EFFECT OF LEPTIN ON IN-VITRO MATURATION OF OOCYTES AND ON EARLY EMBRYONIC DEVELOPMENT IN BUFFALOES

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ABSTRACT

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The present study was aimed to assess the physiological function of Leptin on *in-vitro* maturation of oocytes and early embryonic development to increase the efficiency of *in-vitro* embryo production in buffaloes. The oocytes were maturated in 199 medium supplemented with 10% FBS, 0.5µg/ml FSH, 10IU/ml LH, 1 µg/ml estradiol-17β, 20ng/ml EGF and 50 µM cysteamine in four groups i.e. Group-I (without leptin), Group-II (10ng/ml leptin), Group-III (20ng/ml leptin) and Group-IV (30ng/ml leptin) in CO_2 incubator at 37°C temperature, 5% CO_2 and high humidity. The matured oocytes were fertilized *in-vitro* in modified synthetic oviductal fluid (mSOF) containing 10ng/ml heparin. Presumptive zygotes were cultured in mSOF for 7 days to study embryonic development. The oocytes maturation rates were 76.55, 83.25, 90.14 and 92.16 %, respectively in groups I, II, III and IV, cleavage rates were 48.75, 59.77, 70.83 and 71.50%, embryonic development to morula stage were 41.03, 50.96, 58.82 and 57.34%% and blastocyst development rates were 25.64, 30.77, 44.12 and 44.06 %. The oocytes maturation, cleavage, morula and blastocyst development rates were significantly higher in leptin supplemented groups. From the present study, it may be concluded that leptin at the concentration of 20ng/ml in maturation medium significantly ($P<0.05$) improved maturation rate, cleavage, morula and blastocyst development in buffaloe oocytes.

Key words: Buffalo, Leptin, Oocytes, *in-vitro* maturation, *in-vitro* fertilization, *in-vitro* culture.

INTRODUCTION

Leptin plays an important role in reproduction and development (Cunningham *et al.*, Holness *et al.*, Cervero *et al.*,). Leptin treatment of normal prepubertal female mice accelerates the onset of puberty (Chehab). During the menstrual cycle, there are variations in serum leptin levels with higher concentration reported in the pre-ovulatory and mid-luteal phases and lower in the early follicular phase (Hardie *et al.*, Lukaszuk *et al.*, Henson and Castracane). Leptin mRNA and protein have been identified in human and mouse blastocysts and hatched blastocysts (Gonzalez *et al.*, Kawamura *et al.*,) and it appears to be regulated in a paracrine manner when blastocysts are co-cultured with human endometrial cells (Gonzalez *et al.*,). It also enhances meiotic progression of pig oocytes and subsequent development of parthenogenetic embryos (Craig *et al.*,) In contrast, the evidence for a beneficial effect of leptin during mouse oocyte maturation is controversial. Leptin treatment of mouse cumulus-enclosed oocytes or denuded oocytes (DOs) does not enhance meiotic progression (Swain *et al.*,), the addition of leptin during culture of mouse follicles increases germinal vesicle breakdown (GVBD) (Ryan *et al.*,). The present study was performed to analyse the effect of leptin on oocytes maturation and early embryonic development in buffaloes.

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MATERIALS AND METHODS

Buffalo ovaries were collected from the abattoir at Haldwani, located 25 km away from the G.B. Pant University of Agriculture & Technology, Pantnagar in the Tarai region of Uttarakhand State of India (latitude 28° 53' 24" to 31°27' 50" north and longitude 77°34' 27" to 81°02' 22" east and altitude 243.84 meters). Ovaries were collected in normal saline containing antibiotics (Penicillin 400 IU and streptomycin 400 mg/ml) maintained at 35 to 37°C and brought to laboratory within 2 hours of slaughter. Prior to oocytes aspiration, extra tissues from the ovaries were removed and ovaries were washed several times in normal saline containing antibiotics. After proper washing, ovaries were kept in incubator at 37°C for about 30 min prior to aspiration of follicles.

The oocytes were collected from each ovary by aspiration of follicles using 18G needle attached to 10 ml syringe having 1 ml oocyte collection media (OCM). The oocyte collection media comprised of Dulbecco's Phosphate Buffered Saline with 0.3% BSA and 50 µg/ml gentamicin. All the visible surface follicles of 3-10 mm diameter were aspirated in the OCM and contents of the syringe were gently transferred into a 50 ml sterilized and disposable conical tube (Greiner, Germany). Immediately after collection, oocytes were left in the conical tube for about 10 min to enable them to settle at the bottom. Subsequently, supernatant was gently aspirated and removed and about 5 ml media containing oocytes left at the bottom were poured into a 94x10 mm petridish (Greiner, Germany) and content of the petridish was searched for oocytes under a stereo-zoom microscope (Olympus, Japan). Isolated oocytes were transferred into a 35x10 mm petridish (Greiner, Germany) and washed 2-3 times in OCM and classified as Grade A, B, C and D based on the number of the cumulus cell layers and appearance of cytoplasm.

1. Grade A: COCs with 4 or more than 4 layers of compact cumulus cells investment and evenly granular homogenous ooplasm.
2. Grade B: COCs with 2-3 layers of compact cumulus cells investment and evenly granular homogenous ooplasm.
3. Grade C: COCs with 1 layer of cumulus cells investment.
4. Grade D: Denuded COCs or COCs with highly scattered cumulus cells investment and irregular dark ooplasm.

Total oocytes were equally divided in four experimental groups as per the plan of work. The oocytes were washed three times with control/ experimental IVM medium separately. Only grade A, B and C oocytes were allowed to mature in 50 ml droplets of maturation medium in 35x10 mm petridishes for 22-24 hr in a CO_2 incubator (Thermo Lab System, USA, Thermo Forma Series II water jacketed CO_2 incubator) maintained at 39°C, 5% CO_2 and high humidity. The oocytes were subjected to in-vitro maturation for 22 hrs in Medium-199 supplemented with 10% serum, 0.5µg/ml FSH, 10 IU/ml LH, 1µg/ml Estradiol-17â, 20ng/ml EGF and 50µM Cysteamine in four groups i.e. Group-I (without Leptin), Group-II (Leptin 10 ng/ml), Group-III (Leptin 20 ng/ml), Group-IV (Leptin 30 ng/ml) in CO_2 incubator at 39°C temperature, 5% CO_2 and high humidity. The assessment of maturation was done by the degree of expansion of cumulus cells mass and extrusion of first polar body (PB1) into perivitelline space.

Modified synthetic oviductal fluid media (mSOF) used for *in-vitro* capacitation of the sperm was used for *in vitro* fertilization. For fertilization of matured oocytes, 50 ml droplets of mSOF were taken in a 35x10 mm petridish covered with mineral oil and kept at least for 2 hrs inside CO_2 incubator for equilibration. Frozen semen of Murrah buffalo bull was thawed at 37°C for 30 seconds and the contents were poured in a 15 ml centrifuge tube having about 14.5 ml of sperm washing medium. After gentle mixing, the tube was centrifuged at 110 g (800 rpm) for 10 min. The supernatant was discarded and sperm pellet was dissolved again by adding sperm washing media in the same volume and centrifuged at 110 g (800 rpm) for 10 min. In the meantime oocytes

were washed and kept in the 50 ml droplets of sperm washing media. After second centrifugation, supernatant was discarded and pellet was dissolved in about 2 ml of fertilization media (mSOF supplemented with 8 mg/ml BSA fatty acid free, 10 ml/ml MEM essential amino acid solution 50x stock, 5 ml/ml MEM nonessential amino acid solution 100x stock, 2 ml/ml ITS; Insulin (10 mg/ml), transferrin (5.5 mg/ml) and selenium (5 ng/ml) 100x stock, 2 ml/ml 200mM L-glutamine and 10 mg/ml heparin) and ensure concentration of progressively motile sperm as 1-2 x10^6/ml. The 50 ml droplets containing oocytes were charged with 50 µl of sperm suspension (taken from top 1/3rd portion of sperm suspension) and placed inside CO_2 incubator maintained at 39°C, 5% CO_2 and high humidity for about 22 hrs.

In vitro culture (IVC) of presumptive zygotes

After about 6 hr of oocyte-sperm co-incubation, 80% fertilization media was replaced with embryo culture medium (mSOF supplemented with 8 mg/ml BSA fatty acid free, 10 ml/ml MEM essential amino acid solution 50x stock, 5 ml/ml MEM nonessential amino acid solution 100x stock, 2 ml/ml ITS; Insulin (10 mg/ml), transferrin (5.5 mg/ml) and selenium (5 ng/ml) 100x stock and 2 ml/ml 200mM L-glutamine). Subsequently after 16 hr (total about 22 hr of oocyte-sperm co-incubation) presumptive zygotes were stripped off of the cumulus cells by repeated pipeting, washed with in-vitro culture (IVC) medium and these presumptive zygotes were transferred over 4 days old cumulus cells monolayer (CCM) in 100 µl culture drops. The groups of 4-6 oviductal epithelial cells cylinders were also added in each culture drop and placed in CO_2 incubator. Thereafter, culture media was changed with freshly prepared embryo culture media at every 48 hr. The cleaved oocytes further cultured for development until blastocyst stage or for 9 days extended culture whichever is earlier. The cleavage rate was recorded on day 2 (36 to 48 hours post insemination) of culture and stage of embryonic development was evaluated every 24 hours, until day 9 or blastocyst development whichever is earlier. Observations were made for the cleavage rate and subsequent development of embryos to 4-cell, 8-cell, 16-cell, morula, and different stages of blastocyst development and their hatching.

An embryo was defined to have reached the morula stage when first sign of compaction could be observed but with blastomeres clearly distinguishable on the surface (an appearance referred to as a "Cell Ball" of small blastomeres). The compact morula was defined as the stage at which the blastomeres had coalesced to form a smooth tightly compacted cell mass with minimal diameter. The appearance of early blastocyst stage was characterized by first appearance of a stable confluent blastocoel. Blastocysts were defined as the expanded blastocyst when the diameter of the zona pellucida was increased as a result of expansion of the blastocoel. Finally the expanded blastocysts were identified as hatching when their zona pellucida cracked.

Statistical analysis

The data obtained in the present study were analysed statistically using $\times^2$-test for proportion as described by Snedecor and Cochran.

RESULTS AND DISCUSSION

The results obtained in the present study showing the effect of leptin inclusion in maturation medium used for *in vitro* maturation of buffalo oocytes and their subsequent effect on cleavage rate and early embryonic development are presented in table 1.

In-vitro maturation

A total of 848 oocytes were subjected to *in vitro* maturation in four groups according to plan of work of experiment-2 to study the effect of Leptin. A total of 209, 209, 213 and 217 oocytes were used, respectively in groups I, II, III and IV. A total of 160 (76.55%), 174 (83.25%), 192 (90.14%) and 200 (92.16%) oocytes matured, respectively in groups I, II, III and IV. The overall maturation rate was 85.61%. The oocytes maturation rate in group III (20ng/ml Leptin in IVM) and IV (30ng/ml Leptin in IVM) were significantly ($P<0.05$) higher as

compared to group I (0ng/ml Leptin in IVM) and II (10ng/ml Leptin in IVM).

Cleavage and early embryonic development

The oocytes matured *in vitro* in different Leptin supplemented groups were fertilized *in vitro* using buffalo sperms. The cleavage rate of presumptive zygotes cultured in mSOF at 48 hours post insemination were 48.75, 59.77, 70.83 and 71.50%, respectively with overall cleavage rate of 63.49%. Cleavage rate in group III (20ng/ml Leptin in IVM) and IV (30 ng/ml Leptin in IVM) was significantly ($P<0.05$) higher as compared to group I (0 ng/ml Leptin in IVM) and II (10 ng/ml Leptin in IVM).

The percentage of cleaved zygotes that developed up to morula stage were 41.03, 50.96, 58.82 and 57.34% and up to blastocyst stage, 25.64, 30.77, 44.12 and 44.06%, respectively in group I, II, III and IV. Embryonic development up to Morula and blastocyst stage were significantly ($P<0.05$) higher in group III and IV as compared to group I and II. The percentage of cleaved zygotes that developed to morula as well as blastocyst stage between the group III and IV did not vary significantly indicating that use of 30 ng/ml leptin in IVM leads to almost similar results comparable to 20 ng/ml leptin in IVM. In the present study, Leptin treatment at the rate of 20 ng/ml in IVM gave better results of cleavage rate and blastocyst development.

In the present study, the oocyte maturation rate in group III (20ng/ml Leptin in IVM) and IV (30ng/ml Leptin in IVM) were significantly ($P<0.05$) higher as compared to group I (0ng/ml Leptin in IVM) and II (10ng/ml Leptin in IVM). Cleavage rates were also significantly ($P<0.05$) higher in group III (20ng/ml Leptin in IVM) and IV (30 ng/ml Leptin in IVM) as compared to group I (0 ng/ml Leptin in IVM) and II (10 ng/ml Leptin in IVM). The per cent of cleaved zygotes that developed to morula as well as blastocyst were also significantly ($P<0.05$) higher in group III and IV as compared to group I and II. Morula as well as blastocyst development among the group III and IV did not vary significantly

In agreement with present study, it has been reported that the 10 ng/ml leptin significantly increased the development rate of two-cell stage embryos into blastocysts and hatched blastocysts (Kawamura *et al.*,). It has also been reported that supplementation of culture medium with leptin significantly enhanced the formation of blastocyst and hatched blastocysts in mice and pigs (Craig *et al.*,). It is also reported that leptin supplementation during oocyte maturation reduced the proportion of apoptotic cumulus cells and enhanced subsequent pre implantation development (Paula-Lopes *et al.*,). It is possible that the inhibitory effect of leptin on cumulus cell apoptosis enhances subsequent blastocyst development. Conversely, Swain *et al.* reported that increasing concentrations of murine leptin had no effect on one-cell embryo development, while the study by Fedorcsak and Storeng showed that even low concentrations of leptin inhibited the proportion of embryos reaching the hatched blastocyst stage as the result of induced DNA fragmentation. These conflicting results may have reflected the differences in the strain or species of experimental animal, the culture conditions of the embryos at different developmental stages or the type of exogenous leptin used.

Kawamura *et al.* confirm that leptin improves early embryonic development at physiological concentrations *in vitro*. In contrast, the presence of high leptin concentrations (100 ng/ml) exerts an inhibitory effect on the two and four cell stage embryos developing into advanced stages when cultured *in vitro*. Leptin has a concentration and developmental stage-dependent effect on early mouse embryo development (Muren *et al.*,). They observed that the 100 ng/ml leptin treatment exhibited an inhibitory effect on two-cell embryos.

Leptin plays a role in the cascade of events associated with oocyte maturation. It has been discussed previously, that leptin supplementation during in vitro maturation (IVM) of bovine oocytes exerts long-term positive effects as shown by increased proportion of oocytes that developed to blastocysts, increased blastocyst cell numbers and the reduced proportion of apoptotic cells in the blastocysts (Boelhauve *et al.*,). In agreement with these results, Paula-Lopes *et al.* showed that leptin acts in cumulus-enclosed oocytes to stimulate subsequent development to the blastocyst

stage. Leptin also enhances meiotic progression of pig oocytes and subsequent development of parthenogenetic embryos (Craig *et al.*,). Paula-Lopes *et al.* demonstrated that physiological leptin doses enhanced both oocyte maturation and differentially regulated gene expression in oocytes and cumulus cells. At low doses leptin concentrations enhanced the developmental capacity of oocytes while higher doses have no or inhibitory effect (Boelhauve *et al.*,).

From the present study, it may be concluded that supplementation of Leptin at the rate of 20 ng/ml in *in-vitro* maturation medium significantly increased the cleavage rate and embryonic development up to blastocyst.

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