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Influence of cysteamine supplementation during *in vitro* culture of early stage caprine embryos on blastocyst production

S D KHARCHE[1], S AGRAWAL[2], J PATHAK[3], A K S SIKARWAR[4], CHETNA GANGAWAR[5], RAVI RANJAN[6], A K GOEL[7], S K JINDAL[8] and S K AGARWAL[9]

ICAR-Central Institute for Research on Goats, Makhdoom, P. O. Farah, Mathura, Uttar Pradesh 281 122 India

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A major factor affecting *in vitro* mammalian embryo development is increased oxidative stress. Reactive oxygen species (ROS) are formed continuously in cells as a consequence of both oxidative biochemical reactions and external factors. Therefore, there is a need to minimize the effect of oxidative damage caused by *in vitro* conditions. Effect of application of low molecular weight compounds like cysteamine, and β-mercaptoethanol in *in vitro* maturation medium have been reported to improve the rate of production of blastocyst in many species like mouse (Mohammadi-Roushande *et al.* 2007), cattle (de Matos *et al*. 1995), buffalo (Lojkic *et al*. 2012), sheep (de Matos *et al*. 2002) and prepubertal goats (Urdaneta *et al*. 2004). But limited evidences are available about the effect of cysteamine on the production of blastocyst in adult goat. Therefore, the present study aims to analyze the influence of cysteamine supplementation on *Caprine* blastocyst production.

Recovery of oocytes: Goat ovaries (343) were collected from the local abattoir and oocytes were retrieved by follicular slicing from the goat ovaries. Recovered oocytes were graded as per Kharche *et al.* (2008).

In vitro maturation (IVM)*:* The COCs (1,251) were then washed 5–6 times in TCM-199 containing L-glutamine (100 µg/ml), sodium pyruvate (0.25 mM), gentamycin (50µg/ml), LH 10 µg/ml, FSH 5 µg/ml supplemented with 10% FBS, 3mg/ml BSA. Oocytes were matured in 50 µl droplets of maturation medium covered with sterile mineral oil for time period of 27 hr in humidified atmosphere of 5% CO_2 at 38.5±1°C in CO_2 incubator.

In vitro fertilization (IVF)*: In vitro* fertilization was carried out as per Kharche *et al*. (2011) with slight modifications.

Present address: [1,7,8]Principal Scientist (kharche62@gmail.com, akgcirg2012@gmail.com, jindalmathura@gmail.com), [2]Ph D. Student (subhi.agrawal89@gmail.com), [3]Senior Research Fellow (juhi1690@gmail.com), [4]Research Associate (dranujkumar@rediffmail.com), [5,6]Scientist (chetnaom82@gmail.com, dr_raviranjan@yahoo.co.in), Physiology Reproduction and Shelter Management Division, [9]Director (skagarwalcirg@gmail.com).

In vitro culture (IVC)*:* After 18h of sperm-oocytes co-incubation of oocytes with sperms were washed in embryo development medium to remove sperm cells adhered to zonapellucida. Presumptive zygotes (1,171) were selected and randomly divided into 2 groups. Group 1 (610) the presumptive zygotes were culture in mCR2aa as control for 12 days. Group 2 (561) the presumptive zygotes were culture in mCR2aa medium supplemented with 100 µM cysteamine for 10 days. The cleavage rate for both mCR2aa and mCR2aa cystemine were observed. Oocytes in both the groups washed 8–10 times in respective embryo development medium in order to separate adhering sperm cells. Oocytes were finally transferred into 50µl drop of respective embryo development medium in both the groups for 48 hr in humidified atmosphere of 5% CO_2 at 38.5±1ºC in CO_2 incubator.

After 48 h of post insemination, fertilized oocytes were evaluated under phase contrast microscope for cleavage rate. Cleaved oocytes were cultured in respective embryo development media further for 8–10 days. Development stages of cleaved oocytes were observed and 50% media was replaced every 48 h.

Statistical analysis: The rate of development of embryos to different stages in both the embryo development media were analyzed using Chi-square (÷2) test and value of $P < 0.05$ was considered to be statistically significant (Snedecor and Cochran 1989).

In the present study, 1,251 good quality oocytes were recovered from 343 ovaries, with a recovery rate of 3.64, higher than previously described elsewhere. Results of embryo development are shown in Table 1. Comparatively higher blastocyst production was achieved in embryo development medium supplemented with 100 µM cysteamine. The *in vitro* embryo production system in goat species is still not as efficient as in cattle and buffalo and the results are still unsatisfactory. Therefore, in the present study, the effects of cysteamine on goat *in vitro* embryo development were evaluated. An improvement in the *in vitro* embryo production efficiency was achieved adding that low molecular weight compound during*in vitro* culture. Thiol compounds like â-mercaptoethanol, cysteamine and cystine

Table 1. Effects of cysteamine (added to the embryo development medium) on blastocyst production

Cysteamine (μM/L)	Presumptive zygotes (n)	Cleavage rate (%)	2 Cell (%)	4 Cell (%)	8-16 Cell (%)	Morula (%)	Blastocyst (%)
0 μM	610	36.39	18.46	15.31	39.63	21.62	4.95
100 μM	561	31.72	15.16	20.22	24.71	30.89	8.98

* Values do not differ significantly within column (P>0.05).

are believed to be mediated through an increase in the synthesis of glutathione (GSH) during IVM, IVF and IVC (de Matos *et al*. 1995). GSH is an important scavenger that protects cells against reactive oxygen species. GSH is synthesized by ã-glutamyl cycle, and its synthesis is dependent on the availability of cysteine, a highly unstable aminoacid that is readily oxidized to cystine. The addition of cysteamine in the *in vitro* culture medium of goat zygotes resulted in an increase in GSH levels by reducing oxidized cystine to cysteine in medium and an improvement in embryo developmental rate up to the blastocyst stage. The primary effect of GSH is thought to reduce the oxidative stress due to reactive oxygen species (ROS), which are generated during *in vitro* culture, especially when atmospheric oxygen tension (20%) used (Goto *et al*. 1993). Cysteamine reduces cystine to cysteine and promotes the uptake of cysteine, thereby increasing GSH synthesis. Thus balance between GSH and ROS concentration inside the cell gets maintained. As a result of which the damaging effect of ROS on protein, lipid and nucleic acid of the embryos get minimizes thereby loss of membrane integrity, structural or functional changes in proteins, and damage in nucleic acids get prevented (Tamura *et al*. 2012). This fact suggests that the beneficial effects of cysteamine on subsequent embryo development were mediated by GSH. The intracytoplasmic glutathione concentration varies during pre-implatation development of *in vitro* produced bovine embryos. The lowest level of GSH was found in 2–8 cell embryos as the *de novo* synthesis of GSH begins to increase at the 8–16cell stage embryos following the highest level found in hatched blastocyst. The fact that early stage embryos are probably more sensitive to oxidative stress because of exhausted GSH pool synthesized during IVM inspired us to supplement the *in vitro* culture media with cysteamine during the presumptive zygote stages of embryo development.

The several researchers demonstrated that the presence of 100 μM cysteamine during maturation of bovine oocytes significantly increased the number of morula and blastocysts on Day 9 of IVC. This is in agreement with results obtained by others (de Matos *et al*. 1995) who showed that the addition of low molecular weight thiols, like cysteamine, during IVM improves the rate of blastocyst development. This results could be explained by the improved protection against oxidative stress during IVF and early stages of embryo development since it was demonstrated that cysteamine can stimulate *de novo* GSH synthesis during IVM and that presumptive zygotes maintain high intracellular GSH levels decreasing to control level in the 6–8 stage cell embryos (de Matos *et al*. 2002). de Matos *et al*. (2002) demonstrated that the addition of cysteamine to maturation media and 2–8-cell bovine embryos results in an increase of embryo quality and blastocyst production. The beneficial effect of cysteamine present during the early stages of embryo development is probably acquired through increased glutathione concentration,which could help overcome the developmental block at 8- to 16–cell stage embryos (Lee *et al*. 2000).

Our study demonstrated that the addition of 100 μM cysteamine to the *in vitro* culture medium resulted in comparatively higher blastocyst production (8.98% vs 4.95%) than with control group. At this concentration, cysteamine significantly improved the efficiency of *in vitro* embryo production in this species: an increased proportion of tight morula and blastocyst-stage embryos developed from presumptive zygotes were observed and, more interestingly, embryos obtained under the new tested conditions were of a better quality. Also, at the highest concentration, the compound did not exert a toxic effect on embryos as no differences were observed compared with the control group. However, no beneficial effect was recorded on cleavage rate. This in agreement with the result reported by de Matos *et al*. (1995), Lee *et al.* (2000), de Matos *et al*. (2002), and Lojkic *et al*. (2012). Blastocoel fluid contains hydrogen peroxide which is cytotoxic and can induce apoptosis in inner cell mass cells. Treatment with hydrogen peroxide applied to oocytes or embryos decreased the blastocyst development and increased the induction of permanent embryo arrest or apoptosis in a stage-dependent manner (Bain *et al*. 2011). The increase of inner cell mass cells could, therefore, be explained with the fact that cysteamine addition to the IVC media increases intracellular glutathione content and since glutathione is involved in the removal of hydrogen peroxide, inner cell mass cells would not become apoptotic Lojkic *et al*. (2012). In conclusion, we found that the addition of cysteamine to the IVC medium stimulates caprine embryo development and enhances embryo quality, which could lead to the optimization of the *in vitro* system for blastocyst production.

SUMMARY

The objective of this study was to evaluate the effect of cysteamine supplementation on embryo development in mCR2 media. The COCs (1251) were matured in TCM–

199 medium containing FSH (5µg/ml), LH (10µg/ml), follicular fluid (10%), FBS (10%) with 3 mg/ml BSA for 27h at 38.5°C and 5% CO_2 in an incubator. Matured oocytes were co-cultured with 1×10^6 spermatozoa/ml collected from a Sirohi buck in fertTALP (10% FBS+ 4mg/ml BSA and 10 µg/ml heparin) for 18 h in incubation. After 18 h of sperm-oocytes, co-incubation of oocytes with sperms were washed in embryo development medium to remove sperm cells adhered to zonapellucida. Presumptive zygotes (1,171) were selected and randomly divided into 2 groups. Group 1, the presumptive zygotes (610) were cultured in mCR2aa as a control for 12 days. Group 2, the presumptive zygotes (561) were culture in mCR2aa medium supplemented with 100 µM cysteamine for 10 days. The percentage of cleavage, morula, and blastocyst production in groups 1 and 2 were 36.39% and 31.71% (cleavage); 21.62% and 30.89% (morula); 4.95% and 8.98% (blastocyst) respectively. In conclusion, results indicated that the addition of cysteamine to the IVC medium stimulates caprine embryo development and blastocyst production.

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