EFFECT OF HUMAN ACTIVIN-A ON IN-VITRO MATURATION OF OOCYTES AND EARLY EMBRYONIC DEVELOPMENT IN BUFFALOES

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ABSTRACT

The present study was designed to assess the effect of human Activin-A on *in-vitro* maturation of oocytes and early embryonic development. The oocytes were subjected to *in-vitro* maturation for 22 h 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 Activin-A), Group-II (Activin-A 5 ng/ml), Group-III (Activin-A 10 ng/ml), Group-IV (Activin-A 20 ng/ml) in CO_2 incubator at 39°C temperature, 5% CO_2 and high humidity. The matured oocytes were co-incubated with $1x10^6$ sperms of buffalo in modified synthetic oviductal fluid (mSOF) medium containing 10µg/ml heparin and after 22 h of sperm-oocyte incubation, fertilized oocytes were stripped off cumulus cells and cultured in mSOF medium for 7 days to study the embryonic development. A total of 665 oocytes were subjected to *in-vitro* maturation in four groups 165, 167, 166 and 167, respectively in groups I, II, III and IV and 148 (89.69%), 156 (93.41%), 157 (94.57%) and 158 (94.61%) oocytes were matured, respectively in groups I, II, III and IV with overall maturation rate of 93.08%. The cleavage rates were 39.86%, 48.07%, 53.50%, and 59.49%, respectively with overall cleavage rate of 50.40%. It was significantly ($P<0.05$) higher in group IV compared to group I and II (39.86 vs 48.07%). It was also significantly ($P<0.05$) higher in group III compared to group I (53.50 vs 39.07%). Embryonic development up to blastocyst stage was also significantly ($P<0.05$) higher in group IV as compared to group I and II (36.17 vs 16.95 and 21.33%). From the present study, it may be concluded that human Activin-A at 20ng/ml in maturation media significantly improved the cleavage rate and embryonic development up to blastocyst stage in buffalo.

Key words: Buffalo, human activin-A, oocytes, *in vitro* maturation, *in-vitro* fertilization, *in-vitro* culture.

Activin-A is a member of the transforming growth factor β (TGFβ) super family (Kingsley). It formed by disulfide linkage of two β subunits, resulting in either activin A (βAβA). TGFβ family members have a variety of actions on cell growth, differentiation and function from early embryonic stages through adulthood (Rombauts *et al.*, Halvorson and DeCherney). Activins bind a type II receptor (ActRII or ActRIIB), which then recruits and transphosphorylates a type I receptor (ALK4) leading to phosphorylation of downstream smads (Mathews and Vale). Activin promote oocyte maturation (Alak *et al.*,). Activin has been shown to enhance progesterone and inhibin production as well as FSH-induced aromatase activity (Xiao *et al.*,). Activin receptors were characterized some years ago and were found in several types of cell including granulose, cumulus and oocytes (Sadatsuki *et al.*, Izadyar *et al.*,) indicating that activin may exert autocrine or paracrine roles in follicle and oocyte development. Indeed, evidence from functional studies *in vitro* supports a role for these peptides in the regulation of bovine oocyte maturation and acquisition of developmental competence (Stock *et al.*, Silva and Knight, Silva *et al.*,).

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Several attempts have been made to assess the effect of activin and follistatin on oocyte maturation but the results so far have been conflicting. In the majority of studies, observations have been restricted to effects on nuclear maturation while some authors showed no effect of activin on nuclear maturation in rat (Tsafriri *et al.*,1989) and bovine (Van Tol *et al.*,1994) oocytes, others reported that activin increased the percentage of germinal vesicle breakdown in rat (Itoh *et al.*,1990) and primate (Alak *et al.*,1996) oocytes. In these studies, only nuclear maturation was assessed and cytoplasmic maturation, as indicated by the ability of fertilized oocytes to develop to the blastocyst stage was not monitored. Izadyar *et al.* reported that addition of activin-A to bovine oocyte maturation media did not affect the proportion of blastocysts formed but the dose of activin-A used (10 ng/ml) was very low compared with the endogenous levels (3 µg/ml) shown to be present in bovine follicular fluid (bFF) (Knight *et al.*,1995). Silva and Knight reported that activin-A did not significantly affect post fertilization cleavage rate indicating a lack of effect on bovine oocyte nuclear maturation. This observation is consistent with a previous report of Van Tol *et al.* showing that the same doses of activin-A (0.1 and 0.5 µg/ml) tested had no effect on nuclear maturation of bovine oocytes. It suggest that activin-A can modulate cytoplasmic maturation of oocytes since their competence to develop into blastocysts is dependant on proper cytoplasmic maturation (Ectors *et al., 1995*). The present study was performed to analyse the differential effect of activin-A on oocytes maturation and early embryonic development in buffaloes.

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 h 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 medium (OCM). The oocyte collection medium 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.

Total oocytes were 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 used for maturation in 50 µl droplets of maturation medium in 35x10 mm petridishes for 22-24 h 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 h 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 Activin-A), Group-II (Activin-A 5 ng/ml), Group-III (Activin-A 10 ng/ml), Group-IV (Activin-A 20 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) was used for *in-vitro* capacitation of the sperm to be used for *in vitro* fertilization. For fertilization of matured oocytes, 50 µl droplets of mSOF in a 35x10 mm petridish covered with mineral oil was pre-equilibrated in CO_2 incubator for 2h. Frozen semen of Murrah bull was thawed at 37°C for 30 seconds and the contents were poured in a 15 ml centrifuge tube containing 14.5 ml of sperm washing medium (mSOF). After gentle mixing, the tube was centrifuged at 110 g (800 rpm) for 10 min (Remifuge, Mumbai, India). The supernatant was discarded and sperm pellet was re-suspended 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 re-suspended in 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 non essential 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). The 50 µl droplets containing oocytes were charged with 50 µl of sperm suspension (taken from top 1/3rd portion) having concentration of progressively motile sperm as 1-2 x10^6/ml and maintained at 39°C, 5% CO_2 and high humidity for about 22 h.

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 non essential 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 transferred over cumulus cells monolayer (CCM) in 100 ml 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 blastocyt development and their hatching.

A total of 165, 167, 166 and 167 oocytes were used respectively in group I, II, III and IV, and a total of 148 (89.69%), 156 (93.41%), 157 (94.57%) and 158 (94.61%) oocytes matured in group I, II, III and IV, respectively. The overall maturation rate was 93.08%. The maturation rate among different groups did not vary significantly ($P<0.05$).

The oocytes matured *in vitro* in different activin-A supplemented groups were fertilized *in-vitro* using buffalo sperms. The cleavage rates of presumptive zygotes cultured in mSOF at 48 hours post insemination were 39.86%, 48.07%, 53.50% and 59.49%, respectively in groups I, II, III and IV. The overall cleavage rate was 50.40%. Cleavage rate in group IV (Activin-A, 20 ng/ml in IVM) was significantly ($P<0.05$) higher as compared to group I (Activin-A, 0 ng/ml in IVM) and II (Activin-A, 5 ng/ml in IVM). It was also significantly ($P<0.05$) higher in group III (Activin-A, 10 ng/ml in IVM) compared to group I.

The percentage of cleaved zygotes that developed up to morula stage were 57.62, 50.67, 59.52 and 61.70% and up to blastocyst stage were 16.95, 21.33, 26.19 and 36.17%, respectively in groups I, II, III and IV. Embryonic development up to blastocyst stage was also significantly ($P<0.05$) higher in group IV as compared to group I and II. In the present study, dose dependent increases in cleavage rate as well as percentage of cleaved presumptive zygotes that developed to morula and blastocyst stages were

observed. Activin-A treatment at the rate of 20 ng/ml in IVM gave better results of cleavage rate as well as morula and blastocyst development.

In the present study the oocyte maturation rates between the treatment groups as well as control group did not vary significantly (P<0.05). This is in agreement with Izadyar *et al.* who reported that activin-A had no effect on bovine oocyte maturation. Silva and Knight (1998) also indicated that activin-A lacks significant effect on nuclear maturation of bovine oocytes. This observation is consistent with a previous report of Van Tol et al. (1994) os showing that the activin-A (0.1 and 0.5 µg/ml) had no effect on nuclear maturation of bovine oocytes.

Cleavage rate in group IV (Activin-A, 20 ng/ml in IVM) was significantly (P<0.05) higher as compared to group I (Activin-A, 0 ng/ml in IVM) and II (Activin-A, 5 ng/ml in IVM). It was also significantly (P<0.05) higher in group III (Activin-A, 10 ng/ml in IVM) compared to group I. Percentage of cleaved embryos that developed to blastocyst stage were also significantly (P<0.05) higher in group IV (Activin-A, 20 ng/ml in IVM) compared to group I and II. In the present study, dose dependent increase in cleavage rate as well as percentage of cleaved embryos which developed to morula and blastocyst stages were observed. Activin-A treatment at 20 ng/ml in IVM gave better results of cleavage and blastocyst development.

The results are in concurrent with the study of Stock *et al.*, (1997) they observed that the addition of activin-A (10 and 100 ng/ml) during *in vitro* maturation significantly improved postcleavage development. Additional support to beneficial role of activin-A on oocyte maturation is also provided by observation of a positive correlation between endogenous activin-A concentration in IVM droplets and the proportion of oocytes developing to the blastocyst stage (Silva and Knight,1998). It suggests that activin-A can modulate cytoplasmic maturation of oocytes since their competence to develop into blastocysts is dependant on proper cytoplasmic maturation (Ectors *et al.*,1995). The presence of exogenous activin-A during COC maturation significantly increased the developmental potential of oocytes as expressed by the proportion of either total oocytes or cleaved oocytes reaching the blastocyst stage (Silva and Knight,1997).

Activin receptors mRNA were expressed in several types of cell including granulose, cumulus cells and oocytes (Sadatsuki *et al.,* 1993 Izadyar *et al., 1996*) which suggests the role of activin in follicle and oocyte development. It may exert its effect via autocrine or paracrine manner in the regulation of oocyte maturation. Evidence from *in-vitro* studies supports a role for activin in the regulation of bovine oocyte maturation and acquisition of developmental competence (Stock *et al.*, Silva and Knight, 1998 Silva *et al., 1999*). In other species it is reported that activin increased the percentage of germinal vesicle breakdown in rat (Itoh *et al.*) and primate (Alak *et al.,*1996) oocytes. It has also been reported that activin increases the number of FSH receptors on rat granulosa cells (Nakamura *et al.*1993). It is therefore, possible that activin-induced increase in FSH receptors on cumulus granulosa cells, lead to enhanced gonadotropin action during *in-vitro* COC maturation could account for the positive effect of activin on oocyte developmental competence observed by Silva and Knight.

From the present study, it may be concluded that supplementation of human Activin-A at 20 ng/ml in *in-vitro* maturation medium significantly increases the

cleavage rate as well morula and blastocyst development. However, dose dependent increase in cleavage rate as well as morula and blastocyst development rate was observed.

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