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Effect of culture temperature and gaseous phase on embryo development in goat

R K SINGH[1], SADHAN BAG[2], B C DAS[3] and A C MAJUMDAR[4]

Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

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ABSTRACT

The present study was carried out to study the effect of different gaseous phase and culture temperature on *in vitro* oocyte maturation, fertilization and embryo development in goat. Goa oocytes were matured in TCM-199 with HEPES, 10% FBS, 10% follicular fluid, 0.5 µg/ml FSH, 10 IU/ml LH, 0.5 µg/ml estradiol for 27 h. The mature ooctyes were fertilized with freshly ejaculated sperm in TALP media and cultured in mSOF media without any cell supplementation. The *in vitro* maturation, fertilization and culture (IVMFC) were done at different gas phases (C_1=5% CO_2, 20% O_2; C_2=5% C_2, 10% O_2; C_3=7.5% CO_2, 20% O_2; C_4=7.5% CO_2, 10% O_2) at 38.5°C as well as 40.5°C in CO_2 incubator with maximum humidity. The oocyte maturation rate was 82.78, 87.57, 75.71 and 75.65%, respectively, when oocytes were cultured at C_1, C_2, C_3 and C_4 at 38.5°C. The oocyte maturation rate was significantly higher in C_2 as compared to C_3 and C_4. Although there was no significant variation of cleavage rate among the 4 gaseous conditions, it was comparatively higher in C_1 (9.44%) as compared to C_2, C_3 and C_4.

The development of 4–8 cell and morula stage embryos were also not significantly different among these different gas phases. However, the number of 4–8 cell and morula stage embryos was comparatively higher in C_1 condition. The percentage development of 4–8 cell stage embryos was comparatively lower in C_4 conditions as compared to C_1, C_2 and C_3. Similarly, the rate of development of morula stage embryos was comparatively lower (1.13%) in C_2 condition as compared to C_1, C_3 and C_4. It was also observed that the oocyte maturation rate, cleavage rate and embryo development was lower in 40.5°C as compared to 38.5°C. The present study indicated that alteration of gaseous phase did not have any significant effect on oocytes maturation and embryo development although the maturation rate was significantly higher in 5% CO_2 and 10% C_2 in goat when oocytes were cultured at 38.5°C. Further it was also observed that higher culture temperature had significantly detrimental effect on the success of IVMFC in goat.

Key words: Embryo development, Gas phases, Goat, High temperature, IVMFC

Decreasing O_2 percentage and increasing CO_2 percentage improved the *in vitro* development of embryos in different species (Carney and Bavister 1987, Karja *et al.* 2004). Although, Brinster (1971) found no significant differences in development when mouse embryos were cultured under 1, 5 or 10% CO_2, but Carney and Bavister (1987) reported an approximate doubling in the percentage of embryos developed to the blastocyst stage upon raising the concentration of CO_2 gas phase from 5 to 10% CO_2 at 37°C. Oxygen tension during culture was one of the important factors affecting both oocyte maturation and embryo development (Khurana and Niemann 2000, Van Soom *et al.* 2002). The efficiency of oocyte or embryo culture could be improved by decreasing oxygen tension (Berthelot and Terqui 1996), whereas some reported contradictory results (Machaty *et al.* 1998, de Matos *et al.* 2002). Park *et al.* (2005) porcine (23% vs 13%) after IVF than the low oxygen (7%). Heat stress has been considered as one of the most important factors that reduced the fertility in different fertility by heat stress is early embryonic mortality (Roman-Ponce *et al.* 1978, Gwazdauskas *et al.* 1970, Trout *et al.* 1988). Similarly, increasing culture temperature during IVMFC has also been reported to cause generation of free radicals and peroxide production in embryos during *in vitro* culture at higher temperature (Arechiga *et al.* 1994, Ealy *et al.* 1992). Exposure of maturing oocytes to elevated temperatures disrupted spindle formation during metaphase I in mice (Baumgartner and Chrisman 1987). In the present study the effect of varied CO_2 and O_2 tension in different temperature on *in vitro* oocyte maturation and embryo development were carried out in goat.

MATERIALS AND METHODS

Ovaries were collected from local slaughterhouse, Bareilly, from goats of unknown reproductive status. They were carried to the laboratory in normal saline solution

Present address: [1]Ph.D. Scholar, [2,3]Senior Scientist, [4]Principal Scientist, Physiology and Climatology Division.

(0.85%) fortified with antibiotics (5 µg/ml gentamicin) in a thermos flask within 2 h of slaughter at 35–37°C. In the laboratory, ovaries were washed 5-times with normal saline solution fortified with antibiotics. Oocytes were aspirated from all visible non-atretic follicles with 18 gauge needle attached to 5 ml sterile plastic syringe containing oocyte collection medium (OCM). The cumulus oocytes complexes (COCs) along with follicular fluid were pooled into a 50 ml sterile plastic tube and were allowed to settle for 10 min. About 35 ml of supernatant was gently replaced with 35 ml of fresh OCM. This process was repeated twice. Finally, the sediments wee taken in a large Petridish (90 mm) and oocytes were searched under a sterio-zoom microscope. The cumulus oocyte complexes (COCs) were isolated, evaluated and graded. Only excellent (>5 layers of cumulus cells and evenly granulated cytoplasm) and good (>3 layers of cumulus cells and evenly granulated cytoplasm) COCs were collected and were washed in a sterile petri-dish containing 4 ml of OCM. The COCs were further drop washed 5–6 times with maturation medium. Throughout the experiment, two CO_2 water jacketed incubators were used. The temperature and CO_2 level were changed in both incubators during the experiment as follows:

1. 38.5°C temperature and 5% CO_2 , 20% O_2 (C_1)
2. 38.5°C temperature and 5% CO_2 , 10% O_2 (C_2)
3. 38.5°C temperature and 7.5% CO_2 , 20% O_2 (C_3)
4. 38.5°C temperature and 7.5% CO_2 , 10% O_2 (C_4)
5. 40.5°C temperature and 5% CO_2 , 20% O_2 (C_5)
6. 40.5°C temperature and 5% CO_2 , 10% O_2 (C_6)
7. 40.5°C temperature and 7.5% CO_2 , 20% O_2 (C_7)
8. 40.5°C temperature and 7.5% CO_2 , 10% O_2 (C_8)

The level of oxygen tension was maintained by replacing air in incubator with nitrogen gas. The level of carbon-di-oxide (CO_2) was maintained by CO_2 cylinder connected with incubator. In each culture condition at least three experiments were repeated. The washed COCs were divided into 2 groups for both CO_2 incubators and cultured for 27 h to obtain the optimum rate of fertilization. They were cultured in 50 µl droplets (15–20 oocytes/droplets) of maturation medium (TCM-199 with HEPES, 10% FBS, 10% follicular fluid, 0.5 µg/ml FSH, 10 IU/ml LH, 0.5 µg/ml estradiol) in a 35 mm sterile petri-dish. The droplets were covered with warm non-toxic mineral oil and cultured at different culture conditions as above gas phases and temperatures. The evaluation of matured oocytes after *in vitro* culture for 27 h was based on the visual assessment of degree of cumulus expansion under inverted microscope as per criteria described by Kobayashi *et al.* (1994). The semen was freshly collected from adult buck of proven fertility maintained in the Divisional Animal shed. After collection and transportation to the laboratory at 38°C within 10 min, the semen quality were assessed by the gross motility and wave like motion. The semen with > 70% initial motility and +4 motion were used for IVF. About 20 µl of ejaculated semen was taken in a centrifuge tube. About 5 ml of fertilization TALP was added to it and centrifuged at 1000 rpm for 5 minutes. Supernatant was discarded. The sperm pellet was reconstituted with 5 ml of fresh medium and centrifuged at the same rate. The supernatant was discarded again and sperm pellet was then reconstituted to 2 million spermatozoa/ml. The sperm number was counted by counting chamber. Semen sample was kept at 38.5°C and 5% CO_2 in humified air until co-incubation with oocytes. After maturation, the oocytes (degree 1 and 2 of cumulus expansion) were washed several times in fertilization TALP groups were transferred into 50µl of fertilization- TALP medium and were inseminated with 50 µl of processed spermatozoa. The droplets were covered with warm paraffin oil and they were kept in CO_2 incubator as above (2.5). The co-incubation was done for 18 h. After 18 h of sperm-oocyte co-incubation, the oocytes (presumptive zygotes) were separated and cultured in modified synthetic oviductal fluid (mSOF) supplemented with 0.8% BSA. The cleavage rate was observed after 42–44 h of post insemination. The cleaved embryos (2–4 cell stage) were separated and put for culture in the same media and same environment from which they were separated as detailed out in the experimental design for a period of 7 days. Every alternate day, the embryos were examined under an inverted compound microscope to evaluate the developmental stages.

Analyses of the data were done as per the standard procedure (Snedecor and Cochran 1989). The percentage proportion of agreement was compared statistically through normal deviate test.

RESULTS AND DISCUSSION

When oocytes were cultured at different gaseous phases, viz. C_1, C_2, C_3 and C_4 at 38.5°C, the oocyte maturation rate was 82.78, 87.57, 75.71 and 75.65% respectively (Table 1). The oocyte maturation rate was significantly ($P<0.01$) higher

Table 1. Effect of different gas phases on *in vitro* oocyte maturation in goat at 38.5°C

Gas phase	Total no. of culturable oocyte	Total no. of matured oocytes	Total number of oocyte cleaved	No. of 4–8 cell embryos	No. of morula
C_1	180	149^{ab} (82.78%)	17 (9.44%)	9 (5.00%)	8 (4.44%)
C_2	177	155^{a} (87.57%)	14 (7.90%)	7 (3.95%)	2 (1.13%)
C_3	140	106^{b} (75.71%)	9 (6.43%)	5 (3.57%)	4 (2.86%)
C_4	115	87^{b} (75.65%)	7 (6.08%)	2 (1.74%)	4 (3.48%)

C_1=5% CO_2, 20% O_2; C_2=5% C_2, 10% O_2; C_3=7.5% CO_2, 20% O_2; C_4=7.5% CO_2, 10% O_2. Values with common superscript in column are not significantly different to each other.

in C_2 as compared to C_3 and C_4. There was no significant variation of oocyte maturation rate among C_1, C_3 and C_4. The cleavage percentage was 9.44, 7.90, 6.4 and 6.08%, respectively in gaseous phase of C_1, C_2, C_3 and C_4 (Table 1). Although there was no significant variation of cleavage rate among the 4 gaseous conditions during IVMFC, it was comparatively higher in C_1 (9.44%) as compared to C_2, C_3 and C_4 (Table 1). The development of 4–8 cell and morula stage embryos were also not significantly different among these different gas phases. However, the number of 4–8 cell and morula stage embryos was comparatively higher in C_1 condition. The percentage development of 4–8 cell stage embryos was comparatively lower in C_4 conditions as compared to C_1, C_2 and C_3. Similarly, the rate of development of morula stage embryos was comparatively lower (1.13%) in C_2 condition as compared to C_1, C_3 and C_4 (Table 1).

When oocytes were cultured at 40.5°C, the oocyte maturation rate was 68.15%, 44.88%, 40.00% and 50.29%, respectively in C_1, C_2, C_3 and C_4 (Table 2). The oocyte maturation rate was significantly higher ($P<0.01$) in C_1 as compared to C_2, C_3 and C_4.

There was no significant variation of rate of oocyte maturation in C_2, C_3 and C_4. However, the lowest oocyte maturation was observed in C_3 condition as compared to C_1, C_2 and C_4 (Table 2). When IVMFC was done in different gaseous phases at 40.5°C, there was no significant difference of cleavage rate and 4–8 cell stage embryo development. The cleavage rate and embryo development was very much lower in different gaseous phase. There was no development of morula stage embryo in C_1 and C_3. Similarly, in C_2 and C_4, the morula stage embryo development was about 1% only (Table 2).

The maturation rate, % of cleaved oocytes, % of 4–8 cell and morula stage embryos were better in C_1 than other gas phases although no significant differences were observed.

Table 2. Effect of different gas phases on *in vitro* oocyte maturation and embryo development at 40.5°C in goat

Gas phase	Total no. of culturable oocytes	Total no. of matured oocytes	Total oocytes cleaved	No. of 4–8 cell stage embryos	No. of morula
C_1	157	107[a] (68.15%)	4 (2.55%)	2 (1.27%)	0 (0%)
C_2	127	57[b] (44.88%)	4 (3.15%)	1 (0.79%)	1 (0.79%)
C_3	165	66[b] (40.00%)	4 (2.42%)	2 (1.21%)	0 (0%)
C_4	175	88[b] (50.29%)	7 (4.00%)	2 (1.14%)	2 (1.14%)

C_1=5% CO_2, 20% O_2; C_2=5% C_2, 10% O_2; C_3=7.5% CO_2, 20% O_2; C_4=7.5% CO_2, 10% O_2. Values with common superscript in column are not significnatly different to each other.

Several reports suggest that decreasing O_2 percentage and increasing CO_2 percentage improve the *in vitro* development of embryos in different species (Carney and Bavister 1987, Karja *et al.* 2004). Brinster (1971) found no significant differences in development when mouse embryos were cultured under 1%, 5% or 10% CO_2, however, Carney and Bavister (1987) reported an approximate doubling in the percentage of embryos developed to the blastocyst stage upon raising the concentration of CO_2 gas phase from 5 to 10% CO_2 at 37°C. Increased partial pressure of oxygen generates excessive amounts of cytotoxic reactive oxygen species (ROS) and may directly affect the viability of gametes (Johnson and Nasr-Esfahani 1994). On the other hand, oxidative stress, depending on its severity, could lead to either cell necrosis or apoptosis. The hypoxic oxygen tensions (below the physiological level of 5%) do not seem to harm the embryo as shown in mice (Umaoka *et al.* 1991) and rabbits (Li and Foote 1993). In contrast, several studies have shown that the efficiency of oocyte or embryo culture could be improved by decreasing oxygen tension (Berhelot and Terqui 1996, Izquierdo *et al.* 1998, izquierdo *et al.* 1999), whereas others were contradictory (Machaty *et al.* 1998, Khurana and Niemann 2000, Hashimoto *et al.* 2000, de Matos *et al.* 2002). In the present study, it was observed that changes of gaseous phase did not have any significant effect on the rate of *in vitro* maturation and embryo development in goat which is in agreement of above reports.

In the present study the oocyte maturation was very poor at 40.5°C. It may be due to the effect of elevated temperature causing disrupted spindle formation during metaphase I and reduction of oocytes progressed to metaphase II as has been observed in mice (Baumgartner and Chrisman 1987) and cattle (Lenz *et al.* 1983). Further, the cleavage rate and development of 4–8 cell and morulae stage embryos were very low at 40.5°C. As stated earlier, it might be due to the damage of cytoskeleton or other intra cellular organelles at higher temperature. In addition embryos formed via fertilization with heat damaged spermatozoa had lower developmental potential (Ulberg and Burfening 1967).

In summary, the present study indicated that at normal temperature (38.5°C) alteration of gaseous phase did not have any significant effect on embryo development although the maturation rate was significantly higher in 5% CO_2 and 10% O_2 in goat. Further the results also indicated higher culture temperature had significantly detrimental effect on the success of IVMFC irrespective of different gaseous phases in goat.

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