Sex Linked Developmental Rate Differences in Murrah Buffalo (*Bubalus bubalis*) Embryos Fertilized and Cultured *In Vitro*

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ABSTRACT : The aim of the present study was to determine the effect of paternal sex chromosome on early development of buffalo embryos fertilized and cultured *in vitro*. Embryos were produced *in vitro* from abattoir derived buffalo oocytes. The cleaved embryos were co-cultured with buffalo oviductal epithelial cells and evaluated on day 7 under the phase contrast microscope to classify development. The embryos which reached the morula/blastocyst stage were fast developing, the embryos which were at 16-32 cell stage were medium developing and the embryos below 16 cell stage were slow developing. The embryos which showed some fragmentation in the blastomeres or degenerated blastomeres, were degenerating. Sex of embryos (n = 159) was determined using PCR for amplification of a male specific BRY. 1 (301 bp) and a buffalo specific satellite DNA (216 bp) fragments. The results thus obtained show that 1) X and Y chromosome bearing sperms fertilize oocytes to give almost equal numbers of cleaved XX and XY embryos, 2) male embryos develop faster than female embryos to reach advanced stage and 3) degeneration of buffalo embryos is not linked with the paternal sex chromosome. We suggest that faster development of males is due to differential processing of X and Y chromosome within the zygote for its activation and / or differential expression of genes on paternal sex chromosome sex chromosome during development of buffalo embryos fertilized and cultured *in vitro* which may be attributed to a combination of genetic and environmental factors.
(**Key Words**: Sexual Dimorphism, Early Development, Sex Ratio, Embryo Survival)

INTRODUCTION

For decades it has been accepted that all phenotypic sexual differentiation is secondary to gonadal development. Recently, however, several reports indicate that sexual dimorphism occurs even before differentiation of gonads begins, as XY embryos develop faster than their XX rivals. Based on the sex ratio of live young after transfer of fast and slow cleaving blastocysts to pseudopregnant mice, Tsunoda et al. (1985) concluded that mouse male embryos develop faster than female embryos. Subsequent studies with cattle embryos using cytogenetic analysis (Avery et al., 1991; Yadav et al., 1993) or polymerase chain reaction (Avery et al., 1992) to determine the sex, supported the assumption that *in vitro* male bovine embryos develop faster than female embryos. However, the overall sex ratio of bovine embryos produced *in vitro* doesn't differ from the expected value (Iwasaki et al., 1989; King et al., 1991). There is no report which indicates whether a correlation exists between the rate of development and sex of preimplantation buffalo embryos produced either *in vitro* or *in vivo*. Therefore, the present work was undertaken to study the effect of paternal sex chromosome on 1) whether X and Y chromosome bearing sperms fertilize oocytes to give equal or different number of cleaved XX and XY embryos 2) developmental pattern of male and female embryos, and 3) sex linked survival of buffalo embryos, fertilized and cultured *in vitro*.

MATERIALS AND METHODS

In vitro production of embryos

Buffalo ovaries were collected from an abattoir and transported to the laboratory in sterile isotonic saline at 32 to 37℃ within 4 h. Follicular oocytes (2 to 6 mm in diameter) were aspirated with 19 gauge needle attached to 5 ml syringe. The aspiration medium consisted of TCM-199 supplemented with 10% FBS and phosphate buffered saline supplemented with 0.3% BSA in 1:1 ratio. Cumulus oocyte complexes (COCs) with an unexpanded cumulus and homogenous cytoplasmic granulation were chosen for *in vitro* maturation. Briefly, after washing with

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culture medium (TCM-199 + 10% FBS + 5 μg/ml FSH-P) the COCs were placed in 50 μl droplets of culture medium (10-12 COCs per droplet), covered with paraffin oil in a 35 mm petridish and cultured for 24 hours in CO_2 incubator (5% CO_2 in air) at 38.5℃ (Madan et al., 1994). The sperm used for IVF throughout the present study was from the same batch and the same donor and had been tested for IVF earlier. The sperm was prepared for insemination as described by Chauhan et al. (1997). Briefly, two straws of frozen thawed ejaculated buffalo semen were washed with BO medium (Bracked and Oliphant, 1975) containing heparin (10 μg/ml) without BSA. The sperm was suspended for swim up in BO medium with 10 μg/ml heparin and 10 mM caffeine. Progressively motile sperm were placed in 100 μl droplet of BO medium containing 0.5% BSA, 10 μg/ml heparin and 5 mM caffeine in a petridish, covered with mineral oil and placed in CO_2 incubator for one hour at 38.5℃ before insemination to *in vitro* cultured oocytes. After 24 hours of *in vitro* culture, the oocytes were washed in BO medium and introduced into 100 μl droplets of processed buffalo sperm (10-12 COCs per droplet) and were left for 6 hours in CO_2 incubator at 38.5℃. At the end of sperm-oocyte incubation, the oocytes were separated from the sperm droplet and washed with TCM-199 supplemented with 10% FBS and cultured for 42 h (the beginning of the oocyte-sperm incubation was designated as day 0) after which these were examined for cleavage.

Buffalo oviducts were collected from freshly slaughtered animals having ovaries with freshly formed corpora lutea and brought over to the laboratory in isotonic saline at 4℃. The oviducts were freed from ligaments and adjoining blood vessels and 2 cms of ampullary and isthmic regions were excised. Using a pair of foreceps as a clamp, epithelial cells were expressed from the isthmus towards the ampulla into a petridish containing TCM-199 with 10% FBS. The cells were disintegrated into small clusters by repeated flushings through 26G needle and the resultant cell suspension was allowed to settle at the bottom of the petridish. The cells were picked up from there and washed twice with 5 ml of TCM-199 containing 10% FBS. The cell suspensions were transferred to 200 μl droplets of TCM-199 with 10% FBS and cultured in CO_2 incubator at 38.5℃. After 24 hours the cell clusters were washed three times in TCM-199 containing 10% FBS and transferred to each of the culture droplets (approximately 50 cells per 50 μl droplet) to be used subsequently for co-culture with cleaved embroyos. Cleaved embryos were co-cultured at 38.5℃, 5% CO_2 in air and evaluated under phase contrast microscopy on day 7 to classify as fast, medium, slow developing and degeneration embryos.

Sex determination

Pure genomic DNA from buffaloes of known phenotypic sex was prepared for use in PCR to serve as positive controls. Peripheral blood from male and female buffaloes was collected in ACD solution and buffy coat was separated to isolate genomic DNA using proteinase K : SDS digestion, phenol extraction followed by ethanol precipitation (Sambrook et al., 1989). DNA was dissolved in TE and stored at 4℃. EDTA was removed prior to its use in PCR. *In vitro* producted embryos (n = 159) at different stages of development (2-cell to blastocyst) were washed serially in 5-6 separate drops of DMPBS to finally aspire in 2 μl DMPBS and transferred into 0.6 ml eppendorf tube containing 2 μg of proteinase K in 18 μl autoclaved and UV irradiated triple glass distilled water overlaid with a drop of mineral oil. The embryos were incubated at 55℃ for min followed by 10 min inactivation of proteinase K at 99℃ and used in PCR.

PCR sexing (Apparao et al., 1993) was conducted with modifications to favor optimum amplification. Two pairs of primers BRY.1 (301 bp male specific for a fragment present on Y chromosome of buffalo) and satellite DNA (A control pair of primers which amplifies the satellite DNA sequence, 216 bp, in both the male and the female) were employed. Amplification of satellite sequence in both the sexes served as an internal control to validate the success of PCR in case the sample happened to be female. The sequence of the primers were; BRY.1 sense: ggATCCgAgACACAgAACAgg, antisense: gCTAATCCATCCATCCTATAg (Reed et al., 1988); and satellite DNA sense: TggAAgCAAAgAACCCCgCT and antisense: TCgTgAgAAACCgCACACTg (Pluciennicczak et al., 1982). HPLC purified Primers were used to avoid spurious amplification. PCR was conducted according to standard procedure (Saiki et al., 1988) for amplification of a male specific 301 bp and buffalo specific 216 bp fragments. The reaction mixture contained 1X PCR buffer, 200 μM of each dNTP, 0.2 μM of each of the primers, 1 U Taq polymerase and 0.1% Triton-X-100 in a 50 μl reaction. The pH of 100 mM dNTPs stock solution was neutral. All reagents were premixed to form a sexing reagent which was divided into aliquots containing adequate reagent for 10 sexing reactions (300 μl each aliquot) and stored in a separate section of the freezer. Thirty μl of this sexing reagent was added to 20 μl PCR sample during the initial denaturation to give 'hot start' for avoidance of primer dimerization (Erlich et al., 1991). Thermocycling was performed on minicycler (MJ Research Inc., MA) for 40 cycles with denaturation at

95℃ for 30 sec, annealing at 58℃ for 1 min and extension at 72℃ for 40 sec. Thermocycling started with initial denaturation at 95℃ for 5 min and ended with final extension at 72℃ for 5 min. The reaction mixture was brought to room temperature and analyzed immediately using constant field agarose gel electrophoresis. Twenty μl of PCR amplified products were analyzed on 2.5% agarose gel. The separated fragments, stained with Ethidium Bromide, were visualized under UV lights.

Statistical analysis

Chi square analysis was performed to compare observed sex ratio (both overall and at each development rate) with the expected sex ratio of 1 : 1.

RESULTS

Buffalo preimplantation embroys (n = 159) were producted from abattoir derived oocytes matured and fertilized *in vitro*. All cleaved embryos could be obtained up to 42 hours post-insemination (hpi) and no cleavage was observed after 42 hpi when observed till 72 hpi. The cleaved embryos, co-cultured with buffalo oviductal epithelial cells, were evaluated on day 7 under the phase contrast microscope with DIC attachment (figure 1). The criteria to classify development rate was that the embryos which reached the morula/blastocyst stage were fast developing, the embryos which were at 16-32 cell stages were medium developing and the embryos below 16 cell stage were slow developing. The embryos which showed

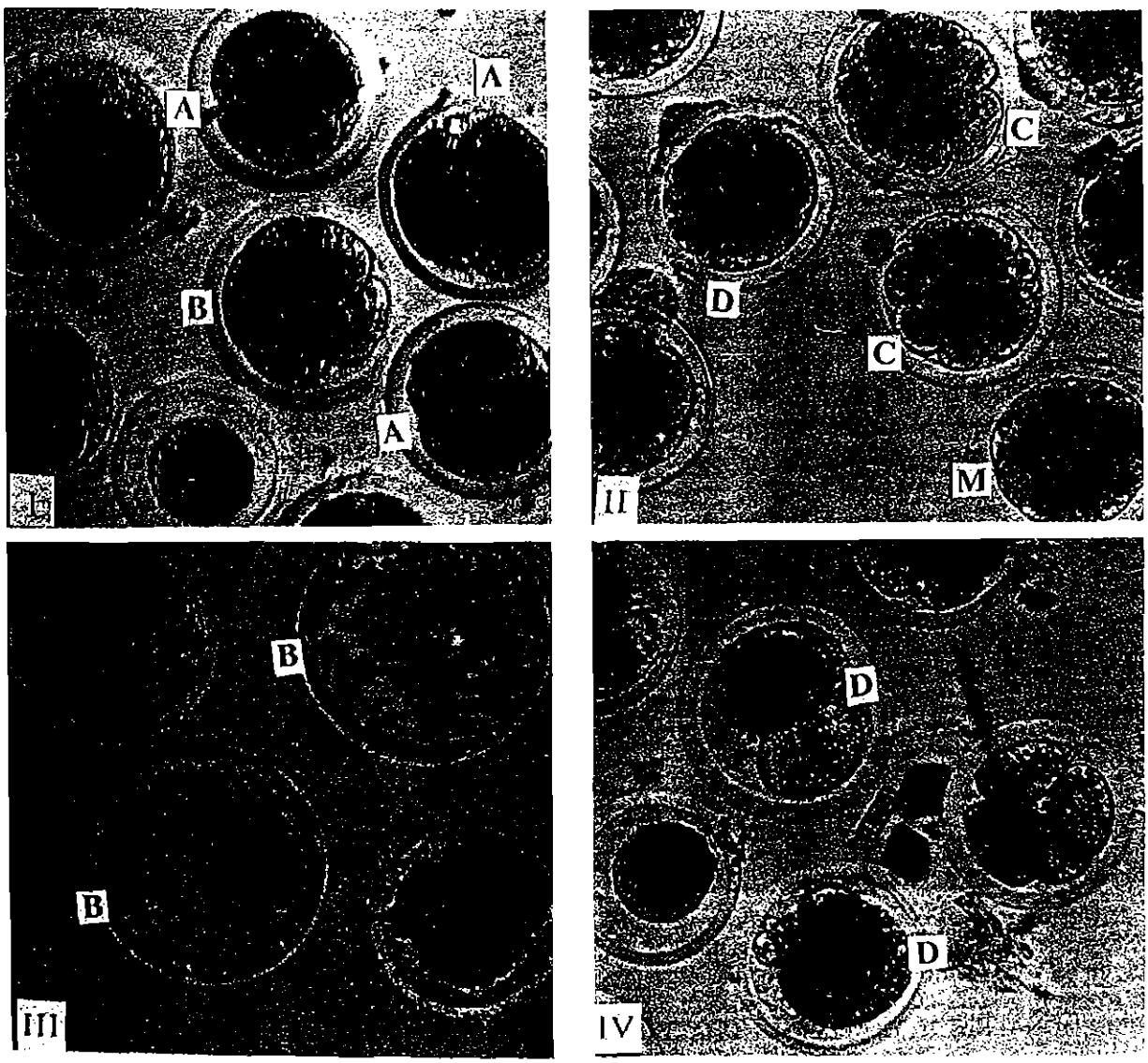

Figure 1. *In vitro* produced buffalo embryos at different stages of their development Ⅰ) 2-cell [A] and 4-cell [B]; Ⅱ) 8-cell [C] 16-cell [D] and morula [M]; Ⅲ) Blastocyst [B] and; Ⅳ) degenerated [D].

the fragmentation in the blastomeres or degenerated blastomeres, were degenerating. On day 7 fast growing morula/ blastocyst, medium growing 16-32 cell and, slow growing 2-15 cell and degenerating embryos were together observed. The morphology of cultured embryos were normal. The blastomeres were round with completely filled cytoplasm in all embryos except degenrated embryos when observed on day 7 of culture.

PCR was applied to buffalo embryos for amplification of male specific BRY.1 DNA (301 bp) present on Y-chromosome. A control pair of primers, which amplifies the satellite DNA sequence (216 bp) in both the female and the male buffaloes, was also included in PCR amplification (multiplex PCR). Amplification of this sequence validated the success of PCR and ascertained that embryo sample was not lost while its transfer to PCR tube, in case the sample happened to be female. A negative control (NDC for no DNA control) alongside two positive controls, i.e. a male (MC) and a female (FC) control (pure genomic DNA from known phenotypic sex), were set up simultaneously to check any possibility of contamination. Expected pattern for NDC, FC and MC were obtained. Therefore, the assay was valid and there was no contamination. Results were interpreted accurately by direct comparison of the sample with controls i.e. male pattern in the sample for male, female pattern in the sample for female. NDC never showed FC or MC pattern, which meant that there was no contamination of either naturally arising DNA or carry over contamination. In all the 159 embryos either both male specific 301 bp and buffalo specific 216 bp (male embryo), or only buffalo specific 216 bp (female embryo) fluorescent bands of amplified DNA fragment were observed (figure 2). Sex ratio which is defined as the observed number of male embroys in total number of embryos may have any value from zero (if all females) to one (if all males) and significantly different higher sex ratio was observed at fast development rate and advanced development stage (figure 3). No. significant differences were observed between male and female degenerating and; male and female surviving embryos (figure 4) which show that the degeneration of the buffalo embryos produced *in vitro* is

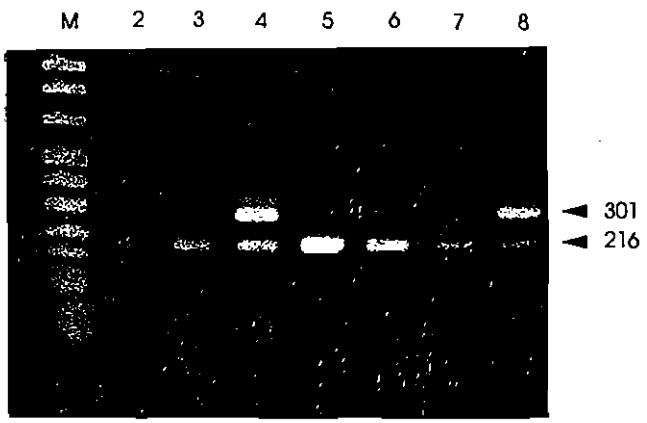

Figure 2. Agarose gel electrophoresis analysis of multiplex PCR products amplified with buffalo samples in the presence of BRY.1-specific (male) and satellite sequence-specific (control) primers. Lane M- molecular size marker (pUC BM 21 DNA, Hpa II + pUC BM 21 DNA. Dra I + Hind III), lane 2- No DNA control, Lane 3- Female control, Lane 4- Male control, Lane 5, 6, 7- female embryos and Lane 8- male embryo.

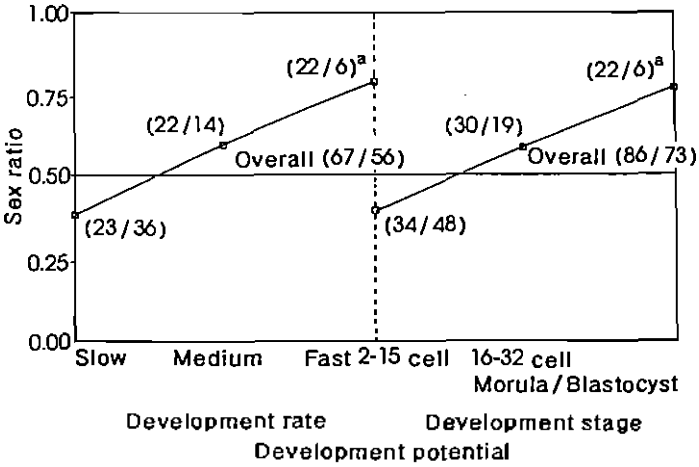

Figure 3. Sex ratio determined by PCR analysis of buffalo embryos fertilized and cultured *in vitro*.

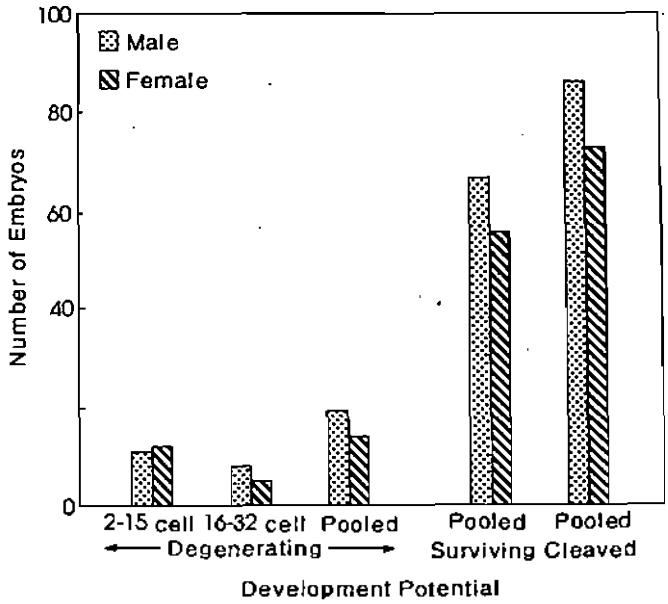

Figure 4. Sex linked survival of buffalo embryos generated *in vitro*.

not controlled by paternal sex chromosome. Overall sex ratio was not significantly different from the expected value.

DISCUSSION

The process of sexual dimorphism is dependent on the activity of sets of genes in complex interacting networks or pathways. Within these pathways, specific genes may be rate-limiting or act as a switch, such that their activity seems to cause a development event. In the mouse, it was concluded for embryos produced both *in vitro* (Valdivia et al., 1993) and *in vivo* (Tsunoda et al., 1985) that male embryos develop faster than female embryos. In bovine embryos, contradictory findings have been reported about the sex linked developmental rate differences among *in vitro* (Avery et al., 1991; Xu et al., 1992; Yadav et al., 1993) and *in vivo* fertilized embryos (Avery et al., 1989). However, for *in vitro* produced bovine embryos, there is an agreement that male embryos develop faster than females. Their is no report in buffalo embryos to either support or contradict the hypothesis that male embryo develop faster than their female colleagues *in vitro*, although Totey et al. (1996) reported that the overall sex ratio deviated from the expected value in favor of either males or females depending upon the bull. However, our results could not confirm this as almost equal numbers of cleaved male and female embryos were obtained. In the present study, established *in vitro* techniques to mature and fertilize oocytes followed by *in vitro* culture of embryos (Chauhan et al., 1997) were used to synchronize the start of the development as accurately as possible. The results thus obtained show that males develop faster than female buffalo embryos. The present results are supported by our recent observation, in which we transferred *in vitro* generated fast developing embryos (blastocysts) to recipient females and out of seven calves born, six were males (Chauhan, MS, unpublished).

Significant trends towards higher sex ratio at advanced stages and fast growing buffalo embryos (figure 3) can have three possible explanations. Firstly, that semen used for *in vitro* fertilization contained more Y chromosome bearing sperms. Secondly, that the number of Y chromosome bearing sperm which penetrate the oocyte is more. Both of these seem unlikely as overall sex ratio (0.517) was not different from the expected value (0.5). The plausible explanation seems to be that by the time the embryos were sexed, males reached the advanced stage of development at faster rate. This might be due to differential processing of X and Y chromosome bearing sperm within the zygote and/ or; to very early differential expression of genes derived from X and Y (including cis-acting regulatory elements) chromosomes; or genes on autosomal chromosomes controlled by a gene on X or Y chromosome (paternal sex chromosomal trans-acting regulatory elements). In the mouse, differential behaviour of paternal sex chromosome within early embryos was found to increase the development gap between male and female embryos. Thornhill and Burgoyne (1993) suggested that X chromosome has a retarding effect whereas others (Burgoyne, 1993; Tsunoda et al, 1985) attributed faster development of males to an accelerating effect of Y chromosome. Transcription of Sry, the only Y linked gene required to give rise male development (Koopman et al., 1991) has been shown to start at as early embryonic stage as 2-cell (Zwingman et al., 1993), which suggest that paternal Y chromosome is activated after the first cleavage. It has been reported that mouse X linked enzyme (HGPRT) increase rapidly after 3rd cleavage (Monk and Handyside, 1988). In bovines also, it was suggested that activation of paternal X chromosome seems to occur after 3rd cleavage as HGPRT activity rapidly increases after 8-cell stage (Kratezer and Gartler, 1978) and has been confirmed recently (Kita and Imai, 1993). Therefore there seems to be a differential processing of X and Y chromosome leading to their activation at different stages of development, and this might be involved in the initiation of developmental rate differences between male and female embryos. This falls in line with Yadav et al. (1993) who were led to conclude that sex differences in embryonic development probably become apparent before activation of the embryonic genome.

Expression of X- and Y-chromosome related genes at different stages of development demonstrate the differences in the gene expression in male and female embryos at the very early stages leading to differential activities of one or more metabolic pathways. Total glucose metabolism in male bovine embryos collected directly from donor cattle 7 days after estrus is greater than in female embryos, and it has been suggested that this may be related to more rapid dvelopment of males (Tiffin et al., 1991). Also, it has been reported that glucose control sex related growth rate differences and is responsible for early cleavage (Peippo and Bredbacka, 1996) and fast cleavage (Bredbacka and Bredbacka, 1996) of male bovine embryos cultured *in vitro* and raised the possibility that the sex difference is linked with growth stimulating effects of oxygen radicals. The levels of these may be unusually low in female as a result of glucose dependent control of the X linked enzymes like HPRT and G6PDH which are overexpressed prior to X

chromosome inactivation (Bredbacka and Bredbacka, 1996). Since glucose was also present in our *in vitro* embryo culture system (M-199), the fast development of males in buffalo may also be attributed to glucose controlled development.

CONCLUSION

Buffalo embryos produced *in vitro* show that more number of male embryos reached the advanced stage of development showing their faster development than the female. However, the number of cleaved embryos is likely to have equal numbers of male and female embryos. Also, it is shown that degeneration as well as survival of cleaved embryos produced *in vitro* is not controlled by paternal sex chromosome. We suggest that the fast development of male embryos may be due to differential processing of X and Y chromosome within the zygote for its activition and / or differential expression of genes on paternal sex chromosome during development of buffalo embryos fertilized and cultured *in vitro* which might be due to a combination of genetic and environmental factors and remains to be clarified.

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