INFLUENCE OF GLUCOSE DURING *IN VITRO* CULTURE ON THE SEX OF PREIMPLANTATION BUFFALO EMBRYOS

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ABSTRACT

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The influence of glucose supplementation on growth rate differences between sexes of *in vitro* produced preimplantation buffalo embryos was assessed by culturing presumptive zygotes *in vitro* with glucose supplemented (0.5mM) and glucose free synthetic oviduct fluid (SOF) medium. Of the 220 presumptive zygotes cultured in SOF medium plus glucose 48 cleaved of which, 29 were males and 19 females with a sex ratio of 1.5:1. Of the 221 presumptive zygotes cultured in SOF medium minus glucose 49 cleaved of which, 25 were males and 24 females with a sex ratio of 1:1. In conclusion, though the supplementation of glucose resulted in an increase in number of males (29 vs. 25) this difference was not statistically significant (P = 0.87) indicating that it might be difficult to use this metabolic parameter as a sole criteria for sex selection of buffalo embryos.

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

INTRODUCTION

Sex determination of preimplantation buffalo embryos have been historically performed by embryo biopsy followed by sex chromosome identification either by fluorescent in situ hybridization (FISH) (Munne et al., 1993), polymerase chain reaction (PCR) (Martinhago et al., 2010), single nucleotide polymorphism (SNP) (Treff et al., 2010) or comparative genomic hybridization (CGH) (Fragouli et al., 2008). However, such approaches are time consuming and invasive. Alternatively, new non invasive metabolomic technologies may provide an opportunity to generate predictive information based on sex related differences in embryo physiology.

These sex specific differences in developmental kinetics are apparently mediated by embryo metabolism. Glucose metabolism appears to be a pivotal metabolic biomarker especially at blastocyst stage (Gardner *et al.*, 2010). Bredbacka and Bredbacka (1996) reported that male embryos developed faster than female embryos only in the presence of exogenous glucose and explained this fact by differential gene expression in male and female bovine embryos. Kumar *et al.* (2012) have reported that the requirement of glucose for maturing oocytes and growing embryos appeared to be species specific with buffalo embryos requiring glucose throughout the *in vitro* maturation and culture steps. Hence, the present study was undertaken with the objective to study the influence of glucose in culture media on growth rate differences between sexes in *in vitro* produced preimplantation buffalo embryos.

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MATERIALS AND METHODS

Buffalo ovaries were obtained from the slaughterhouse and transported to the Assisted Reproductive Technology Laboratory, Department of Animal Reproduction, Gynaecology and Obstetrics, Madras Veterinary College, Chennai in 0.9 per cent normal saline containing penicillin (100 IU/ml) and streptomycin (50 mg/ml) at 30-35° C in a thermos flask within 2 h of slaughter.

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 ±g/ml of Folltropin (FSH), 0.02 IU/ml of Luteinizing Hormone (LH), 1 ±g/ml of estradiol and 10 ng/ml of epidermal growth factor (EGF). A group of ten to fifteen COCs were transferred to a 50 ±l 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 cultured for 24 h at 38.5□C in a humidified atmosphere of 5 per cent CO_2 in air.

The maturation rate was assessed based on the degree of cumulus expansion (a criteria used for assessing the degree of cumulus cell 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 semen were separated by swim up method (Parrish *et al.*, 1986). The fertilization droplets of 75 ±l of *in vitro* fertilization (IVF-TALP) medium supplemented with heparin (10±g/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 (5 ±l) suspension obtained by swim up technique was inseminated into the IVF droplets containing oocytes to achieve the final concentration of 2 million sperm /ml and co-incubated for 18-24h 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 to remove the loosely attached spermatozoa, cellular debris and chemical residues, respectively and transferred separately into pre-equilibrated 50 ±l IVC droplets (10-15/droplet) containing two-step Synthetic oviduct fluid (SOF), supplemented with Streptomycin (100±g/ml) and penicillin (100 U/ml) at 38.5°C in 5 per cent CO_2 in air. The presumptive zygotes were divided in to 2 groups viz., one cultured in media containing glucose (SOF + 0.5mM glucose) and another without glucose (SOF without glucose). 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±l 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 (For.5«GGATCCGA-GACACAGAACAGG3«; Rev.5«GCTAATCCATCCATCCTATAG3«), BOV 97M (For.5«GATCTTGTGATAAAAAGGCTATGC3«;Rev.5« GATCACTATACATACACCACTCTC 3«) and satellite gene (For. 5«TGGAAGCAAAGAACCCCGCT3«; Rev.5«TCGTGAGAA-ACCGCACACTG3«) fragment of buffalo. The following thermofile was followed for amplification: Initial denaturation - 97°C 3 min; denaturation - 94°C 30 sec; annealing - 58°C 45 sec; extension - 72°C 45 sec for 40 repeated cycles and final extension at 72°C for 5 minutes

RESULTS AND DISCUSSION

Supplementation of glucose in culture medium has been reported to have an important role for oocyte and embryo culture in many species (Kumar *et al.*, 2012). Several studies in cattle utilizing *in vitro* produced embryos have shown that early cleaving embryos did not require glucose as an energy source; on the contrary, it was even detrimental (Matsuyama *et al.*, 1993). However unlike cattle, buffalo embryos required glucose supplementation throughout *in vitro* development (Novoa *et al.*, 2010; Kumar *et al.*, 2012).

Of the 220 presumptive zygotes cultured in SOF medium plus glucose 48 cleaved resulting in a cleavage rate (MeanØ SE) of 21.85Ø 0.84 per cent. Of the 221 presumptive zygotes cultured in SOF medium minus glucose 49 cleaved resulting in a cleavage rate (MeanØ SE) of 22.21Ø 1.14 per cent. Our results concur with the findings of Bredbacka and Bredbacka (1996) who have reported no differences in cleavage rates of bovine embryos cultured in glucose free media and media containing 5.56 mM glucose (69.9 per cent versus 63.8 per cent). According to Lonergan *et al.* (1999) there was no relationship between the metabolic activity of presumptive zygotes at 21-24 hpi and the time of first cleavage (27-42 hpi), thus indicating that the time of first cleavage was independent of the metabolic activity of the zygote, or that the metabolic requirements for first cleavage have been fulfilled before 21-24 hpi. However, Kumar *et al.* (2012) found that nuclear maturation rates of buffalo oocytes were significantly improved in the presence of optimum level of 5.56 mM glucose. They concluded that the presence of glucose was obligatory throughout the IVM and IVC steps and a constant presence of 5.56 mM glucose yielded maximum blastocyst rates. This was a significant deviation from the cattle system, which required far lesser amounts of glucose. The concentration of glucose in the present study was taken based on the results of Novoa *et al.* (2010) who opined that the cleavage rate of buffalo embryos was higher between 0.15mM and 1.5 mM glucose concentration during *in vitro* early embryo culture. The contrary findings of Kumar *et al.* (2012) to the present study could be attributed to the difference in the glucose levels added to the culture medium (5.56 Vs 0.5mM).

In the present study, of 48 embryos that cleaved in the medium containing glucose, 29 were males and 19 were females with a sex ratio of 1.5:1, while of 49 embryos that cleaved in medium without glucose 25 were males and 24 were males with a sex ratio of 1:1. Several authors (Xu *et al.*, 1992; Gutierrez *et al.*, 2001) have reported that in culture, male embryos develop faster than female embryos after *in vitro* fertilization, suggesting an effect of the culture environment on the development of male versus female embryos. The expression of X- and Y-chromosome related genes at different stages of development contributes to differential activities of one or more metabolic pathways. Also, it has been reported that glucose controlled sex related growth rate differences and was responsible for early cleavage (Peippo and Bredbacka, l996) and fast cleavage (Bredbacka and Bredbacka, 1996) of male bovine embryos cultured *in vitro*. Glucose metabolism has been proposed to differ between male and female embryos mainly because the enzyme catalyzing the first and rate limiting step of the pentose phosphate pathway (PPP), glucose-6-phosphate dehydrogenase (G6PD), was encoded by the X-chromosome and more highly expressed in female blastocysts compared with their male counterparts in mice (Kobayashi *et al.*, 2006) and bovine (Wrenzycki *et al.*, 2002).

The influence of glucose supplementation on growth rate differences between sexes in the present study revealed an increased number of male embryos (60.41 per cent) than female embryos (39.58 per cent) when the presumptive zygotes were cultured in SOF medium supplemented with glucose while, *in vitro* culture of presumptive zygotes in SOF medium without glucose resulted in 51.02 per cent male embryos and 48.98 per cent female embryos. Although supplementation of glucose resulted in an increase in sex ratio, the difference was not statistically significant probably due to the fact that the number of embryos analysed was relatively small. Bredbacka

and Bredbacka (1996) also reported a non-significant increase in males compared with females when glucose was added to the culture media.

In conclusion, the results of the present study showed that, the supplementation of glucose in SOF medium during *in vitro* culture of buffalo embryos for 48 hpi could lead to a higher percentage of cleavage among the male embryos. However, the magnitude of increase in the present study was too small and insignificant to throw light on the fact that the metabolic parameter glucose could not be used as a sole criterion for sex selection of *in vitro* produced preimplantation buffalo embryos.

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