Effect of Supplementation of Polyvinylpyrrolidone in Extender on Buffalo Semen Parameters

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ABSTRACT
The current study was planned to evaluate the supplementation of Polyvinylpyrrolidone in extender on cryopreservation of Nili-Ravi buffalo bull semen. The semen samples were collected from Nili-Ravi buffalo (Bubalus bubalis) bull kept at SPU Qadirabad, District Sahiwal, Pakistan. Qualifying semen ejaculates having motility >60%, volume ≥5-6ml and concentration >0.5 billion/ml were diluted 50 × 10⁶ motile sperm/ml approximately at 37°C in Tris-citric acid extender supplemented with different concentrations of PVP (0.01, 0.05, 0.1 mM). The extender without PVP was kept as control. Semen was stored at 4°C for a period of 2 h and kept at 4°C for 4 h. Semen was filled in 0.5 ml French straws using suction pump at 4°C, plunged and stored in liquid nitrogen (-196°C). Semen straws were rewarmed at 37°C for 30 seconds and assessed for sperm motility, plasma membrane integrity (PMI), dead sperm percentage and the live sperm percentage. The data on the role of PVP on different parameters of semen quality were analyzed by using ANOVA and RCBD. Higher percentage (P< 0.05) of sperm motility (66.1±7.51 and 59.4±10.72) and PMI (72.9±5.39 and 75.7±6.5) was observed in extenders having 0.05 mM and 0.1 mM PVP compared to extenders having 0.5 mM PVP and control. The percentage acrosomal integrity was observed greater (P< 0.05) in extended semen containing 0.1 mM (68.2±0.50) PVP compared to extenders having 0.01 and control.

INTRODUCTION
In the economy of Pakistan, livestock is a major sector accounting for 49.1% of agriculture. The most important dairy breeds are Nili-Ravi buffalo and Sahiwal cattle that are most adaptable and versatile of all the domesticated animals in Asia. For agricultural operations, buffalo plays a major role in social development through contributions of milk, meat, and hides. In the Pakistan, the main dairy breed is Nili-Ravi buffalo but it has no more improvement in breeding practices as these benefits are depending upon the efficiency of the artificial insemination (AI) system (Foote, 1975). The composition of the extender in which the semen is diluted before freezing is one of the main factors that influence the success of cryopreservation. Extenders containing lipids, sugars and amino acids exert a stabilizing effect on the sperm plasma membrane, which is needed to maintain the physiologic integrity of the spermatozoon after thawing. In addition, extenders should contain compounds with high osmolarity, like glycerol, to protect the sperm cell during freezing. In contrast, the presence of glycerol during thawing reduces the fertilizing capacity of cryopreserved semen by inducing osmotic shock or premature capacitation (Holt and Medrano, 1997).

Different antioxidants have been added to protect spermatozoa from the deleterious effects of cryopreservation. The oxidative chain reaction is broken by antioxidants. These agents reduce the oxidative stress. Supplementation of antioxidants in extenders have been shown to have a positive effect in cryopreservation of bull, goat, ram, canine, boar, and human semen quality. After thawing antioxidants improved the quality of semen parameters, integrity of plasma membrane and motility of sperm (Kumar and Mahmood, 2001). In 1993, it was reported that 5% and 10% polyvinylpyrrolidone (PVP) cause a significant decrease in the amount of intact bovine spermatozoon after freezing and thawing. By the addition of PVP the percentage of motile spermatozoon becomes decreased in unfrozen semen, and post thaw quality of spermatozoon was not significantly upgraded. A positive effect of PVP on cryopreservation of bovine semen is expected if it is used in low percentages. Sucrose and PVP combination is commonly used as stabilization of structure in cryoultramicrotomy (Leeuw et al., 1993).

PVP is one of the many macromolecules widely used for cryopreservation of normal cells, oocytes and embryos in the hamster. PVP provides a lower rate of zona

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disruption and avoids the need for screening for pathogenic contaminants, such as human immunodeficiency virus and hepatitis B and C. They also showed that inclusion of PVP in the freezing medium for 2-cell stage mouse embryos reduced the incidence of zona damage and increased the survival rate of embryos following in vitro culture after warming (Titterington and Robinson, 1996).

PVP, a constituent of cryoprotectant consisting of sucrose, could be used to prevent cryoinjury in vitrified-warmed embryos. To avoid any potential damaging effects of PVP, some techniques have been developed which do not force slowing sperm motility (Tsai et al., 2000).

For cryopreservation, semen is diluted and sperm become more vulnerable to ROS by decreasing components of natural antioxidant. Therefore, to restore the normal level, antioxidants like ascorbate and alpha tocopherol are added to the extenders which have a protecting effect on metabolism and viability of frozen thawed bull semen. In the extended semen inclusion of BHT, cysteine and glutathione enhanced the quality of buffalo bull spermatozoa (Andrabi et al., 2008).

Evaluation of bull fertility is essential for the industry of artificial insemination (AI). To increase the effectiveness of the breeding program, the semen from young bulls is collected as early as possible then tested for volume, concentration and motility in the laboratory and tested for fertility in herds that are enrolled in a milk-recording program. Pedigree information and production data in the breeding program, the semen from young bulls of known fertility and similar age were shifted immediately to the laboratory for initial assessment. After semen collection, samples were collected for three weeks intervals. Semen samples were collected for three weeks for further assessment. After semen collection, samples were taken from three mature Nili-Ravi breeding bulls maintained at Semen Production Unit (SBU) Qadirabad, District Sahiwal, Pakistan. The semen was cryopreserved and transported to Animal Physiology Laboratory, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, where semen samples were studied for the effect of PVP in cryopreservation.

**Semen processing**

**Extender preparation**

Experimental extenders were prepared by using buffer of *Tris*-citric acid (pH 7.0, osmotic pressure 320 mOsmol Kg⁻¹). Glycerol 7%, fructose 0.2% weight/volume and egg yolk 20% volume/volume were added to the extender. Strepotymmetric-penicillin combination (1000 µg/ml, 1000 unit/ml) was used as antibiotics in extender. PVP was used at the rate of 0, 0.5, 1.0 and 1.5 mM to make experimental extenders. The extender without PVP supplementation was kept as control.

**Collection of semen and assessment**

Semen samples were taken from three mature Nili Ravi breeding bulls of known fertility and similar age and two repeated ejaculates were collected at weekly intervals. Semen samples were collected for three weeks for further assessment. After semen collection, samples were tested for volume, concentration and motility in the laboratory for initial evaluation. Sperm motility was examined with the help of olmier microscope (X400) at 37 °C and concentration of sperm was assessed with the help of neubauer haemocytometer. Briefly, a drop of thoroughly mixed 200 fold diluted (with 0.9% of NaCl) semen was put on the Makler chamber. Under a phase contrast microscope the grid was located with 200X magnification. Spermatozoa were counted in 100 squares with the help of manual counter. Spermatozoa concentration was calculated as described by (Christensen et al., 2005). The neat semen samples were used for further evaluation.
dilution having more than 60% motile spermatozoa.

Semen extension and cryopreservation

Semen aliquots were diluted with experimental extenders at room temperature having \(50 \times 10^6\) motile spermatozoa ml\(^{-1}\). The amount of extender was calculated by applying the formula:

\[
\text{Extender} = \text{volume} \times \text{concentration} \times \text{motility} \times \frac{1}{\text{required concentration of sperm (50\times10^6/ml)}}
\]

Semen sample after dilution was stored at 4 °C for a period of 2 h then it was kept at 4 °C for 4 h. Then with the help of suction pump sample was stored in 0.5 ml. French straws at 4 °C in cold cabinet unit. The straws were positioned above liquid nitrogen (LN\(_2\)) for 10 minutes and were stored in liquid nitrogen container at -196 °C. Semen straws were re-warmed at 37 °C for 30 seconds. For each extender, semen from three straws were pooled and incubated at 37 °C for analysis of different parameter of semen.

Assessment of semen quality parameters

Progressive motility

Semen (a drop) was put on a warm glass slide (37 °C) and then put cover slip on it. Visual motility percentage was evaluated microscopically at 400X.

Plasma membrane integrity

Hypo-osmotic swelling (HOS) test was used to assess PMI (Jeyendran, 1984). Hypo-osmotic swelling solution was prepared by addition of sodium citrate (0.73 g) and fructose (1.35 g) in 100 ml distilled water (~190 mOsmol Kg\(^{-1}\)). To assess the PMI, mix 50 μl of sample with 500 μl of HOS solution and for 30-40 min incubated at 37 °C. A drop of solution (10 μl) was put on a warm slide and observed under light microscope (400X magnification). Two hundred spermatozoa were counted for each treatment. Intact and biochemically active sperm membranes are indicated by swollen or bent tails, while straight and un-swollen tails were considered to have disrupted plasma membrane (Chan et al., 1991).

Statistical analysis

Data on cryopreserved buffalo semen by the supplementation of different levels of PVP was analyzed for each bull and overall by the analysis of variance (ANOVA) using MSTAT-C, least significant difference (LSD) was applied to sort out the best concentration of PVP for cryopreserved buffalo bull semen by comparing treatment means.

RESULTS AND DISCUSSION

Polyvinyl pyrrolidone (PVP) acts as an antioxidant in the control of browning of tissue and enhanced organogenesis and buds shoot growth during micro propagation in different plants. The current study was programmed to conduct the role of PVP as antioxidant to enhance the cryopreserved buffalo semen quality.

Sperm progressive motility

In bull 1, progressive motility of sperm was significantly higher (P<0.05) in extenders having PVP at 0.05mM (73.3±2.89) and 0.1mM (71.7±7.64) compared to control (56.7±7.64) while, it remained similar in extenders containing PVP 0.01mM (65.0±5.0) and control (56.7±7.64) (Fig. 1). In bull 2 and bull 3, the sperm motility percentage was similar (P>0.05) in extenders having PVP at 0.01mM (53.3±11.55 and 51.7±17.56), 0.05mM (58.3±7.64 and 66.7±7.64), 0.1mM (51.7±7.64 and 55±13.23) and control (46.7±2.89 and 56.7±5.77) (Figs. 2 and 3). The overall effect of PVP on post thaw percent sperm motility was better (P<0.05) in extending extender having 0.05mM (66.1±7.51) compared to 0.01mM (56.6±7.26) and control (53.3±5.77). However, sperm progressive motility remained similar in extending containing PVP 0.05mM (66.1±7.51) and 0.1mM (59.4±10.72) (Fig. 4).

Statistical analysis

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It is reported that cystine, glutathione and thioglycol in tris citric acid extender improved the post thaw motility in buffalo bull sperms. Incorporation of vitamin C or E in tris-citric acid buffer (TCA) based extender improved the motility of liquid buffalo bull semen. The greater number of motile spermatozoa present in samples frozen with natural antioxidants would increase the fertilizing potential of post-thaw spermatozoa (Ansari et al., 2011).
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Fig. 2. Effect of polyvinylpyrrolidone addition in extended semen on post thaw motility (Mean±SE) of Nili-Ravi buffalo bull (2) semen. Bars did not differ significantly (P>0.05).

Fig. 3. Effect of polyvinylpyrrolidone addition in extended semen on post thaw motility (Mean±SE) of Nili-Ravi buffalo bull (3) semen. Bars did not differ significantly (P>0.05).

Fig. 4. Effect of polyvinylpyrrolidone addition in extended semen on post thaw motility (Mean±SE) of Nili-Ravi buffalo bull (n=3) semen. Bars having dissimilar letters differ (P<0.05) significantly.

In present study extender containing 0.05mM PVP concentration showed significant improvement in sperm motility in buffalo bull. PVP has never been tested for its effect as an antioxidant in animal cell.

Sperm plasma membrane integrity

The quality of cryopreserved semen is disturbed by reactive oxygen species, the increased production of which can cause damage to sperm bio membrane systems (Kolm and Aurich, 2005).

The effect of PVP on percentage PMI of cryopreserved Nili-Ravi buffalo bull semen is presented in Figures 5, 6, 7 and 8. In bull 1, post thaw percentage of sperm plasma membrane was similar in extenders containing PVP at 0.01mM (74.0±1.8), 0.05mM (76.5±0.5) and 0.1mM (79.2±5.0) that was greater (P<0.05) compared to control (58.3±3.8). The trend remained the same in bull 2 where post thaw percentage of sperm PMI remained similar in extenders having PVP at 0.01mM (63.8±1.6), 0.05mM (66.7±7.0), 0.1mM (68.2±2.8) that was higher compared to control (55.0±1.8). In bull 3, sperm PMI was improved by supplementation of PVP at 0.01 (73.2±2.8), 0.05 mM (75.5±2.2) compared to control (61.7±2.3). However, it remained highest at 0.1 mM (79.7±0.8) compared to 0.01mM, 0.05 mM PVP and control. The overall percentage of sperm PMI was highest (P<0.05) in extender having PVP 0.1mM (75.7±6.5) compared to 0.01mM (70.3±5.67) and control (58.3±3.35). However, percentage PMI remained similar in extender containing PVP 0.05mM (72.9±5.39) and 0.1mM (75.7±6.5).

Previously, it was reported that PMI of buffalo bull spermatozoa was improved in TCA egg yolk glycerol extender containing vitamin C and vitamin E, cysteine, glutathione, and thioglycol. The present study showed improvement in plasma membrane integrity by supplementing PVP up to 0.1mM (Andrabi et al., 2008).
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Live sperm percentage

The effect of PVP on percentage of live sperm is presented in Figures 9, 10, 11 and 12. In bull 1, percentage of live sperm was similar in extenders having PVP at 0.01mM (78.3±3.40), 0.05 mM (72.8±3.21) and control (72.2±4.53), while, it remained higher in extender having 0.1(81.8±2.52) mM PVP compared to control. In bull 2, percentage of live sperm did not improve by supplementation of PVP at 0.01 mM (76.2±4.93), 0.05 mM (68.7±6.33) and 0.1 mM (67.0±0.50) compared to control (70.0±4.27).

However, live sperm percentage in bull 2 was greater (P>0.05) in extender containing PVP at 0.01mM (76.2±4.93) compared to 0.1 mM (67.0±0.50). In bull 3, the percentage of live sperm was improved (P<0.05) in extender having PVP at 0.01mM (79.7±2.52) and 0.05mM (80.5±3.91) compared to control (66.7±1.04), while at 0.1
mM PVP supplementation it (70.7±3.51) did not differ (P > 0.05) from control. The overall percentage of live sperm of Nili-Ravi buffalo bull (n=3) remained similar (P>0.05) in extender containing PVP 0.01 mM (78.1±1.76), 0.05 mM (74.0±5.99), 0.1 mM (73.2±7.70) and control (69.6±2.76). In this study live sperm percentage was not significantly improved by addition of PVP.

**CONCLUSION**

From present study it was concluded that seasonal variations have significant as well as non significant affect on testicular volume and seminal parameters.

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**Statement of conflict of interest**

The authors have declared no conflict of interests.

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