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Influence of exogenous supplementation of IGF-I, cysteamine and their combination on *in vitro* caprine blastocyst development*

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One of the features of *in vitro* produced (IVP) mammalian embryos is the high frequency of early developmental failure; thought to be brought on by sub-optimal culture environments (Fevetta *et al.* 2004 a,b). Apoptosis, in response to inappropriate culture conditions and stress, is a common physiological process during *in vitro* embryo development, contributing to progressive loss of embryos during the *in vitro* embryo production procedure (Hao *et al.* 2003). These reproductive events are controlled by a complex interplay of several hormones and metabolites acting according to the physiological state of the animal in order to maintain normal homeostasis (Velazquez *et al.* 2008). Insulin-like growth factor-I, a component of the complex IGF superfamily (70-amino acid peptide structurally related to insulin), plays an essential role in mammalian reproduction (Kadakia *et al.* 2001) and affects cell growth, differentiation and has anti-apoptotic effects during *in vitro* embryo development (Stefanello *et al.* 2006). Similarly, thiol compounds viz. cysteamine (Cys) stimulates glutathione synthesis and decrease hydrogen peroxide levels, improving embryo development (de Matos *et al.* 2002). Glutathione is one of the non-protein sulphydryl compounds, major free thiol which protects the culture from oxidative stress (Tetemoto *et al.* 2001). *In-vivo* oocytes and embryos seem to be protected against oxidative stress by oxygen scavengers present in follicular and oviductal fluids by eliminating or reducing the effect of ROS (Goncalves *et al.* 2006). Oxidative stress has also been implicated in the etiology of defective embryo development which can alter cell function, minimize cell survival with deleterious effects under *in vitro* culture condition (Goncalves *et al.* 2006). Therefore, it is imperative to focus on sub-optimal culture supplements to inactivate ROS, maintain normal growth and development and optimize the blastocyst formation competence. Relatively meager work has been carried out to study the development of caprine presumptive zygotes in culture media supplemented with growth factors and antioxidants synergistically. Keeping in view paucity of work, in the present study, effect of IGF-I, cysteamine, and combination of both for synergistic effect was studied on *in vitro* embryo development potential/blastocyst production in goats.

Goat ovaries (396) were collected from a local abattoir located at Agra and transported to the laboratory in a thermos flask containing sterilized physiological saline solution at 30–35°C within 3 h. Ovaries were washed in fresh, sterile physiological saline to remove contaminants and rinsed in 70% ethyl alcohol for 3–4 min to eliminate surface microorganisms. Finally ovaries were washed (5–6 times) with warm saline fortified with antibiotics prior to further processing.

The oocytes were recovered from each ovary into a petri plate containing oocyte collection media (Dulbecco's phosphate-buffered saline with 3 mg/ml BSA) by follicle puncture technique with the help of a B.P. blade. Each petri dish containing oocytes was evaluated under a stereo zoom microscope and oocytes with at least 2–4 intact layers of cumulus cells and homogenous cytoplasm were selected for *in-vitro* maturation (IVM). A total of 1,043 oocytes were recovered from 396 ovaries. Selected oocytes were washed four to five times in Oocyte Holding Media (TCM-199 with Hepes modification, 10% FBS, 0.25 mM Sodium Pyruvate, 50 µg/ml Gentamycin, 100 µg/ml Glutamine, 3 mg/ml BSA). Recovered oocytes were graded as excellent (A), good (B), fair (C) and poor (D) quality as per Kharche *et al.* (2008). The cumulus oocyte complexes (COCs) were then washed three times with Oocyte Maturation Media (TCM-199 supplemented with 10% FBS, 0.25 mM Sodium Pyruvate, 100 µg/ml Glutamine, 10 µg/ml LH, 5 µg/ml FSH, 10 ng/ml EGF, 3 mg/ml BSA and 50 µg/ml Gentamycin. The (COCs) were assigned for *in vitro* maturation in maturation media covered with sterile mineral oil for 27 h in a humidified atmosphere of 5% CO_2 at 38.5±1°C in a CO_2 incubator.

In-vitro fertilization was carried out as per Kharche *et al.* (2011) with minor modifications. After 27 h of culture, oocytes were separated out from the cumulus cells by treating the COCs with 0.1% hyaluronidase enzyme and

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passing it repeatedly through a fine pipette. Denuded oocytes were washed 10–20 times with Fert-TALP medium containing 10% FBS, 8 mg/ml fatty acid free BSA and 50 µg/ml heparin. Approximately 15–20 oocytes were transferred in each 50 µl drop of Fert-TALP medium covered with sterile mineral oil in a culture Petri dish in humidified atmosphere of 5% CO_2 at 38.5±1° C for 1 h in CO_2 incubator.

Fresh semen was collected in the artificial vagina from a fertile pure breed adult Sirohi buck. The first and second seminal ejaculates were examined for volume, colour, consistency and gross and progressive sperm motility. A sample of 100 µl fresh semen was diluted with 5 ml of sperm TALP medium containing 4 mg/ml fatty acid free BSA and washed by centrifugation at 1,200 rpm for 5 min. The supernatant was discarded and semen pellet was again dissolved in 5 ml sperm TALP and again centrifuged at 1,200 rpm for 5 min. Final washing was done with 1 ml Fert TALP medium and 100 µl of pellet was added in 900 µl of Fert TALP medium. Sperms were then kept for incubation in a CO_2 incubator in humidified atmosphere of 5% CO_2 at 38.5±1ºC for 45 min. Finally Fert TALP medium drops containing the matured oocytes were inseminated with 25–50 µl of the final diluted semen so as to obtain a sperm concentration of 1×10^6 sperm/ml. After *in vitro* insemination, the oocytes and sperms were co-incubated for 18 h at 38.5±1°C with 5% CO_2 in humidified atmosphere.

After 18 h of post insemination, fertilized oocytes were washed in order to separate adhering sperm cells. Presumptive zygotes were grouped and cultured in embryo development medium (EDM; mCR_2aa medium containing 10% FBS and 3 mg/ml BSA) with different supplementations (IGF-I and cysteamine) for 8–10 days in humidified atmosphere of 5% CO_2 at 38.5±1°C in a CO_2 incubator. In Group 1, 2, 3 and 4, presumptive zygotes were cultured in mCR_2 aa supplemented with 100 ng/ml IGF-I, 50 µM cysteamine, 100 ng/ml IGF-1 + 50 µM cysteamine and without supplements (control), respectively for 8–10 days in humidified atmosphere of 5% CO_2 at 38.5±1°C in a CO_2 incubator.

Presumptive zygotes were observed after every 48 h for embryo development potential and 50% media was replenished every 48 h up to 12 days. Embryos were morphologically evaluated under inverted phase contrast microscope.

The data so generated experimentally were subjected to statistical analysis using Chi-square test (χ^2). Cleavage rate and embryo development among the treatment groups were compared using the Chi-square test. A probability of $P<0.05$ was considered to be statistically significant (Snedecor and Cochran 1989).

In the present study, a total of 1,043 culturable oocytes were recovered from 396 ovaries with mean recovery rate of 2.63. The effects of different supplements (growth factors and antioxidants) on cleavage, morula and blastocyst production rates are shown in Table 1. In the present study, statistical analysis revealed that supplementation of IGF-I, cysteamine, and combination of cysteamine and IGF-I in embryo development media (EDM) improved blastocyst production as compared to non-supplemented group (control), but did not improve the cleavage rate. Statistical analysis revealed that significantly higher percentage ($P<0.05$) of embryos were arrested at 2-cell stage in control group as compared to IGF-I supplemented group. However, a non-significant difference ($P>0.05$) in the formation of 4 and 8–16 celled stage embryos was observed between the treated groups.

Analysis of data also revealed that morula production was significantly ($P<0.05$) higher in culture media supplemented with IGF-I as compared to control and IGF-1 + cysteamine supplemented group. However, no significant difference ($P>0.05$) in the morula production was observed between the IGF-I and cysteamine supplemented groups. Furthermore, supplementation of growth factors and/or antioxidant significantly ($P<0.05$) increased the proportion of blastocyst production in comparison to non-supplemented control (Table 1). Addition of both (IGF-I or cysteamine) either singly or in combination (IGF-1+ cysteamine) improved the percentage of embryos that reached blastocysts stage ($P<0.05$) as compared to control group.

Several retrospective studies reported the addition of various chemical mediators, growth factors and antioxidants which favor *in vitro* embryo development potential. Insulin-like growth factor I (a 70-amino acid peptide structurally related to insulin), affects cell growth and differentiation and has anti-apoptotic effects during *in vitro* embryo

Table 1. Effects of IGF-1 and cysteamine on cleavage and blastocyst production rate of *in vitro* matured caprine oocytes

Treatment group	No. of oocytes (n)	Cleavage rate (%)	2-Cell (%)	4 -Cell (%)	8-16 Cell (%)	Morula (%)	Blastocyst (%)
Group 1 (IGF)	278 (101)*	107 (38.49)^a	7 (6.54)^a	14 (13.08)^a	33 (30.84)^a	36 (33.64)^a	17 (15.89)^a
Group 2 (Cysteamine)	260 (97)*	114 (43.85)^a	10 (8.77)^a	18 (15.79)^a	34 (29.82)^a	34 (29.82)^a	18 (15.70)^a
Group 3 (IGF + Cysteamine)	262 (119)*	104 (39.69)^a	13 (12.50)^b	19 (18.27)^a	33 (31.73)^a	22 (21.15)^b	17 (16.35)^a
Group 4 (Control)	243 (79)*	99 (40.74)^a	15 (15.15)^b	21 (21.21)^b	35 (35.35)^b	21 (21.21)^b	7 (7.07)^b

*Indicates number of ovaries processed for oocyte retrieval. Values bearing different superscripts within the column differ significantly ($P<0.05$).

development (Stefanello *et al*. 2006). Similarly, thiol compounds such as cysteamine (Cys) stimulates glutathione synthesis and decrease hydrogen peroxide levels, improving embryo quality and blastocyst production development (de Matos *et al.* 2002).

In the present study, supplementation of embryo development medium either with IGF-I or cysteamine or both (IGF-I + cysteamine) significantly increased the percentage of embryos that reached blastocysts stage ($P<0.05$) as compared to control group. Present findings are in agreement with the retrospective work carried out by Kitagawa *et al.* (2004) who reported that the addition of vitamin E, a fat-soluble antioxidant, to the culture medium improved developmental competence to the blastocyst stage and increased the cell number in porcine IVF embryos by suppressing peroxidation of membrane lipids. Similarly, the supplementation of β-mercaptoethanol in bovine IVF embryos (Takahashi *et al.* 2002) and cysteamine in caprine IVF embryos (De *et al.* 2011, Kharche *et al.* 2016) resulted in a significant increase in the rate of development to the blastocyst stage embryos in comparison to other supplements. Our study demonstrated that the addition of 50 µM cysteamine to the *in vitro* culture medium resulted in higher blastocyst production (15.70%) as compared to earlier report (8.98%) in similar species by using 100 µM cysteamine (Kharche *et al.* 2016).

Hao *et al*. (2003) suggested that apoptosis might contribute to the progressive loss of embryos during the *in-vitro* production. Oxidative stress can cause numerous types of embryo damage due to the fact that reactive oxygen species (ROS) easily pass through cell membranes; alter most kinds of cellular molecules viz. lipids, proteins and nucleic acids, resulting in mitochondrial damage, embryo cell block, ATP depletion, and apoptosis. In the present study, significantly lower blastocyst yield in control group revealed the importance of supplementation of antioxidant (cysteamine) by protecting embryos from ROS *in vitro.*

Analysis of results on the effect of growth factor on blastocyst development revealed that proportion of embryos that reached blastocyst stage were significantly ($P<0.05$) higher in culture media supplemented with IGF-1 compared to control. This supports the earlier findings of Lima *et al.* (2006) who reported that supplementation of embryo culture media with IGF-I resulted in a significantly greater number ($P<0.05$) of 2–4 cell stage bovine embryos developing into blastocysts, expanded blastocysts and hatched blastocysts. Similarly, Sirisathien *et al.* (2003) found putative beneficial effects of adding insulin-like growth factor-I (IGF-I) for bovine embryo culture in chemically defined media and concluded that IGF-I could independently enhance bovine pre-implantational development and might play a mitogenic role during early bovine development. The effects of IGF-I are mediated mainly through type I IGF receptor. Transcripts for IGF-I receptor are present in embryos from zygote to blastocyst stages (Yaseen *et al.* 2001). IGF-I transcripts are also detectable in both bovine oviductal epithelium of all three oviductal regions (Pushakumara *et al.* 2002) and endometrium (Robinson *et al.* 2000). Bovine oviductal cell primary cultures also secrete IGF-I into culture medium (Winger *et al.* 1997).

In the present work, supplementation of IGF-I, cysteamine or both (IGF-I + cysteamine) in embryo development media did not improve the cleavage rate but significantly increased ($P<0.05$) the blastocyst production as compared to control. However, difference in blastocyst production was non-significant ($P>0.05$) among the supplemented groups (IGF-1, cysteamine, IGF-1 + cysteamine). Contrary to it, a synergistic effect of cysteamine, IGF-1 and EGF on the production of morula and blastocyst stage embryos as compared to IGF-I, EGF and cysteamine alone in ovine species was reported by Shabankareh and Zandi (2010). It may be due to difference in specific requirement of culture conditions of caprine and ovine embryos during different stages of development under *in vitro* conditions.

SUMMARY

The present study was carried out to investigate the putative beneficial effects of insulin-like growth factor-I (IGF-I) and cysteamine supplementation alone or their combination on *in vitro* embryo development competence of fertilized goat oocytes. Presumptive zygotes (18 h post insemination) were randomly assigned for *in vitro* embryo development in embryo development medium (EDM) supplemented with IGF-I (Gr. 1), Cysteamine (Gr. 2), IGF-I + Cysteamine (Gr. 3) and Control containing only EDM (Gr. 4). Statistically non-significant difference was observed in cleavage rate among all the treated groups. Morula formation rate was significantly higher in IGF-I supplemented group compared to IGF-I + cysteamine supplemented and non-supplemented (control) groups. Furthermore, supplementation of IGF-I, cysteamine and IGF-I + cysteamine in embryo culture medium significantly improved blastocyst formation rate compared to control. However, a nonsignificant difference in blastocyst formation was observed among the supplemented groups. These findings lead to the conclusion that under *in vitro* conditions, supplementation of IGF-I and cysteamine alone or combination in IVC media were equally effective in embryo development and blastocyst production, however, this effect was significantly higher as compared to non-supplemented group (control).

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