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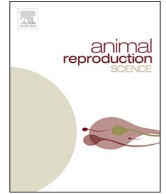

Sperm sexing in Nili-Ravi buffalo through modified swim up: Validation using SYBR® green real-time PCR

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ABSTRACT

Sperm sexing through flow-sorting technology is relatively expensive, requires considerable technical support and is actually not practicable in many developing countries. The aim of this study was to investigate the feasibility of producing enriched pools of X or Y chromosome-bearing sperm by a modified swim-up method. For this purpose semen was collected from five mature Nili-Ravi buffalo bulls for a period of six weeks. The qualifying ejaculates were divided into two aliquots for further processing through modified swim-up or control (untreated). After processing, semen was cryopreserved in tris citric acid extender using standard techniques. Semen quality was assessed at pre dilution, post dilution and post thawing. Validation of technique was done by using SYBR® green real time PCR using two sets of primers, PLP and SRY for X and Y chromosome of buffalo genes, respectively. Sperm recovery rates, pre freeze and post thaw sperm quality were found significantly higher in X chromosome bearing sperm fraction than Y chromosome bearing fraction and control. Mean fold relative expression of X bearing sperm was significantly higher (4–5 fold) in X chromosome bearing fraction of supernatant than Y chromosome bearing fraction (0.06 fold), similarly mean fold relative expression of Y chromosome bearing sperm was significantly higher in Y chromosome bearing fraction (4 fold) of supernatant than X chromosome bearing fraction (0.15 fold) compared to control (1.00). In conclusion, a modified swim up method proved to be an effective method for Nili-Ravi buffalo sperm sexing as validated by real time PCR.

1. Introduction

Sex pre-selection plays an important economic role in animal production industries where females are requisite for the dairy industry while males are preferred for beef production (Seidel, 2007). To achieve this, the technique for sorting of X (female) or Y (male) chromosome-bearing spermatozoa has been suitably developed and is being used commercially for cattle in several countries. The technique relies on flow cytometric separation of X and Y chromosome bearing spermatozoa (based on DNA difference) and accuracy is greater than 90% (Morrell et al., 1988; Garner et al., 1983). In addition to being expensive, challenging, and time-

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consuming, the technique itself is damaging to sperm (Seidel, 2003) and induces alterations in mRNA expression patterns of some genes in embryos (Morton et al., 2007). This is the reason that the sperm sex sorted through flow-cytometry yielded lower fertility rates *in vitro* (Cran et al., 1993,1994; Merton et al., 1997; Lu et al., 1999; Xu et al., 2009) *in vivo* (Schenk et al., 1999; Maxwell et al., 2003; Bodmer et al., 2005) and even the abnormal embryonic development if the fertilization is successful (Maxwell et al., 2004).

Buffalo is the main dairy animal of South Asia; Pakistan and India holds the largest buffalo populations of the world where it is kept as large and small herds. Unfortunately, only few attempts have been made for sperm sexing in buffalo that were based on flow-cytometric separation for subsequent use in *in vitro* fertilization (Lu et al., 2006, 2007). For artificial insemination in buffaloes, the sex selection of spermatozoa has been compromised due to higher cost of flow-cytometric sex sorted semen doses to poor dairy farmers (Mota et al., 2013). Alternative sperm sexing methods could therefore be of relevance for the benefit of small dairy farmers both in biological and economic terms (Mota et al., 2013). These sperm sexing techniques are based on size and weight (Bhattacharya et al., 1996), electrical surface charge (Shirai et al., 1974), immunological properties (Hendriksen et al., 1993; Blecher et al., 1999; Sang et al., 2011) and swimming ability (Madrid-Bury et al., 2003) of X and Y chromosome bearing sperm. These techniques include differential separation through percoll (Machado et al., 2009), albumin gradients (Machado et al., 2009; Wolf et al., 2008) and modified swim up (Azizeddin et al., 2014). Swim-up technique, routinely used to prepare sperm for *in vitro* fertilization, when modified as having vertical swim-up in a long narrow tube, the swimming ability of bull spermatozoa was challenged. Owing to their smaller size, Y chromosome bearing spermatozoa swim faster than X chromosome bearing sperm, and move to the upper portion of the swim-up tube while, sperm with X chromosomes tend to reside in the lower portion of the tube (Azizeddin et al., 2014). Sperm sexing through conventional swim up technique, has been done in human with success rate of 81% (Check et al., 1989; Khatamee et al., 1999).

Once the spermatozoa are sex sorted, the presumptive X or Y chromosome bearing sperm population are evaluated for sex ratio to avoid any carry-over errors of sexing method (Colley et al., 2008). These probable techniques include sex determination by PCR (Reynolds and Varlaro, 1996; Tan et al., 2006), quinacrine mustard staining for Y chromosome bearing sperm (Ogawa et al., 1988), DNA quantification of sex sorted sperm (Welch and Johnson, 1999), fluorescence in situ hybridisation (FISH) (Rens et al., 2001) and real-time quantitative PCR (qPCR) (Joerg et al., 2004). Among these, FISH and qPCR are most reliable, however, FISH is complicated, laborious, time-consuming, and requires highly skilled technicians, which limits its use. Real-Time PCR assay is of relevance due to its sensitivity and simplicity. The first study on qPCR technique was done by Joerg et al. (2001) and subsequently Parati et al. (2006) using TaqMan probes to the X and Y chromosomes for proteolipid protein gene (PLP), and male sex determination gene (SRY) respectively. Sex determination of spermatozoa by qPCR offers the possibility of simultaneous amplification of the segment of interest and measurement of the amount of resultant DNA molecules through reaction cycles. Through the amplification of the signal with real time PCR, it is possible to quantify the X and Y chromosome bearing spermatozoa. Further, the SYBR® Green is more economical compared to Taqman probe and provides an equally accurate approach to qPCR, due to its high specificity and artifacts such as minimal primer dimers (Granfar et al., 2005). SYBR® Green dye tends to bind to all double-stranded nucleic acid molecules (Whittwer et al., 1997), hence the accumulation of primer dimers and the amplification of non-specific PCR products can be detected in SYBR® Green (Deprez et al., 2002).

Development of alternative, less deleterious and feasible sperm sexing procedures are highly desirable for domestic animals and specifically for buffalo. Hence, the present study was planned for pre-freeze separation of X and Y chromosome bearing spermatozoa using the modified swim- up technique, and to assess the recovery rate after separation, post-thaw sperm quality (progressive motility, plasma membrane integrity, acrosome integrity, viability and live/dead ratio) and finally the validation of sperm separation techniques through quantitative SYBR® green RT-PCR.

2. Materials and methods

2.1. Experimental design

Semen was collected from five mature Nili-Ravi buffalo bulls maintained at the Semen Production Unit, Qadirabad, District Sahiwal, Pakistan. Two consecutive ejaculates per week were collected from five buffalo bulls with an artificial vagina (42 °C) for a period of six weeks (replicates). At least one of the two consecutive ejaculates from each bull/replicate did qualify the set criteria (volume > 1 mL, sperm concentration > 1.5 billion/mL, motility > 60%, abnormalities < 20%). A total of 30 qualifying ejaculates (6 ejaculates/bull) were processed. The qualifying ejaculates were divided into two aliquots; the first aliquot was untreated (control) and processed for cryopreservation using routine procedure, while the second aliquot was processed for sperm sexing using modified swim-up. On the average each bull produced 44 unsorted straws from first aliquot while 7 straws of X sorted sperm samples and 2 straws of Y bearing sperm samples from second aliquot. After sorting, semen was assessed for recovery rates and then diluted in tris citric acid extender, checked for sperm quality (progressive motility, plasma membrane and acrosomal integrity) and cryopreserved using standard techniques. Cryopreserved treated (sexed) and untreated (control) semen samples were analyzed for post thaw sperm motility, plasma membrane integrity, acrosomal integrity, viability and live/dead ratio.

2.2. Extender preparation

All sperm suspensions were diluted in tris-citric acid extender (pH 7.0) prepared as follows: 3.0 g Tris-(hydroxymethyl)-aminomethane and 1.56 g citric acid dissolved in 73 mL distilled water, and supplemented with 0.2% Fructose, 7% glycerol, and 20 mL egg yolk (osmotic pressure 320 mOsmol/kg). Antibiotics (1000 μg/mL streptomycin sulphate and 1000 IU/mL benzyl

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