oocytes were tested. A beneficial effect of β-mercaptoethanol, but not hypnotaurine supplementation on progression to metaphase II and glutathione content in resulting metaphase II oocytes was observed, suggesting that scavenging of free oxygen radicals and (or) other cellular functions linked to glutathione, may impact nuclear and cytoplasmic maturation of prepubertal goat oocytes. Hypotaurine is a potent antioxidant (Aruoma et al., 1988), but was without effect on progression to metaphase II in the current study. However, additional experimentation (including dose response studies) would be required to determine whether proposed beneficial effects of elevated glutathione levels (in response to β-mercaptoethanol treatment) on progression to metaphase II are independent of its antioxidant properties. While beneficial effects of β-mercaptoethanol supplementation on progression to metaphase II and glutathione content in oocytes matured in defined medium (lacking FBS) were observed, it is important to note that overall rates of progression to metaphase II for treated oocytes were less than observed for control oocytes matured in medium containing FBS in other experiments reported here. Hence, further experimentation will be required to enhance overall maturation rates of oocytes matured in defined medium or to confirm beneficial effects of β-mercaptoethanol treatment on progression to metaphase II of oocytes cultured in the presence of FBS in our system.

Conclusions

In summary, results of the present study support our hypothesis and indicate that oocyte selection and antioxidant (β-mercaptoethanol) supplementation positively influence percentage of oocytes from prepubertal Boer goats progressing to metaphase II stage during in vitro maturation. However, no significant effect of culture time on nuclear maturation was observed and supplementation with high concentrations of estradiol was found to be inhibitory. Development of effective procedures for in vitro maturation of oocytes from prepubertal animals is a limiting factor in application of in vitro embryo production as a tool for propagation of Boer goat genetics. Results of described study enhance our knowledge of factors that influence ability of oocytes of 1-6 month-old Boer goats to reach metaphase II stage during in vitro maturation. Results also form the platform for future studies to test interactions between described treatments to further optimize success of in vitro maturation procedures and to test factors that influence in vitro fertilization and development of embryos (derived from prepubertal Boer goat oocytes matured in vitro) to a stage of development appropriate for laparoscopic transfer into the uterus.

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