



Effects of selective extenders on buffalo semen quality at physical and molecular level

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Received: 26 November 2019; Accepted: 1 January 2020

ABSTRACT

This study was designed to investigate the effects of selective extenders on buffalo semen quality at physical and molecular level for screening a suitable extender for cryopreservation of buffalo semen. Semen was collected from four indigenous buffalo bulls using artificial vagina method twice in a week. After initial evaluation, each semen sample was divided into three aliquots and diluted with three different extenders (Tris-egg-yolk extender, Soya-milk extender and Andromed extender). After initial quality assessment the semen was frozen with liquid nitrogen vapor with a programmable bio freezer and finally stored at -196°C liquid nitrogen. Post thaw semen quality evaluation was performed after 24 h of storage. The expression of aldo-ketoreductase family 1 member B1 (AKR1B1) and A-kinase anchoring proteins (AKAP4) transcripts in fresh and three extenders groups (post thaw) of buffalo sperm were observed. The total, progressive, static and slow motility and hypoosmotic swelling (HOS) reactivity of pre-freeze and post thaw sperms were varied significantly among Andromed, Tris-egg-yolk and Soya-milk based extender. Similar variations were also observed for different kinetic parameters of pre-freeze and post thaw buffalo sperm. However, no significant variations were found in AKR1B1 and AKAP4 genes expression among Andromed, Tris-egg-yolk and Soya milk extender groups considering pre-freeze and post thaw sperms characteristics. It may be concluded that, Tris-egg-yolk semen extender might be used for cryopreservation of buffalo semen at efficiency level similar as commercially available semen extender like Andromed.

Keywords: AKAP4, AKR1B1, Buffalo semen, Cryopreservation, Extender

The global buffalo population is 185.29 million where Asian buffalo dominates the world population, representing 97% (179.75 million) of the total buffalo population (Singh *et al.* 2018). However, in Bangladesh, the total buffalo population is only 1.457 million (Hamid *et al.* 2016). One of the major constraints of this phenomenon is lack of a good buffalo semen cryopreservation system. The cryopreservation of semen is considered as a major tool for the propagation of superior germplasm with desired genetic traits. Moreover, the quality of frozen semen determines the success of Artificial insemination (AI) program but the success of artificial insemination (AI) in buffalo is still low (Herbowo *et al.* 2019). Though semen cryopreservation is routinely practicing for cattle, it is not common for buffalo in Bangladesh. Therefore, development of a suitable semen cryopreservation protocol is a mandatory to produce more F_1 crossbred heifers at field level. Various studies have revealed that cryopreservation induces capacitation like changes

(cryocapacitation) in spermatozoa (Kumar and Atreja 2011). Semen cryopreservation efficiency largely depends on semen extender and cryoprotectant used in the extenders among others (Foote 1970). Different types of semen extenders available for cryopreservation of semen. However, controversial results were observed in their efficiency of semen cryopreservation. Latest reports have indicated the presence and expression of mRNA transcripts in the fresh and frozen spermatozoa (Ostermeier *et al.* 2005) in the light of their prognostic and diagnostic significance. Thus, analysis of the mRNA in the semen of farm animals is highly warranted and proposed in the studies on cryopreserved spermatozoa. There are different mRNA populations in mature sperm that are highly associated with sperm motility, capacitation, and other parameters (Lambard *et al.* 2004). Presence of different transcript levels in sperm of different motility has been revealed independently (Platts *et al.* 2007 and Steger *et al.* 2008). The mRNA expression of selected buffalo genes responsible for sperm motility and fertility will definitely help to reach a conclusion about the semen quality as well as to find out an effective extender for the cryopreservation of buffalo semen.

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MATERIALS AND METHODS

Animal Selection and management: The study was conducted on semen of four healthy indigenous buffalo bulls (*Bubalus bubalis*). The bulls were maintained uniform standard nutritional and managerial practices at the Buffalo Research Farm of Bangladesh Livestock Research Institute, Savar, Dhaka, Bangladesh which is located in a sub-tropical region.

Semen collection and evaluation: Selected bulls were under twice in a week semen collection schedule using an artificial vagina (Walton 1945). Semen collections were made in the early morning between 7.30 h and 8.30 h from March to May. After collection, the ejaculates were immediately transferred in to a water bath at around 37°C and evaluated for gross quality, motility, morphology and kinematics using a Computer Assisted Semen Analysis System (Hamilton Throne, IVOS II). Fresh semen drop was diluted with saline solution (NaCl) in a ratio of 1 : 100 and then put into 20 micron standard count 4 chambered leja slide for analysis.

Preparation of extenders: Extenders were prepared according to the compositions described in the Table 1. Tris-egg yolk extender was prepared according to the Chaudhari *et al.* (2015) and soya milk was extracted in the laboratory according to El-Keraby *et al.* (2010).

Freezing Protocol: After mixing with the extenders, diluted semen was placed in a cold handling cabinet (Minitube, Germany) for 4 hours at 4°C for equilibration. The semen was diluted with the extender to give a sperm concentration of 20 million/dose. The semen samples were filled and sealed in standard printed straws (0.25 ml) using an automated sealing-filling machine (Minitube GmbH, Germany). After equilibration, freezing of straws was carried out in liquid nitrogen (LN₂) vapour using a programmable bio-freezer (Minitube, Germany). The straws

were then plunged in LN₂ (–196°C) for overnight storage.

Post Thaw evaluation of semen: Semen straws were thawed next day in a water bath at 37°C for 30 seconds and the sperm quality was further evaluated using the same protocol as described for fresh semen.

Hypoosmotic swelling (HOS) test: The plasma membrane integrity of spermatozoa was assessed using HOS test employing 150 mOs/L solutions of sodium citrate and fructose with 30 minutes of incubation at 37°C.

Gene expression analysis: Total RNA was extracted from sperm using the Maxwell®16 LEV simply RNA Cells Kit (Cat # AS1270) according to the manufacturer's guidelines and using Maxwell® 16 Instrument (Cat # AS2000). RNA samples were quantified using nanodrop instrument (Thermo Fisher Scientific, Wilmington, USA). RNA samples showing 260 to 280 ratio of 1.8–2 were considered for cDNA preparation. The GoScript™ Reverse Transcription System was used for cDNA preparation. The cDNA was prepared as per manufacturers guidelines. Primer sequences (Table 2) were selected from Deb (2011). All primer pairs were checked for expected product size by electrophoresis on 1.5% agarose gel in 0.5x Tris-borate-EDTA buffer (45-mM Tris base, 45-mM boric acid, 1-mM EDTA; pH 8.0) containing 0.5 mg/mL ethidium bromide at least twice during the experiment. The qPCR was performed according to Deb (2011) in triplicates in a 7500 Fast instrument (Applied Biosystems, Japan) using a 20 µL reaction mixture containing 0.2-mM of each bovine-specific primer (0.5 µL forward and 0.5 µL reverse primers), 10 µL of SYBR green master mix (Maxima SYBR Green/Fluorescein qPCR Master Mix, Thermo Fisher Scientific), 2.0 µL of diluted cDNA sample and 7 µL of nuclease free water. A negative control (NTC) was used for each gene. PCR products that exhibited only a single fusion temperature, which confirms a unique PCR product, were retained for further quantitative analysis. The target genes

Table 1. Composition of different semen extenders

Tris-egg yolk-citrate extender components		Soya milk extender components		Andromed extender
Tris (w/v)	3.028 g	Tris (w/v)	3.028 g	20% Andromed and 80% Mili Q Water as per manufacturers guidelines
Citric acid (w/v)	1.678 g	Citric acid (w/v)	1.678 g	
Fructose (w/v)	1.200 g	Fructose (w/v)	1.200 g	
Egg yolk (v/v)	20.0%	Soy milk (v/v)	25.0%	
Glycerol (v/v)	6.4%	Glycerol (v/v)	6.4%	
Streptomycin	660 µl	Streptomycin	660 µl	

Table 2. Primers used in gene expression studies

Gene	Primer sequence	
	Forward	Reverse
GAPDH (control)	5'-AGGTCGGAGTGAACGGATTC-3'	5'-GGAAGATGGTGTATGGCCTTT-3'
AKR1B1	5'-TGCAACCCAAATACTCTTTT-3'	5'-AAAAGCCTAGCTGAAAGGAT-3'
AKap-4	5'-TAGTTACGAAGCTGGGATGT-3'	5'-CTTCTCCAGTTGTCCATCAT-3'

Table 3. Effect of different semen extenders on motility parameters (mean±SE) of buffalo sperms

Freezing stage	Types of extender	Motility (%)			
		Total	Progressive	Static	Slow
Fresh semen	—	92.9±0.3	74.6±2.8	7.14±0.3	1.9±0.40
Pre-freeze	Andromed	77.1 ^a ±1.0	59.8 ^a ±1.0	22.94 ^a ±1.0	2.1±0.40
	Tris-egg-yolk	72.3 ^b ±0.6	53.66 ^b ±1.3	27.7 ^b ±0.6	2.32±0.40
	Soya milk	65.3 ^c ±1.3	48.70 ^c ±1.2	34.7 ^c ±1.3	3.18±0.49
Post-thaw	Andromed	52.3 ^p ±1.1	47.42 ^p ±1.3	47.7 ^p ±1.1	2.18 ^p ±1.12
	Tris-egg-yolk	46.0 ^q ±1.5	39.82 ^q ±0.5	53.1 ^q ±0.7	2.70 ^p ±0.18
	Soya milk	33.5 ^r ±0.7	27.12 ^r ±0.5	66.5 ^r ±0.7	4.36 ^r ±0.70

*n=20. Means bearing different superscripts among extenders at pre-freeze (a,b,c) and post-thaw (p, q, r) stage differ significantly (P<0.01).

were quantified by the $\Delta\Delta C$ (t) method. Normalization was performed against *GAPDH* reference gene. The mean of minimum three biological replicates was used for statistical analysis.

Artificial insemination: Cryopreserved semen straws were used for artificial insemination (AI) of naturally estrus buffalo. All the animal were Bangladeshi indigenous buffalo aged between 3.5–4.5 years with body weight between 370–395 kg and they were at their second parity averagely and at the postpartum stage all of them were at sound health condition. AI was done once after detection of heat following general procedure of AI. Pregnancy was diagnosed after 60 days by rectal palpation.

Data analysis: Data were analyzed by one way ANOVA and were presented as mean±SE.

RESULTS AND DISCUSSION

Post thaw motility: The total, progressive, static and slow motility of pre-freeze and post thaw sperms were varied significantly (P<0.01) among Andromed, Tris-egg-yolk extender and Soya-milk extender (Table 3). In general, motility parameters were significantly higher in semen diluted with Andromed extender compared to Tris-egg-yolk extender and Soya-milk extenders at all refrigeration storage intervals. The findings of the present study are in agreement with Meena *et al.* (2010) who also found higher sperm motility and viability in Tris-egg yolk based extender than Soybean based extender. Pre-freeze total and progressive motility and post-thaw total and progressive motility in case of tris-egg yolk extender are comparable with the findings of Akhter *et al.* (2011), Sing *et al.* (2013), Chaudhari *et al.* (2015), and Kumar *et al.* (2016). The findings of this study regarding motility parameters are also comparable with the reports of Singh *et al.* (2012), Singh *et al.* (2013) and Rehman *et al.* (2014). In case of Soya milk extender, motility parameters were lowest. Poor efficiency in case of soya milk extender was also reported in previous studies (Dhami *et al.* 1993, Rana *et al.* 2003). However, some researchers reported superiority of soya milk extender over egg yolk extender on bovine semen preservation (Thun *et al.* 2002, Aires *et al.* 2003, Stradioli *et al.* 2007, Cresilho *et al.* 2012).

Table 4. Effects of selective extenders on plasma membrane integrity of buffalo sperm (mean±SE)

Freezing stage	Extender	HOS reactive sperm (%)
Fresh		72.32±0.24
Pre-freeze	Andromed	63.23 ^a ±0.32
	Tris-egg-yolk	60.42 ^b ±0.42
	Soya milk	58.65 ^c ±0.37
Post-thaw	Andromed	46.81 ^a ±0.76
	Tris-egg-yolk	43.32 ^b ±0.82
	Soya milk	39.05 ^c ±0.84

Means bearing different superscripts among extenders at pre-freeze and post-thaw (a,b,c) stage differ significantly (P<0.01).

Sperm membrane integrity: The HOS reactive sperm is a clear indication of plasma membrane integrity of sperm as well an indication that sperm is biochemically and functionally active. At pre-freeze and post-thaw stages, sperm plasma membrane integrity among three extenders groups was lower compared to fresh stage (Table 4). Sperm Plasma membrane integrity among three extenders groups at pre-freeze and post-thaw stages differ significantly (P<0.01). Membrane integrity was recorded higher in case of Andromed extender both at pre-freeze and post-thaw stage. Sperm Plasma membrane integrity in case of Tris-egg-yolk extender was almost similar to Andromed extender group. Plasma membrane integrity observed in this study are comparable with the results obtained by Akhter *et al.* (2011), Chaudhari *et al.* (2015) and El-Sisy *et al.* (2016).

Sperm kinematics: Results showed significant variations (P<0.01) for different sperm kinematic parameters among Andromed, Tris-egg-yolk extender and Soya-milk extenders as shown in Table 5. Almost all of the values of kinematic parameters were recorded higher in case of andromed extender and these values in case of tris-egg-yolk extender were very close to it.

Gene expression: The expression of aldo-ketoreductase family 1 member B1 (AKR1B1) and A-kinase anchoring proteins (AKAP4) transcripts in fresh and three extenders groups (post thaw) of buffalo sperm has been shown in Table 6. No significant variations were observed in AKR1B1 and AKAP4 genes expression among andromed,

Table 5. Effect of different extenders on kinematic parameters (mean±SE) of fresh and post thawed buffalo sperms

FreezingStage	Types of extender	VAP (µm/s)	VSL (µm/s)	VCL (µm/s)	STR (%)	LIN (%)	ALHµm	BCFHz
Fresh	–	98.7±0.7	79.7±0.7	140.8±0.7	88.9±0.8	57.9±0.4	7.4±0.6	27.9±0.6
Post-thaw	Andromed	84.7 ^a ±0.5	68.7 ^a ±0.7	139.8±0.7	85.3 ^a ±0.7	54.2 ^a ±0.5	7.0±0.5	26.2 ^a ±0.5
	Egg yolk	82.0 ^b ±0.5	66.5 ^b ±0.5	138.6±0.9	82.4 ^b ±0.6	53.0 ^a ±0.6	7.0±0.3	26.1 ^a ±0.4
	Soya milk	63.2 ^c ±0.7	58.0 ^c ±0.9	139.3±0.9	67.9 ^c ±0.9	46.9 ^b ±0.6	6.6±0.4	19.4 ^b ±0.5

VAP, average path velocity; VSL, straight line velocity; VCL, curvilinear velocity; STR, straightness; LIN, linearity; ALH, Amplitude of lateral head displacement and BCF, beat cross frequency. Means bearing different superscripts (a,b,c) among extenders at post-thaw stage differ significantly (P<0.01).

tris-egg -yolk and soya milk extenders groups. The AKR1B1 transcript determines the fate of the embryo through the involvement in apoptic pathway. Moreover, expression of AKR1B1 in bovine blastocysts reliably predicts implantation, early embryo loss/resorption and normal calf delivery (El-Sayed *et al.* 2006). It is also involved in pregnancy failure through metabolism of progesterone and synthesis of prostaglandin F2α (PGF2α) in the bovine uterine endometrium (Gomez *et al.* 2009). Low expression of the AKR1B1 genes may indicate improved embryo quality (Deb *et al.* 2011). AKAP4 is the major protein in the fibrous sheath and is the product of an X-linked gene (Moss *et al.* 1997) expressed only in spermatids (Fulcher *et al.* 1995) and responsible for the progressive motility of the sperm and thereby fertility (Miki *et al.* 2002). Its function is also associated with semen freezability (Yeste 2016 and Chen *et al.* 2014).

Artificial insemination: Conception rates following AI at BLRI Buffalo Research Farm were higher in Andromed (88.89%) and Tris- egg- yolk extender (88.89%) groups than Soya milk extender group (33.33%) (Table 7). After conception, pregnancy was confirmed by rectal palpation.

Considering pre-freeze and post-thaw sperms

Table 6. Expression patterns of AKR1B1 and AKAP4 transcripts in buffalo sperm cryopreserved using different semen extenders.

Semen group		Expression of genes in fold	
		AKR1B1	AKAP4
Fresh		1.00	1.00
Post-thaw	Tris-Egg-Yolk	1.38	1.47
	Soya milk	1.24	0.79
	Andromed	1.22	0.81

Table 7. Effect of different extender on conception rate after AI using cryopreserved buffalo semen

Extender type	No. of animal inseminated	No. of animal conceived	Conception rate (%)
Andromed	9	8	88.89
Tris egg yolk extender	9	8	88.89
Soya milk based extender	9	3	33.33

characteristics this study suggests that, Tris-Egg-Yolk semen extender might be used for cryopreservation of buffalo semen at the same efficiency level compared to commercially available semen extender like Andromed.

ACKNOWLEDGEMENTS

The authors are thankful to the ‘Buffalo Development Project (Component-B)’ of Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka for funding this research work. We are also thankful to the Division of Biotechnology, Bangladesh Livestock Research Institute (BLRI), Savar, Dhaka, for providing laboratory support and providing animals for conducting this research.

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