SEXING OF *IN VITRO* PRODUCED PREIMPLANTATION BUFFALO EMBRYOS BASED ON EARLY CLEAVAGE RATE

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ABSTRACT

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The present study aimed to determine the sex of *in vitro* produced preimplantation buffalo embryos in relation to the time of first cleavage, by polymerase chain reaction (PCR). Abattoir derived buffalo oocytes fertilized *in vitro* were monitored from 18 hours post insemination (hpi) to establish the time of first cleavage. The early cleaved 2-cell embryos were collected at specific time points viz. 18, 24 and 36 hpi, snap frozen and sexed to study the time of cleavage versus sex of *in vitro* produced buffalo embryos. Of the 113 embryos cleaved, the percentage of embryos that cleaved at 18, 24 and 36 hpi was 2.65, 6.19 and 91.15, respectively. The percentage of male and female embryos that cleaved at 18, 24, and 36 hpi were 66.67 and 33.33, 50 and 50, and 40 and 60, respectively. Thus, it could be concluded that the time of first cleavage in buffalo embryos occurred as early as 18 hpi with male embryos cleaving at a faster rate than the female embryos.

Key words: Sex, Cleavage rate, Preimplantation, Buffalo embryos

INTRODUCTION

In cattle and buffalo industry certain sectors would benefit from a higher proportion of male progeny most notably beef breeders (Masaya Geshi, 2012). With the advent of *in vitro* fertilization (IVF) and the technique of embryo manipulation, sexing of preimplantation embryos has paved the way for manipulating the sex ratio in buffaloes. In cattle, Yadav *et al.* (1993) and Larson *et al.* (2001) have suggested that male embryos develop faster than female embryos.

Sex detection of embryos as early as the 2-cell stage would be more beneficial, pointed to the fact that this difference was indicated by the time of the first cleavage itself (Yadav *et al.*, 1993) and this could be used as an easy noninvasive method for embryo sexing. Although numerous approaches for mammalian embryo sexing have been reported, PCR appeared to be the most practical and reliable for sex determination of embryos by detecting male specific DNA sequences from buffalo embryo biopsies (Bredbacka *et al.*, 1994) and has invaluable advantage of being so fast (Hirayama *et al.*, 2013). Hence the present study was designed with objective of determining the sex of *in vitro* produced preimplantation buffalo embryos in relation to the time of first cleavage, by polymerase chain reaction (PCR).

MATERIALS AND METHODS

Buffalo ovaries were obtained from the local abattoir and transported to the Assisted Reproductive Technology Laboratory, Department of Veterinary Gynaecology and Obstetrics, Madras Veterinary College, Chennai in 0.9 per cent normal saline

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containing penicillin (100 IU/ml) and streptomycin (50 mg/ml) at 30-35° C in a thermos flask within 2 h of slaughter.

Experiment I was designed to assess the early cleavage time of buffalo embryo. Preliminary trials (n=6) were carried out wherein, the fertilized oocytes were screened from 18 hpi to establish the time of first cleavage.

Experiment II was designed to study the sex of early cleaved embryos. Based on results of experiment I, trials (n=6) were carried out wherein, early cleaved 2-cell embryos were collected at specific time points and snap frozen for sexing.

Cumulus oocyte complexes (COCs) retrieved by slicing of ovaries were screened and graded as A, B, C, D and E based on their cumulus cells investment and ooplasm homogeneity. Only COCs of grades A, B and C were matured *in vitro*. The A, B and C grade oocytes were washed in TCM 199 + 10 per cent fetal bovine serum (FBS - GIBCO: Invitrogen, USA) and finally in *in vitro* maturation (IVM) medium, composed of TCM-199 supplemented with 10 per cent FBS, 1 µg/ml of Folltropin (FSH), 0.02 IU/ml of Luteinizing Hormone (LH), 1 µg/ml of estradiol and 10 ng/ml of epidermal growth factor (EGF). A group of 10-15 COCs were transferred to a 50 µl droplets of maturation medium in a 35 mm petridish, pre-equilibriated with the IVM medium for 2h at 38.5°C under 5 per cent CO_2 in air and *in vitro* cultured for 24 h at 38.5°C in a humidified atmosphere of 5 per cent CO_2 in air.

After 24 h of IVM, the maturation rate was assessed based on the degree of cumulus expansion as Degrees 2, 1 and 0. The COCs with 2 and 1 degrees of cumulus expansion were considered as matured and utilized for *in vitro* fertilization. The motile sperms from frozen thawed semen were separated by swim up method. Concentration of the final sperm pellet was determined with a haemocytometer and the sample was diluted with spTALP (sperm tyrode's albumin lactate pyruvate) to yield a concentration of 1-2 $\times 10^6$ sperm/ml. The fertilization droplets of 75 µl of *in vitro* fertilization (IVF-TALP) medium supplemented with heparin (10 µg/ml) in 35 mm petridish overlaid with sterile mineral oil was pre-equlibrated at 38.5°C under 5 per cent CO_2 in air. The matured COCs were washed in pre-equilibriated spTALP and oocytes were washed in IVF-TALP medium and transferred to the pre-equilibrated IVF droplets such that each droplet contained 10-15 oocytes. The motile sperm suspension obtained by swim up technique were inseminated into the IVF droplets containing oocytes to achieve the final concentration of 2 million sperm/ml and co-incubated for 18-24 h at 38.5°C in a humidified atmosphere of 5 per cent CO_2 in air.

At the end of insemination period, the presumptive zygotes were washed three times in IVC medium and transferred separately into pre-equilibrated 50 µl IVC droplets (10-15/droplet) containing synthetic oviduct fluid (SOF), supplemented with streptomycin (100 µg/ml) and penicillin (100 U/ml) and *in vitro* cultured for 36 hpi. Early cleavage to 2-cell embryo and cleavage rate were assessed at specific point of time viz. 18, 24 and 36 hpi. The cleaved embryos were washed thrice in sterile PBS and snap frozen in 2 µl nuclease free water for sexing.

Sex of the embryos was determined by the amplification of male specific sequences in buffalo genomic DNA using primers from Y chromosome specific repeat sequences. The embryonic DNA obtained from *in vitro* produced 2-cell stage buffalo embryos were used for sexing. Direct duplex PCR was carried out for amplifying the BRY.1, BOV 97M and satellite gene fragment of buffalo.

Repeat Sequence	Primer Sequence	PCR product length (bp)
Satellite Sequence	**5' TGGAAGCAAAGAACCCCGCT 3'** **5' TCGTGAGAAACCGCACACTG 3'**	216
BOV 97M sequence	**5' GATCTTGTGATAAAAAGGCTATGC 3'** **5' GATCACTATACATACACCACTCTC 3'**	150
BRY.1 sequence	**5' GGATCCGAGACACAGAACAGG 3'** **5' GCTAATCCATCCATCCTATAG 3'**	300

The following thermofile was followed for amplification,

Step 1	Initial denaturation	97°C 3 min	
Step 2	Denaturation	94°C 30 sec	40 cycles
	Annealing	58°C 45 sec	
	Extension	72°C 45 sec	
Step 3	Final extension	72°C 5 min	
	4°C thereafter		

RESULTS AND DISCUSSION

The timing of first zygotic cleavage means the time frame from the oocyte insemination to the first mitotic division (Van Soom *et al.*, 1997). It is evident that in cattle as in humans timing of the first zygotic cleavage is a valuable parameter of intrinsic embryo quality (Lonergan *et al.*, 1999 and Lonergan *et al.*, 2000). The experimental design of several studies spans the period of time corresponding to the first division between 24 hpi and 48 hpi with varying selection time points. The precise timing of events during the first cell cycle is however hard to establish. The time to the first zygotic cleavage varies by 8 h in humans (between 22 and 30 hpi, Payne *et al.*, 1997) and more than 20 h in cattle (between 22 and 48 hpi, Grisart *et al.*, 1994) with the peak of 2-cell stage embryos at 36 hpi. In the present study, out of 1100 oocytes cultured in experiments I and II, 933 showed cumulus expansion with mean maturation rate of 84.48± 0.98.

In experiment I, the fertilized oocytes were screened from 18 hpi to determine the time of first cleavage. Of the 113 embryos cleaved, the percentage of embryos that cleaved at 18 hpi was 2.65 (3/113). Perusal of available literature showed no reports on cleavage rate at this specific point of time. The time chosen for the selection of first cleavage embryo in the present study was based on the previous report of Gupta (2003) who reported 23 per cent cleavage before 24 hpi and 77 per cent cleavage after 24 hpi. Of 113 embryos that cleaved, 6.19 per cent (7/113) cleaved at 24 hpi while 91.15 per cent (103/113) cleaved at 36 hpi. The results of the present study were in accordance with results of Lonergan *et al.* (1999) who stated that more than 75 per cent of zygotes underwent the first cleavage by 36 hpi and these embryos accounted for more than 90 per cent of all blastocysts produced. Totey *et al.* (1996) reported that the first cleavage in the she buffalo occurred *in vitro* between 24 and 30 hpi although some of the embryos underwent their first cleavage outside of this range. They also reported that 71 per

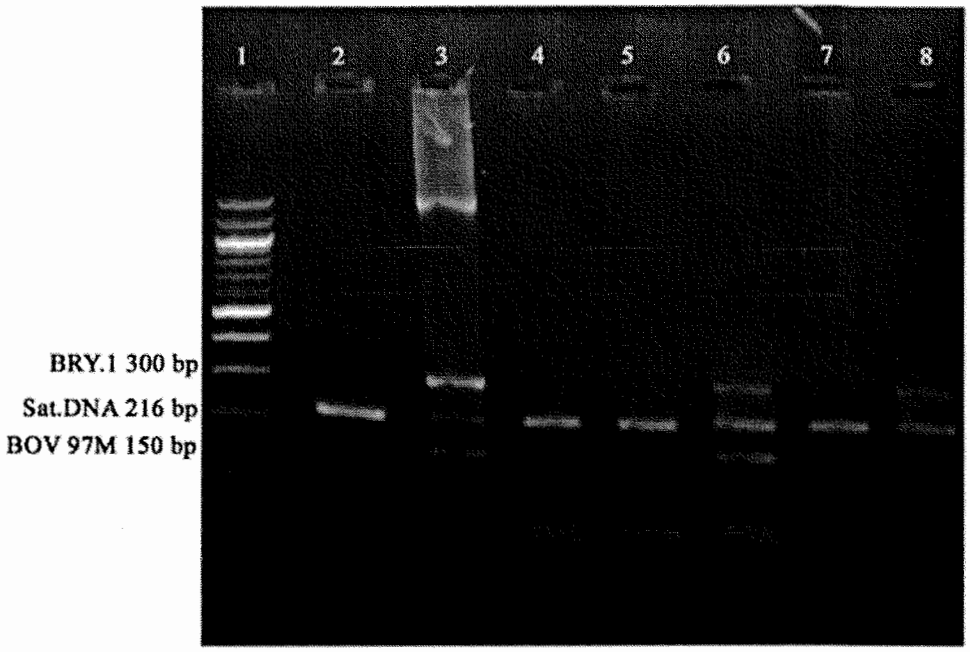
cent had cleaved before 30 hpi and 29 per cent had cleaved after 30 hpi. The discrepancy in the result from the present study might be due to differences in cut off time points i.e. 24 hpi versus 30 hpi with the possibility of more number of cleavage between 24-30 hpi. Although reasons for the observed variation in the timing of the first zygotic cleavage are still not clear, they might be related to the culture conditions and some intrinsic factors and/or sperm to stimulate calcium transients, paternal effect on the duration of the S-phase or chromosomal abnormalities (Sakkas *et al.*, 1998).

Several reports indicated that sexual dimorphism occurred even before differentiation of gonads had begun. Burgoyne (1993) suggested that X chromosome had retarding effect whereas Tsunoda *et al.* (1985) attributed faster development of males to an accelerated rate of Y chromosome. Transcription of Sry, the only Y linked gene had been observed to start as early as 2-cell stage (Zwingman *et al.*, 1993) which suggested that paternal Y chromosome was activated after the first cleavage.

In experiment II, sexing was carried out at 2-cell stage as the prime objective of the study was only to evaluate the time of first cleavage versus sex of buffalo embryos as it would be more beneficial. It was observed in the study that out of the 3 embryos that cleaved at 18 hpi 66.67 per cent (2/3) were males and 33.33 per cent (1/3) were females as against the 33.33 per cent males and 66.67 per cent females reported by Gupta (2003). The higher percentage of males before 24 h in the present study was probably related to the more specific time point of 18 hpi taken for the study. Among all the 8 embryos that cleaved at 24 hpi and sexed, 50 per cent (4/8) were males and 50 per cent (4/8) females. Only 20 embryos out of 98 embryos that cleaved at 36 hpi were sexed, of which 40 per cent (8/20) were males and 60 per cent (12/20) females. Valdivia *et al.* (1993) reported that in mouse, male embryos developed faster than female embryos *in vitro*. In bovine embryos, contradictory findings have been reported about the sex linked developmental rate differences among *in vitro* (Yadav *et al.*, 1993) and *in vivo* fertilized embryos (Avery *et al.*, 1989). However, there are only scarce reports in buffalo embryos to either support or contradict the hypothesis that male embryos developed faster than the female embryos *in vitro*. Totey *et al.* (1996) reported that the overall sex ratio deviated from the expected value in favour 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. Similar to our study Lonergan *et al.* (1999) also reported a skew in sex ratio when embryos were sexed at 2-cell stage; however, the deviation in sex ratio was not as marked as on day 8.

In conclusion, the results of the present study showed that the time of first cleavage in buffalo embryos occurred as early as 18 hpi with more proportion of male embryos cleaving at a faster rate than the females. The higher occurrence of males among fast cleaved embryos could be used as an easy non-invasive method for embryo sexing in buffaloes.

DIRECT DUPLEX PCR FOR BUFFALO EMBRYO SEXING USING BRY.1, BOV 97M AND SATELLITE DNA PRIMER



Agarose gel electrophoresis analysis of direct duplex PCR products amplified with whole buffalo embryos at two cell stage cleaved at 18 hpi (Lanes 7 & 8) and at 24 hpi (Lanes 4, 5 & 6) in the presence of BRY.1, BOV 97M and bovine satellite DNA primers. Lane 1: 100 bp ladder. Lanes 4, 5 & 7 showing only satellite DNA band (216 bp) indicating the samples to be female. Lanes 6 and 8, showing bands for BRY.1 (300bp), BOV 97M (150 bp) and satellite DNA (216 bp) indicating the samples to be male

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