COMPARATIVE EFFICACY OF COCONUT WATER DILUENT WITH DIFFERENT SEMEN EXTENDERS FOR CRYOPRESERVATION OF NILI RAVI BUFFALO BULL SEMEN

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ABSTRACT

In this study, efforts were made to investigate fresh semen parameters and to select a suitable extender for buffalo semen cryopreservation. Experiment I, fresh undiluted seminal parameters were determined while Experiment II, efficacy comparison of coconut water extender (CWE) with tris citric acid extender (TCAE) and skimmed milk extender (SME) was made. Four bulls single ejaculate was collected weekly for 5 and 10 weeks for Experiment I and II, respectively however osmotic pressure replicates were 16 and 6 for semen and seminal plasma, respectively. In Experiment I, each bull spermatozoa concentration, motility (%), semen volume and pooled semen percentage NAR, PMI, viability and MTT (3-(4, 5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction rate was checked. In Experiment II, pooled semen added to extenders and then equilibrated (4°C) and filled to obtain 20×10^6 spermatozoa/0.5 ml straws before plunging in liquid nitrogen. Percentage thawed spermatozoa viability, normal acrosomal ridge (NAR), motility, DNA damage, plasma membrane integrity (PMI) and lipid peroxidation (nM) recorded. In Experiment I, seminal parameters as spermatozoa concentration (1226.43±71.48 million/mL), semen volume (2.84±0.14 mL), viability (90.05±0.71%), motility (77.13±0.71%), PMI (86.23±0.34%), NAR (94.67±0.30%) and MTT reduction rate (0.290±0.06) while osmotic pressure of seminal plasma (294.83±3.87 mOsm/kg) and semen (290.87±2.58 mOsm/kg) was recorded. In Experiment II, TCAE higher ($P<0.05$) sperm motility noted compared to CWE and SME whereas percentage viability, NAR, PMI, DNA damage was non-significant. Lipid peroxidation compared to SME and TCAE was higher ($P<0.05$) in CWE. In conclusion, based on sperm motility and lipid per oxidation, TCAE was more efficient for cryopreservation of buffalo semen.

Keywords: *Bubalus bubalis*, buffaloes, spermatozoa, semen, cryopreservation, extender

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INTRODUCTION

World has 177.247 million buffalo population out of which 97% are present in Asia (Presicce, 2007) while Pakistan has major proportion of world buffalo population which is 31.7 million (Anonymous, 2010-2011). Out of total milk produced in country, 68% is contributed by buffalo (Bilal *et al*., 2006).

Present reports for osmotic pressure of buffalo semen are contentious; an osmotic pressure of 293.33±3.39 (Ibrahim *et al*., 1985), 268.81±1.17 (Khan and Ijaz, 2008) and 289.4 mOsm/kg (Mughal *et al*., 2013) has been reported.

Now world human population has increased which demands to rise per animal milk production which is possible by the use of modern biotechnology of artificial insemination (A.I.) with cryopreserved semen in buffaloes. Semen extender increases the semen volume and during cryopreservation also maintains fertility. As in cryopreservation motile sperm number are decreased due to damage to plasma membrane (Yildiz *et al*., 2007) that causes 40 to 50% spermatozoa to die (Watson, 2000) due to which cryopreserved semen has less fertility than fresh semen. For better fertility with cryopreserved semen in buffalo, it is necessary to develop an appropriate semen extender for buffalo as their spermatozoa are most vulnerable to cryopreservation compared to cattle (Raizada *et al*., 1990).

Changes in spermatozoa membrane integrity (Rasul *et al*., 2001), decreased sperm motility (Tuli *et al*., 1981; Budworth *et al*., 1988), acrosomal damage (Saacke and White, 1972; Akhtar and Chaudry, 1989) are various factors for low fertility in buffaloes with cryopreserved semen. These damages of cryopreservation to buffalo semen can be controlled by developing an appropriate semen extender. Therefore, effort was made to investigate fresh undiluted seminal parameters select and to suitable extender for buffalo bull semen cryopreservation.

MATERIALS AND METHODS

Management of animals and collection of semen

Four bull's semen was collected that were maintained at Semen Prodution Unit, Qadirabad, Sahiwal, Pakistan. Bull's management and feeding has already been mentioned (Wadood *et al*., 2016). Four bulls single ejaculate harvested weekly for 5 and 10 weeks for Experiment I and II (except semen osmotic pressure check up), respectively. Semen was collected by artificial vagina at temperature of 42°C and then holding time of 15 minutes was provided to semen collection tubes in water bath maintained at 37°C.

Experiment I: Semen analysis

MTT reduction assay as mentioned by Iqbal *et al*. (2010) was used to assess metabolically active spermatozoa while photometer (Bovine photometer n° 1119, IMV, France) was used to evaluate spermatozoa concentration. Spermatozoa viability, motility, NAR and PMI rates were determined analogue to Experiment II. However, whole semen and seminal plasma osmotic pressure was noted using the cryoscopic osmometer (Gonotec 030, Berlin, Germany). Whole semen was centrifuged at 4000 rpm for 10 minutes to harvest seminal plasma. For whole semen and seminal plasma, no of replicates were 16 and 6, respectively.

Experiment II: Preparation of extender and processing of semen

TCAE was prepared with some changes to formula of Liete *et al*. (2010), SME (Kommisrud *et al*., 1996) while CWE (Cardoso *et al*., 2003). Extenders composition presented in Table 1. Extenders with pH 7.0 and 300 mOsm/kg osmotic pressures were prepared and osmotic pressure was verified with cryoscopic osmometer (Gonotec 030, Berlin, Germany). Pooled semen of four bulls with ≥70% motility was mixed with three various extenders and then equilibrated semen at 4°C in cold cabinet was filled in 0.5 ml polyvinyl French straw to obtain 20×10^6 spermatozoa/straw. From 4°C to -15°C, straws were cooled 3°C/minutes and then from -15°C to -80°C 10°C/minutes. Prior to plunging into liquid nitrogen (-196˚C), straws were positioned for 10 minutes 4 cm above liquid nitrogen.

Semen analysis

Semen was thawed at 37°C for 30 seconds and procedure of Ijaz *et al*. (2009) was adopted for checkup of spermatozoa motility (%). Procedure of Khan and Ijaz (2008) was adopted to assess percentage PMI (75 mOsm/kg HOS solution) and spermatozoa viability (supravital staining) while methods used by Tejada *et al*. (1984); Rasul *et al*. (2000) were adopted to determine DNA damage (acridine Orange method) and NAR, respectively. For DNA integrity, viability, PMI and NAR rates, two hundred spermatozoa were checked and three observations mean was considered single data point. To verify Malondialdehyde (MDA: stable lipid peroxidation product), Thiobarbituric acid assay as per Ohkawa *et al*. (1979) was carried out by spectrophotometer (UV 2800, BMS, Canada) at 532 nm. Result was noted in nM (MDA).

Statistical analysis

For statistical analysis, statistical Package for Social Science (Windows version 12, SPSS Inc., Chicago, IL, USA) was adopted. Data was shown in form of mean ± S.E. Analysis of variance used to analyze the data; Duncan's Multiple Range Test used to check group differences. Differences of results were noted significant at $P<0.05$.

RESULTS AND DISCUSSIONS

In this study, effort was made to investigate fresh undiluted seminal parameters and to select suitable extender for buffalo bull semen cryopreservation.

Experiment I

In Table 2, undiluted fresh Nili Ravi buffalo bull semen volume, spermatozoa concentration including percentage motility while pooled semen MTT reduction rate, percentage NAR, PMI, and viability are presented. Buffalo osmotic pressure of seminal plasma (294.83±3.87 mOsm/kg) and whole semen (290.87±2.58 mOsm/kg) was recorded (Figure 1). Adeel *et al*. (2009) results for buffalo spermatozoa concentration, mean spermatozoa motility and semen volume are in line to present study; however, for said semen parameters in buffalo, different results were noted by Hashemi *et al*. (2007). Kanwal *et al*. (2000) findings for mean buffalo spermatozoa viability (%) are similar to our results. Relatively lower sperm viability in buffalo was noted by Iqbal *et al*. (2010). Aguiar *et al*. (1994) report for buffalo ejaculates mean spermatozoa acrosomal integrity was with agreement to present study, however, Hashemi *et al*. (2007) results were relatively higher. Rasul *et al*. (2001) results for buffalo ejaculates PMI were in agreement to this

study, however, Singh *et al*. (2007) noted a lower a PMI in buffalo semen. These differences of seminal parameters than our study might be due differences in scrotum circumference, nutrition, experience of analyst, weight and body size, breed or age of bull, semen collection season and procedure and frequency of semen collection and processing. Our results of semen osmotic pressure are in accordance to Ibrahim *et al*. (1985); Mughal *et al*. (2013). However, Khan and Ijaz (2008) reported relatively lower buffalo semen osmotic pressure than present study. This difference in results might be due to expertise of the analyzer, efficacy of the osmometer used or breed of the bull.

Experiment II

In TCAE, significantly higher ($P<0.05$) sperm motility was recorded than CWE and SME whereas percentage viability, NAR, PMI, DNA damage was non-significant among three extenders. However, Lipid peroxidation was higher ($P<0.05$) in CWE than TCAE and SME (Table 3).

In TCAE, similar sperm motility results for buffalo semen cryopreservation were noted (Rasul *et al*., 2001; Andrabi *et al*., 2008; Adeel *et al*., 2009; Ansari *et al*., 2010; Mughal *et al*., 2013). Low motility in SME might be due to low visibility of SME as noted by Foote *et al*. (1993). In TCAE during cryopreservation, zwitterions did not cross the plasma membrane and desiccate spermatozoa more proficiently resulting in less intracellular ice development, Good *et al*. (1966). Moreover, pH changes during cryopreservation were efficiently resisted by zwitterions.

In this study sperm viability in TCAE was relatively higher (15%) than Ijaz *et al*. (2009) that could be because of that Ijaz *et al*. (2009) did not adjusted osmotic pressure of semen extender nevertheless same extender was used in both studies.

A significantly higher acrosomal integrity of bull sperm in isotonic extender was reported by Woelders *et al*. (1997) that is also in line to present study. Similar results to our study noted by Singh *et al*. (2007) but (Andrabi *et al*., 2008; Akhtar *et al*., 2010) at osmotic pressure 320 mOsm/kg of buffer reported a 24% and 13% higher acrosomal integrity, respectively than our study that could be due to variation of osmotic pressure of buffer in both studies. In various semen extenders, difference of acrosomal integrity is due to the fact that various seminal parameters respond differently in different extenders (Songsasen, 2002). At 300 mOsm/kg, higher acrosomal integrity might be due to hypertonic (Recorded osmotic pressure~293 mOsm/kg) extender (Ibrahim *et al*., 1985) as it decreases intracellular ice by proper dehydration of spermatozoa (Mann, 1964; Watson, 1979; Fiser *et al*., 1981, 1982; Fiser and Fairfull, 1986, 1989; Pommer *et al*., 2002).

Contrary to present study, Kumar *et al*. (2011) by commet assay reported 35% higher DNA damage in buffalo bull semen that could be due to difference in technique used. By acridine orange staining technique, higher sperm DNA damage of 4.5% and 10.4% was reported by Nur *et al*. (2010) and El-Sisy *et al*. (2010) in ram and buffalo, respectively that might be due to an-isotonic extender as extender osmotic pressure was not mentioned in these studies. By acridine orange technique, a 7.7% higher buffalo spermatozoa damaged DNA was noted in SME by Waterhouse *et al*. (2010) and variable osmotic pressure extender might be responsible for elevated DNA destruction in Waterhouse *et al*. experiment. Moreover, buffalo plasma membrane higher unsaturated fatty acids trigger oxidative stress in an-isotonic diluent that leads to DNA damage (Aitken and Krausz,

Table 1. Composition of semen extenders.

Ingredients with source	TCAE	SME	CWE
Solution A			
Coconut water	-	-	250 ml
Tri-sodium citrate dehydrate (Merck, 64271 Darmstadt, Germany)	-	-	7.50 gm
Skimmed milk (Millac Foods (Pvt.), Ltd. Pakistan)	-	55 gm	-
Tris (Hydroxymethyl) aminomethane (BDH Laboratory supplies, England)	16.78 gm	-	-
citric acid monohydrate (Merck, Germany)	9.32 gm	-	-
D-Fructose (BDH Laboratory supplies, England)	7.53 gm	-	-
Double distilled water	500 ml	500 ml	250 ml
Solution B			
Solution A	73 ml	73 ml	73 ml
Egg yolk	20 ml	20 ml	20 ml
Glycerol (Scharlab S.L. Spain)	7 ml	7 ml	7 ml
Pencillin (Sinochem, China)	1000 I.U./ml	1000 I.U./ml	1000 I.U./ml
Streptomycin (China National Medicine and Health Products Chongqing, China)	1.0 mg/ml	1.0 mg/ml	1.0 mg/ml

Table 2. Mean Nili Ravi buffalo bull seminal parameters of pooled semen and fresh ejaculates (n=10).

Fresh ejaculates			Pooled semen			
Motility (%)	Volume (mL)	Concentration (million/mL)	MTT reduction rate	Viability (%)	NAR (%)	PMI (%)
77.13±0.71	2.84±0.14	1226.43±71.48	0.290±0.06	90.05±0.71	94.67±0.30	86.23±0.34

Values as mean ± S.E.; n= Number of collections

Table 3. Buffalo mean post thawed spermatozoa parameters in different extenders (n=5).

Paramiters	TCAE	SME	CWE
Motility (%)	54.00±3.66^{B}	39.00±2.85^{A}	39.00±3.69^{A}
Viability (%)	73.37±2.47^{A}	68.07±2.86^{A}	71.10±3.10^{A}
Normal apical ridge (%)	69.78±2.31^{A}	73.14±2.69^{A}	69.36±1.85^{A}
Plasma membrane integrity (%)	59.91±2.96^{A}	60.20±3.40^{A}	56.30±3.80^{A}
Damaged DNA (%)	1.14±0.15^{A}	0.78±0.11^{A}	0.90±0.12^{A}
Oxidative status (nm)	29.30±6.19^{A}	25.13±3.86^{A}	52.30±5.82^{B}

Values presented mean ± S.E.

Seminal parameters with significant difference ($P<0.05$) in rows with alphabets (A-B).

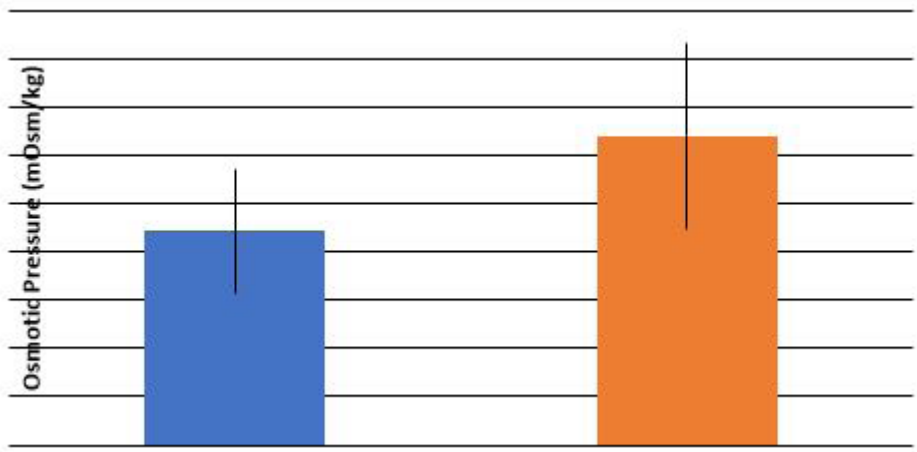

Figure 1. Buffalo seminal plasma and whole semen osmotic pressure.
n = no of replicates

2001; Meyers, 2005). Due to oxidative stress (Lewis and Aitken, 2005) and Intracellular ice formation (Royere *et al*., 1991) sperm chromatin over compaction occur because of covalent bonds formation and DNA strand separation (Cordova *et al*., 2002; Glogowski *et al*., 1994). Sperm with damaged DNA unable to attach fallopian tube and at fertilization did not decondence (Ardon *et al*., 2008) and pass on faulty genentic material (Sakkas and Alvarez, 2010) and ultimately results in failure of fertilization, embryo loss or poor embryo growth.

SME and TCAE has lower ($P<0.05$) lipid per oxidation compared to CWE. In SME, lesser lipid per oxidation might be due to casein proteins etc. that are natural antioxidants in milk (Salamon and Maxwell, 2000). In TCAE, zwitterions are not permeable to plasma membrane and efficiently dehydrate sperm resulting in lower production of intracellular ice that ultimately leads to lower lipid per oxidation that correlates to good quality semen (Chaudhari *et al*., 2008).

In conclusion, based on sperm motility and lipid per oxidation, TCAE was more suitable for buffalo semen cryopreservation. However, fertility trial of cryopreserved semen of these extenders is recommended in future.

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