Animal Science Papers and Reports vol. 38 (2020) no. 1, 21-33
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Efficiency of sucrose density gradient method for sex separation of buffalo spermatozoa as validated by SYBR Green Real Time PCR

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(Accepted December 20, 2019)

The present study evaluates sucrose density gradient centrifugation for separation of X- or Y-chromosome-bearing buffalo spermatozoa. Qualified semen ejaculates from five buffalo bulls were processed through the sucrose density gradient for six consecutive weeks (replicates), while untreated semen constituted the control. Processed semen was diluted in a tris-citric acid extender and evaluated for sperm quality and recovery rates; subsequently it was cryopreserved and analysed for post-thaw quality. The fractions of presumptive X and Y sperm were validated using SYBR green real time PCR through X- and Y-specific sequences of buffalo genomic DNA using two sets of

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primers, PLP and SRY for X- and Y-chromosomes, respectively. Sperm recovery rates, pre-freeze and post-thaw sperm quality were higher (P<0.05) in X-sorted sperm fractions than Y-sorted or the control. The mean fold relative expression of X-bearing sperm was found to be comparable (1.6-fold) in the X-sorted fraction of the supernatant than the Y-sorted fractions (0.3 fold), whereas the mean fold relative expression of Y-bearing sperm was significantly higher in the Y-sorted fraction (2.66-fold) of the supernatant than the X-sorted fractions (0.69-fold) compared to the control (1.00). In conclusion, the sucrose density gradient method proved to be effective as confirmed by real time PCR for Y-sorting sperm sexing of Nili-Ravi buffalo.

KEY WORDS: buffalo / cryopreservation / real time-PCR / semen / sexing / sucrose density gradient

Flow cytometric separation is a well-known technique for sperm sexing [Weigel 2004, Seidel 2007] that is based on the difference between Y- and X-bearing spermatozoa in DNA content (3.6%), surface area (7%) and compatibility for specific fluorescence dyes [Lu *et al*. 2006, Yan *et al*. 2006]. The method is fairly accurate with ~90% of the sperm containing the desired sex [Garner and Seidel 2003, DeJarnette *et al.* 2008]. However, there are many disadvantages of the technique, e.g. a lower concentration of sperm per straw (approximately 2 million), a relatively slow process and potentially a negative effect of the sorting process on sperm, e.g. structural damage [Schenk and Seidel 2007], altered motility patterns [Suh and Schenk 2003, Suh *et al.* 2005], accelerated acrosome reaction [Mocé *et al.* 2006], reduced lifespan [Hollinshead *et al.* 2003] and the risk of cytotoxicity and mutagenic effects of DNA stains due to ultra-laser beam used. These deleterious effects have ultimately resulted in reduced fertility of sexed sperm [Karabinus 2009] when compared with non-sexed semen [Garner and Seidel 2003, DeJarnette *et al.* 2008]. Furthermore, the technique is expensive, which makes it impracticable in most of the least developed and developing countries. Some alternative sperm sexing techniques based on physical properties of sperm such as head volume, density and swimming speed possibly due to differences in DNA content in X- and Y-bearing sperm have also been investigated. The density gradient technique that is capable of separating X- and Y-bearing sperm based on the difference in their DNA content is connected with lower costs and least damage to sperm viability [Kanesharatnam *et al.* 2012]. This technique could be a promising approach for separating X- and Y-bearing sperm [Hossepian de Lima 2007, Cury *et al.* 2009, Aleahmad *et al.* 2009] in buffalo, in which the difference in DNA content is 3.6 % [Resende *et al.* 2009, Lu *et al.* 2007]. The success rate with gradient separation in bovine semen has been reported as 70% with optiprep [Resende *et al.* 2009], 75% with albumin [Beernink *et al.* 1993] and 86% to 94% using Percoll [Lizuka *et al.* 1987, Van Kooij and Van Oost 1992].

Sucrose density gradient centrifugation is a powerful technique for fractionating macromolecules such as DNA, RNA, proteins and cells [Raschke *et al.* 2009]. In this technique the macromolecules are separated with larger ones sedimenting towards the bottom and lighter ones remaining close to the top of the gradient. The method has been particularly successful in the size fractionation of cells having more DNA content

[Sureka *et al.* 2013]. It is also proposed that sucrose density gradient centrifugation enables heavier X-sperm to sediment in the bottom layer of a gradient, whereas lighter Y-sperm remains at the top layer [Kanesharatnam *et al.* 2012]. For the purpose of monitoring the degree of precision for X- and Y-fractions, SYBR green real time-PCR offers the possibility of simultaneous amplification of the segment of interest and measurement of the amount of resulting DNA molecules, thus facilitating quantitation of the X- and Y-chromosome bearing spermatozoa.

Keeping in mind the tremendous efficacy of the sucrose density gradient fractioning technique, it was hypothesised that this method could efficiently separate Y sperm and X sperm in buffalo semen. Therefore this study aimed at: 1) Separation of X- and Y-bearing spermatozoa by sucrose density gradients and assessment of their quality (sperm recovery rate, progressive motility, plasma membrane and acrosomal integrity) after sexing; 2) Cryopreservation of sexed sperm in *tris*-citric acid extender and assessment of post-thaw sperm quality (progressive motility, integrity of plasma membrane and acrosome, viability and live/dead ratio); and 3) Validation of the sucrose density gradient sexing technique by quantitative SYBR® green Real Time-PCR.

Material and methods

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 using an artificial vagina (42ºC) for a period of six consecutive weeks (replicates) for further processing in the experiment. The qualifying ejaculates (volume >1mL, sperm concentration >1.5 billion/mL, motility >60%, abnormalities <20%) were divided into two aliquots; the first aliquot was untreated (control), while the other aliquot was processed by sperm sexing through the sucrose density gradient. After sexing, semen was diluted in the tris-citric acid extender, assessed for sperm progressive motility, plasma membrane integrity, acrosomal integrity and recovery rates and finally it was cryopreserved using a standard protocol. Cryopreserved samples were analysed for post-thaw sperm motility, plasma membrane integrity, acrosomal integrity, viability and the live/dead ratio. Finally this sperm separation technique was validated by SYBR Green RT-PCR.

Extender preparation

All sperm suspensions were diluted in the 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 penicillin) were added to the buffer extender.

Sperm sexing through sucrose density gradient (SDG)

Sucrose was dissolved in 10 mL Sp-TALP solution to make 0.5, 1.5, 2.5, 3.5 and 4.5% sucrose gradients and filtered through 0.2 micron filter units. A discontinuous sucrose density gradient was prepared by layering solutions with successive decreasing sucrose densities (4.5, 3.5, 2.5, 1.5, and 0.5%). Once the 4.5% solution drained into the tube, the 3.5 % solution was loaded into the blue tip and was flown down the inside of the tube and layered on top of the 4.5% solution. This procedure was continued with the 2.5, 1.5 and 0.5% sucrose, respectively. Finally a 20µL semen sample was loaded on the top layer. The layered Falcon tube was centrifuged at 2800 rpm for 12 minutes at room temperature (37ºC) to obtain the separation of X- and Y-bearing sperm. Immediately after centrifugation, the tubes were removed from the rotor in a manner preventing any disturbance of sucrose layers.

The two upper layers were carefully aspirated using a micropipette for sperm bearing the Y chromosome, while the two lower fractions were collected for sperm bearing the X chromosome and centrifuged at 800 rpm for 5 minutes at 37°C. The produced pellet was separated from each tube and re-suspended in the tris-citric acid extender. An aliquot of each suspension was evaluated for motility, volume and concentration. The motile sperm recovery rate was estimated as described by Husna *et al.* [2016].

Sperm freezing and thawing procedures

Diluted suspensions were cryopreserved and thawed using standard techniques as reported by Husna *et al.* [2016]. Briefly, sperm suspensions were adjusted to a final concentration of 60 x 10^6/mL spermatozoa using the Tris-Citric Acid extender at 37ºC. The sample was cooled to 4ºC and further equilibrated at 4ºC for a 4 h period, after which they were filled in 0.5 mL French straws with a suction pump at 4ºC in a cold cabinet unit and kept on liquid nitrogen vapors for 10 minutes. Straws were plunged and stored in liquid nitrogen and transported to the Animal Physiology Laboratory, Department of Zoology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan, where sperm post-thaw quality was evaluated. After 24h, straws were thawed in a water bath at 37ºC for 30 seconds and incubated in a water bath for assessment of post-thaw semen quality.

Post-thaw semen analysis

Sperm progressive motility was evaluated by phase contrast microscopy (400X). Sperm plasma membrane integrity was assessed using the supravital hypo-osmotic swelling test (HOST) [Ansari *et al.* 2011]. The solution for HOST consisted of 0.73 g sodium citrate (Merck) and 1.35 g fructose (Scharlau) in 100 ml distilled water (osmotic pressure, 190 mOsmol/kg). The 50µl volume of the semen sample was mixed with 500 µl of the pre-warmed HOST solution and incubated at 37°C for 30-40 min. After incubation, a drop of the mixture was placed on a slide, cover-slipped and visualised microscopically (400X magnification). A total of 200 spermatozoa per experimental extender were evaluated in five different fields. Swollen tails of sperm

were indicated as intact, biochemically active sperm membranes, while unswollen tails were indicated as disrupted, inactive, nonfunctional sperm membranes [Akhter *et al.* 2008]. Sperm acrosomal integrity was evaluated using a formaldehyde-citrate solution (2.9 g Tri-Sodium Citrate dihydrate and 1 mL of 37% solution of formaldehyde in 99 mL distilled water). Briefly, 100 µL of the sample was mixed with 500 µL of the formaldehyde-citrate solution and 200 sperm were evaluated under a phase contrast microscope at a 1000X magnification using oil immersion. Sperm with an intact acrosome and a clear shinning head were considered to have a normal acrosome, while blunt ended cells were considered to have a disrupted acrosome. Sperm viability and livability were evaluated by means of a dual staining procedure, as previously described [Kovacs and Foote 1992]. Briefly, an equal volume of Trypan-blue and semen were placed on a slide at room temperature and mixed. Smears were air-dried and fixed with formaldehyde-neutral red for 5 minutes. Slides were rinsed with distilled water and Giemsa stain (7.5%) was applied for 4 hours. Slides were rinsed, air dried and mounted with mounting media. Two hundred sperm were evaluated in each smear by phase contrast microscopy at a 1000X magnification and the results were expressed as the percentage of viable spermatozoa in the total number of cells evaluated. Spermatozoa with no staining and purple acrosomes were considered viable with an intact acrosome, while sperm with blue staining were considered dead. Sperm viability was expressed as the percentage of live sperm with intact acrosome and sperm livability as the ratio of live/dead sperm expressed as a percentage.

Validation of sexed spermatozoa by SYBR-Green RT-PCR

Primer design. Pairs of specific primers were designed for the Buffalo Y- and X-chromosome partial sequence using the NCBI web site according to the parameters required for the SYBR Green Real-Time PCR [Dorak 2006]. The 18S rRNA of the buffalo was chosen as the segment to amplify as an internal housekeeping gene (positive control). The Y-specific primer pair (forward: GTTGTGCCAAGACCACATATTC and reverse: ATAGGGTAACATTGGCTACACG) was designed on a conserved region of the buffalo chromosome linked SRY gene. The Y-product amplicon length was 142 bp. The X-specific primers were designed with an amplicon length 104 bp DNA fragment on the buffalo proteolipid protein gene (PLP). The sequences of the forward and reverse primers were GTTCTTCAGGTCACAGGGTAAG and CTCTTTGTGACCCTATGGACTG, respectively. Purity of the X- and Y-chromosome amplification products was evaluated on 2.5% agarose gel.

Semen samples. Three different sets of sorted and unsorted semen samples collected from 5 different buffalo bulls with proven fertility were analysed by Real-Time PCR. The first set was composed of 47 straws of unsorted semen samples. The second set was composed of 24 straws of semen sorted by sucrose density gradient centrifugation for the Y chromosome. The third set was composed of 24 straws of semen sorted using sucrose density gradient for the X chromosome.

DNA sample preparation. The DNA from each semen sample was extracted

by a modified protocol following Chandler *et al.* [2007]. Before DNA extraction a fresh lysis solution was prepared in two steps. First, 0.121 g Tris (pH = 8.0, 10 mM), 0.0293 g EDTA (pH = 8.0, 10 mM) and 80 ml distilled water were brought to pH = 8.0 using NaOH. Then, 0.584 g NaCl, 2 ml 2-mercaptoethanol and 0.5 g SDS were added. The volume was brought to 100 ml with distilled water and the solution was stored at 2-8ºC. Samples were washed twice with a 2.9% sodium citrate solution and re-suspended in a lysis solution (50ºC) and incubated at 50ºC for 30 min. Proteinase K (5 µL; Fermentas, Vilnius, Lithuania; catalogue no. #EO0491) was added to the solution and incubated at 50ºC overnight. One volume unit of a phenol: chloroform mixture was added and the tubes were gently shaken for 15 min at room temperature. Samples were then centrifuged at 15,500 × g for 3 min, the organic phase was aspirated and the phenol: chloroform step was repeated. Two volumes of ice-cold absolute ethanol was added to the remaining aqueous phase and the entire mixture was gently shaken to precipitate the DNA. Samples were centrifuged for 1 min at 15,500 × g and ethanol was carefully decanted. The DNA pellets were dried under vacuum until clear and then re-suspended in sterile water by incubating at 37ºC with gentle agitation until dissolved. Nucleic acid concentration was determined by Nanodrop (Avans cuvdrop AUVS-102).

Quantitative SYBR Green Real Time PCR. Real-Time PCR was performed on a Line Gene K system by Bioer Technology Co. (Line-Gene.K-FQD-48A(A4)/(M2)) using the Maxima TM SYBR Green qPCR Master Mix (catalogue no.K0221; Fermentas, USA). The DNA from each sample was run in duplicate along with the control for every assay and primed in separate wells for the two genes of interest, using a defined volumetric quantity of the same DNA sample (2 µL). Each run was completed with a melting curve analysis to confirm the specificity of amplification and absence of primer dimers. The PCR mixture contained 1 µL of the forward plus reverse primer, 5µL qPCR Master Mix (qPCR Master Mix; Fermentas, USA), 2µL of the template and 4 µL nuclease free water. Amplification for genes was performed applying an optimised protocol (10 min at 95ºC, 40 repeated cycles of two steps at 95ºC for 15 sec, 55ºC X gene/56ºC Y gene for 15 sec and 72ºC for 30 sec).

Statistical analysis

The data on motile sperm recovery rates after sex separation was analyzed by Student's t-test whereas data of pre-freeze and post-thaw sperm quality and validation of sexed semen through real time-PCR was analysed by one-way ANOVA and Student's t-test using the IBM SPSS Version 20.0 statistical software.

Results and discussion

Sperm recovery and quality after sperm sexing

The data on sperm quality parameters, concentration and recovery rate of X- and Y-bearing spermatozoa after sucrose density gradient fractionation of neat semen

samples of Nili-Ravi buffalo bulls is shown in Table 1. The recovery rate (%) of the X-sorted sperm population (6.14±2.14) was significantly higher (P<0.05) than that of the Y-sorted sperm population (0.48±0.09). Sperm progressive motility was higher (P<0.05) in the control (85.00±0.57) followed by the X-sorted (73.83±0.60) and Y-sorted (64.00±0.44) sperm population. A greater (P<0.05) sperm plasma membrane integrity (%) was observed in the X-sorted population (63.83±1.68) compared to the control (59.00±0.44) and the Y-sorted (57.17±1.37) sperm population. Similarly, sperm acrosomal integrity (%) was higher (P<0.05) in the X-sorted population (70.00±1.26) compared to the control (60.20±1.55) and the Y-sorted (62.17±0.79) sperm population. Sperm concentration (million/ mL) was similar (P>0.05) in the control (1323.33±32.29) and the X-sorted (1342.53±191.07) sperm population, being significantly higher (P<0.05) than that of the Y-sorted sperm population (223.57±19.57).

Table 1. Effect of sperm sexing through sucrose density gradient on pre-freeze quality and recovery rates of Nili-Ravi buffalo bull sperm

Item	Progressive motility		Plasma membrane integrity		Acrosomal integrity		Concentration (x 10^6)		Recovery rate (% motile)	
	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
Control	85.00^a	0.57	59.00^b	0.44	60.20^b	1.55	1323.33^a	32.29	-	
SDG-X	73.83^b	0.60	63.83^a	1.68	70.00^a	1.26	1342.53^a	191.07	6.14^a	2.14
SDG-Y	64.00^c	0.44	57.17^b	1.37	62.17^b	0.79	223.57^b	19.57	0.48^b	0.09

abcWithin column means bearing different superscripts differ significantly at P<0.05.
SDG-X – X sorted sperm through sucrose density gradient.
SDG-Y – Y sorted sperm through sucrose density gradient.

Sperm post thaw assessment

The data on the effect of the sucrose density gradient sperm sexing method on post-thaw quality of Nili-Ravi buffalo bull sperm is presented in Figure 1. As indicated by the results, a significantly improved (P<0.05) sperm progressive motility was observed in the X-sorted sperm population (60.83±1.53) compared to the control (54.2±1.53) and the Y-sorted (46.7±1.05) sperm population. Sperm plasma membrane integrity was highest (P<0.05) in the X-sorted (56.3±1.03) sperm population compared to the control (51.8±0.87) and the Y-sorted (55.8±1.49) sperm population. Similarly, sperm livability was greater (P<0.05) in the X-sorted (71.0±2.39) sperm population compared to the Y-sorted sperm population (58.0±2.16) and the control (unsorted). Sperm acrosomal integrity was comparable (P>0.05) in the control (65.3±0.61), X-sorted (67.3±0.80) and Y-sorted (64.5±1.03) sperm populations. Sperm viability was also similar in the control (45.2±1.08), X-sorted (49.5±2.48) and Y-sorted (47.2±1.40) sperm populations.

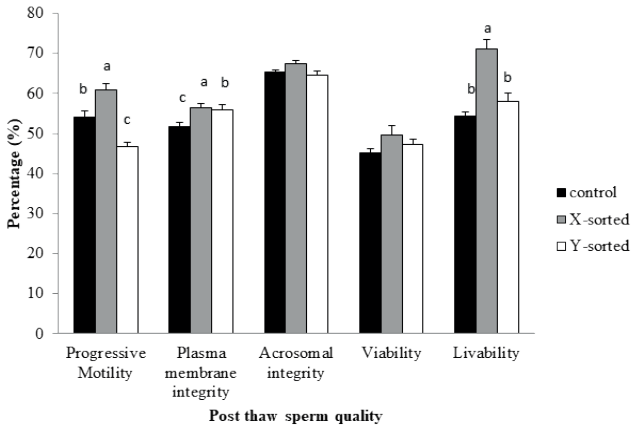

Fig 1. Effect of sperm sexing through sucrose density gradient on post-thaw quality of Nili-Ravi buffalo bulls spermatozoa.

Validation of sex sorting technique by Real-Time PCR

The data on the mean fold relative expression for the X- and Y-sorted sperm contents through real time PCR analysis is shown in Figure 2. The untreated (control) sample was taken as the calibrator. Using the 2 delta CT method, the data is presented as the fold change in gene expression normalised to an endogenous reference gene, i.e. 18S rRNA and relative to the untreated control. For the untreated control sample, delta CT equals to zero and 2 delta equals to one. For the treated samples, evaluation of 2 delta CT indicates the fold change in gene expression relative to the untreated control.

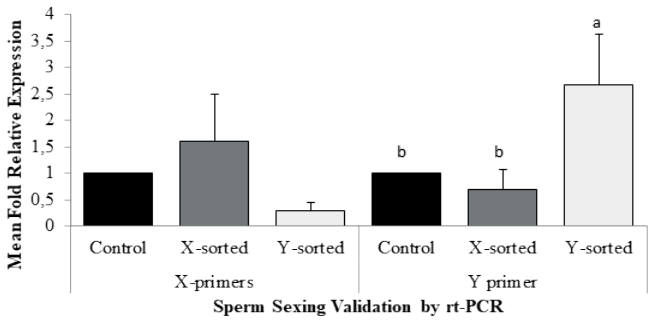

Fig. 2. Expression of SRY and PLP genes in sperm sexed through sucrose density gradients (SDG) of buffalo bull semen.

The mean fold relative expression of the X-sorted sperm population retrieved from lower layers of the sucrose density gradient remained similar ($P>0.05$) to that of the control and the Y-sorted sperm for the X primer and gave a comparable average 2 delta ct value for the X-sorted sperm (1.6 fold) compared to the Y-sorted sperm (0.3 fold) and the control (1.00).

The mean fold relative expression of the Y-sorted sperm retrieved from the upper layers of the sucrose density gradient remained higher ($P<0.05$) for the Y primer and gave a significantly higher (2.66 fold) average 2 delta ct value for the Y primers than the control (1.00). In contrast, the mean fold relative expression of the X-sorted sperm in the upper layer of the sucrose density gradient was significantly lower ($P<0.05$) for the Y primers and gave a lower (0.69 fold; $P<0.05$) average 2 delta ct value for the Y primers as compared to the Y-sorted sperm. Average 2 delta ct values for the X-sorted sperm (0.69 fold) and the control (1.00) for the Y primers were comparable ($P>0.05$).

Sexed semen has brought a revolution to the dairy industry and has successfully been used in bovine species. At present, flow cytometric sexing [Seidel and Garner 2002] is the only reliable technique; however, being much more expensive, time consuming and damaging to sperm viability, it is not actually practicable in many counties [Prakash *et al.* 2014]. Therefore, other sperm sex selection methods have also been attempted to verify their economic feasibility and to enhance the quality of sexed semen. Among these, density gradient centrifugation [Hossepian de Lima 2007] has the potential for separating X- and Y-bearing sperm based on differences in their DNA content and it is believed that the spermatozoa stay separated at the gradient level that matches their density due to their mass and motility [Wolf *et al.* 2008]. Consequently, due to their higher density the X-bearing sperm form a pellet at the bottom, denser layers of the tube [Hadi and Tamim 2013], while the Y-bearing sperm are suspended in the upper, less dense layers. Thus the fractions of semen isolated from the upper layers are expected to be Y-enriched, while the lower layers are expected to be X-enriched. Typically, a sucrose density gradient works on the same principle, the sperm travel through the gradient until they reach the point in the gradient, at which their density matches that of the surrounding sucrose (Raposo, 1996). Sucrose density-gradient separation has previously been used as a low cost and readily available technique for sexing the goat [Sureka *et al.* 2013] and bull sperm [Kanesharatnam *et al.* 2012].

In the present study the sucrose density gradient separation yielded a better motile sperm recovery rate (%) in the X-sorted sperm fraction than the Y-sorted sperm fraction, possibly due to the faster sedimentation velocity of the X-bearing sperm [Kaneko *et al.* 1983]. Furthermore, centrifugation positively influences the X-bearing sperm moving down the gradient, as the effect of sperm motility is minimised and their mass difference effect is maximised, which makes the heavier sperm settle down faster than the lighter sperm [Hadi and Timimi 2013]. Prior to freezing, the values for plasma membrane and acrosomal integrity were higher in the X-sorted sperm than the Y-sorted sperm and the control. After freeze-thawing the X-sorted spermatozoa retained a significantly higher progressive motility, plasma membrane integrity and livability than the Y-sorted and unsorted spermatozoa. It has become apparent that highly motile and intact spermatozoa moved actively in the direction of the density gradients and penetrated the boundary quicker than the poorly motile/ immotile cells with damaged acrosomes, as previously reported by Butt and Chohan [2016]. Furthermore, these

results have clearly demonstrated that the sucrose density gradient technique favours separation of the X-bearing sperm with reduced risks of sperm damage.

The accuracy of sperm sexing techniques has been confirmed by quantitative real time PCR [Parati *et al*. 2006] and FISH [Habermann *et al*. 2005] in bovine species, while FISH was used to verify sexing accuracy in the human [Lin *et al*. 1998 and Yan *et al*. 2006]. In the present study, validation of Nili-Ravi buffalo semen sexed by sucrose density gradient centrifugation was performed using SYBR Green-based Real-Time quantitative PCR. According to the standard meiotic model, in which equivalent numbers of X- and Y-bearing sperm are produced, the mean sex ratio of unsorted buffalo sperm samples was found to be 1.00. This result is consistent with other studies on bovine species [Parati *et al.* 2006]. There was a significant difference ($P<0.05$) in the 2 delta ct value of the Y-sorted semen compared to the control. The mean fold relative expression of the X-sorted sperm in the upper layer of the sucrose density gradient was significantly lower (0.69 fold: $P<0.05$) for the Y primers, whereas the mean fold relative expression of the Y-sorted sperm in the upper layer of the sucrose density gradient was significantly higher (2.66 fold: $P<0.05$) for the Y primers. However, sucrose density gradient separation led to a slight, but non-significant enrichment in the X sperm in the lower layers in our study. The mean fold relative expression of the X-sorted sperm in the lower layer of the sucrose density gradient was comparable (1.6 fold: $P>0.05$) for the X primers as compared to the Y-sorted sperm (0.3 fold) and the control (1.00). The slight differences in the results may be explained by the fact that smaller volumes (0.5-4.5 ml) of the gradients might have been insufficient to promote separation of the X-bearing spermatozoa from the lower layers [Wolf *et al.* 2008]. Furthermore, the protein differences between the X- and Y-sperm [Hendriksen *et al.* 1999] and interactions of both types of sperm with materials (sucrose) in the gradients might have influenced the results with different mechanisms [Rose and Wang 1998]. Previously, sex separation of sperm performed on the basis of differences in DNA contents includes density gradients using Percoll and albumin with a success rate of 94% [Lizuka *et al*. 1987] and 75%, respectively [Beernink *et al*. 1993].

Results of the present study revealed that sucrose density gradient separation may be used in sexing of buffalo semen, more specifically for the Y-bearing sperm fraction, as validated by Real Time PCR through expression levels of the SRY and PLP genes in the sorted fractions. Further studies are suggested for improvement of the sucrose density gradient technique using larger volume gradients, in order to make the Y-bearing, lighter sperm penetration more difficult in denser sucrose gradient layers to facilitate successful X-bearing spermatozoa sexing in buffalo. In conclusion, the sucrose density gradient method is efficient for Y-sperm sorting as validated by real time PCR for the production of male calves. In the light of valuable findings, further research is being suggested to improve methodologies for larger volumes in order to provide greater enrichment of the X and Y fractions and *in vivo* validation through inseminated buffaloes.

Acknowledgments. *Thanks to the administrative staff of the Buffalo Research Institute (BRI) and Semen Production Unit (SPU), Sahiwal for their help and collaboration throughout the sampling period.*

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