

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 50×10^6 motile sperm/ml approximately at 37°C in Tris-citric acid extender supplemented with different concentrations of PVP (0.01, 0.05, 0.1mM). 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 4h. 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 0.1mM PVP compared to extenders having 1.5mM PVP and control. The percentage acrosomal integrity was observed greater ($P < 0.05$) in extended semen containing 0.1mM (68.2 ± 0.50) PVP compared to extenders having 0.01 and control.

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Authors' Contribution

BIK performed laboratory work. S Akhter supervised the study and provided research materials. S Aslam and RE analysed the data and wrote the manuscript.

Key words

Semen extender, Sperm motility percentage, Plasma membrane integrity percentage, Alive sperm percentage and Dead sperm percentage

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 spermatozoa 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 spermatozoa after freezing and thawing. By the addition of PVP the percentage of motile spermatozoa becomes decreased in unfrozen semen, and post thaw quality of spermatozoa 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 first lactation period of each daughter are recorded and used to select genetically superior bulls for purpose of AI service that become parents of the next generation of cows. The quality of frozen-thawed semen can be assessed by fertility rate. However, a limitation of the assessment is that due to the high number of spermatozoa used for AI and variations in the state of sperm cells may not be reflected by the fertility rates (Vale, 1997).

PVP had cryoprotective properties on bovine spermatozoa that are determined by progressive motility. Same results were found for boar spermatozoa (Graham and Crabo, 1972). Addition of various concentration of PVP in a glycerol-containing extender has a cryoprotective effect for ram semen (Schmehl *et al.*, 1986).

The group of antioxidants that are, ascorbic acid, citric acid, DTT, PVP and vitamin C can reduce tissue browning and promote organogenesis, somatic embryogenesis, and shoot growth from buds during micropropagation across different plant species. Therefore, the addition of both 1% PVP and 2 mg/l DTT in coculture medium was found to improve explant viability during and after cocultivation (Yinghui, 2008). In plant transformation, these antioxidants are involved in the control of tissue browning and necrosis (Das *et al.*, 2002).

In laboratories, PVP is used as a reducing agent for analysis, and in the micromanipulation of gametes. In the study of Percoll, it was a well-known selecting agent for spermatozoa, and a cryoprotectant for the conservation of spermatozoa (Leeuw *et al.*, 1993). In assisted reproductive techniques (ART) it has been successfully used to increase the viscosity of the sperm solution, thus facilitating the handling of spermatozoa. In the intracytoplasmic sperm injection ICSI, sperm incubated with PVP for 60 min had a higher fertilization rate. PVP stabilized the sperm plasma membrane and PVP did not affect chromosomal integrity (Dozortsev *et al.*, 1995).

MATERIALS AND METHODS

The semen samples were taken from three Nili-Ravi breeding bulls maintained at Semen Production Unit (SPU) 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. Streptomycin-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 shifted immediately to the laboratory for initial evaluation. Sperm motility was examined with the help of 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

dilution having more than 60% motile spermatozoa.

Semen extension and cryopreservation

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

Extender = volume \times concentration \times motility \times 1 / required concentration of sperm ($50 \times 10^6/\text{ml}$)

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 ($\sim 190 \text{ mOsmol Kg}^{-1}$). 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 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 extender containing PVP 0.05mM (66.1 ± 7.51) and 0.1mM (59.4 ± 10.72) (Fig. 4).

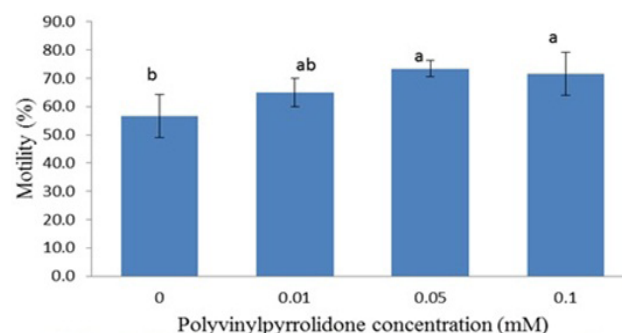


Fig. 1. Role of polyvinylpyrrolidone addition to extender on post thaw motility (Mean \pm SE) of Nili-Ravi buffalo bull (1) semen. Bars with dissimilar letters differ ($P < 0.05$) significantly.

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.

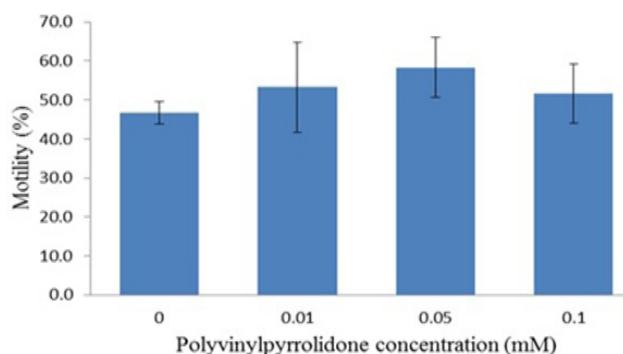


Fig. 2. Effect of polyvinylpyrrolidone addition in extended semen on post thaw motility (Mean \pm SE) of Nili-Ravi buffalo bull (2) semen. Bars did not differ significantly ($P>0.05$).

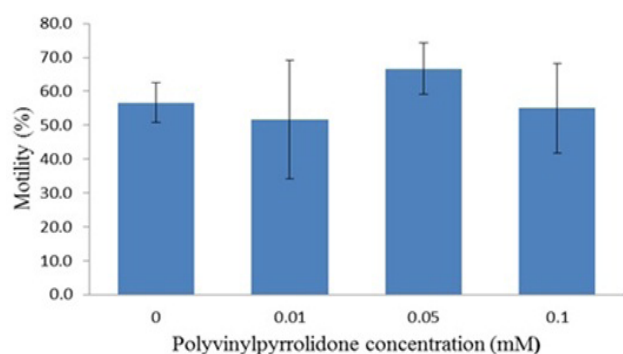


Fig. 3. Effect of polyvinylpyrrolidone addition in extended semen on post thaw motility (Mean \pm SE) of Nili-Ravi buffalo bull (3) semen. Bars did not differ significantly ($P>0.05$).

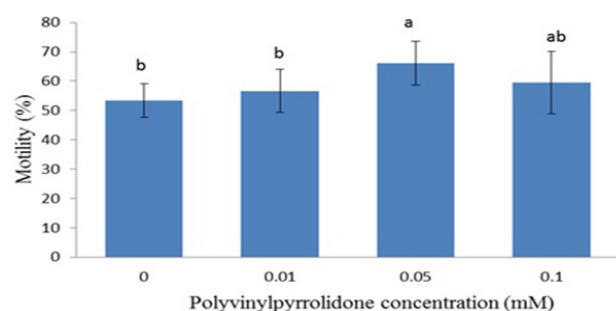


Fig. 4. Effect of polyvinylpyrrolidone addition in extended semen on post thaw motility (Mean \pm SE) of Nili-Ravi buffalo bull (n= 3) semen. Bars having dissimilar letters differ ($P<0.05$) significantly.

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 integrity of sperm plasma membrane was similar in extenders containing PVP at 0.01mM (74.0 \pm 1.8), 0.05mM (76.5 \pm 0.5) and 0.1mM (79.2 \pm 5.0) that was greater ($P<0.05$) compared to control (58.3 \pm 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 \pm 1.6), 0.05mM (66.7 \pm 7.0), 0.1mM (68.2 \pm 2.8) that was higher compared to control (55.0 \pm 1.8). In bull 3, sperm PMI was improved by supplementation of PVP at 0.01 (73.2 \pm 2.8), 0.05 mM (75.5 \pm 2.2) compared to control (61.7 \pm 2.3). However, it remained highest at 0.1 mM (79.7 \pm 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 \pm 6.5) compared to 0.01mM (70.3 \pm 5.67) and control (58.3 \pm 3.35). However, percentage PMI remained similar in extender containing PVP 0.05mM (72.9 \pm 5.39) and 0.1mM (75.7 \pm 6.5).

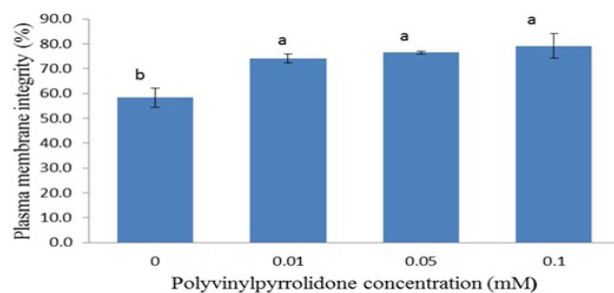


Fig. 5. Role of polyvinylpyrrolidone addition in extender on post thaw PMI (Mean \pm SE) of Nili-Ravi buffalo bull (1) semen. Bars with dissimilar letters differ ($P<0.05$) significantly.

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).

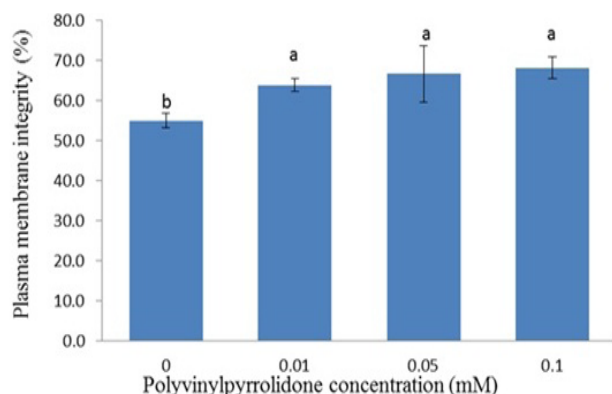


Fig. 6. Role of polyvinylpyrrolidone addition in extender on post thaw PMI (Mean \pm SE) of Nili-Ravi buffalo bull (2) semen. Bars with dissimilar letters differ ($P<0.05$) significantly.

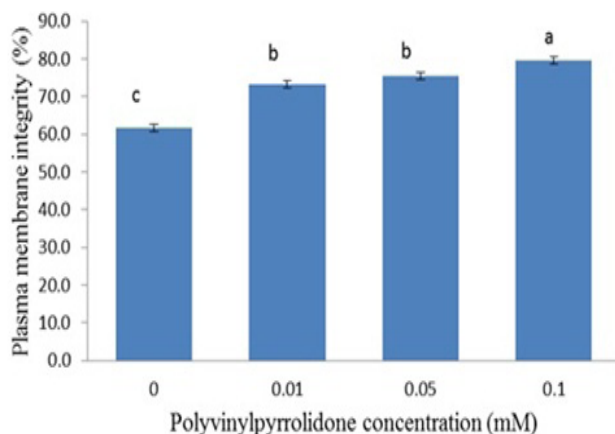


Fig. 7. Role of polyvinylpyrrolidone addition in extender on post thaw PMI (Mean \pm SE) of Nili-Ravi buffalo bull (3) semen. Bars with dissimilar letters differ ($P<0.05$) significantly.

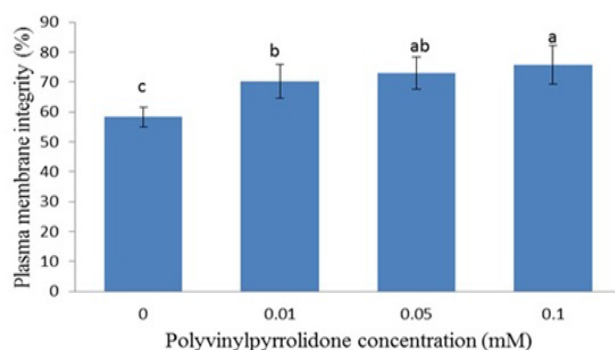


Fig. 8. Role of polyvinylpyrrolidone addition in extended semen on post thaw PMI (Mean \pm SE) of Nili-Ravi buffalo bull (n=3) spermatozoa. Bars with dissimilar letters differ ($P<0.05$) significantly.

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 ± 4.54), 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).

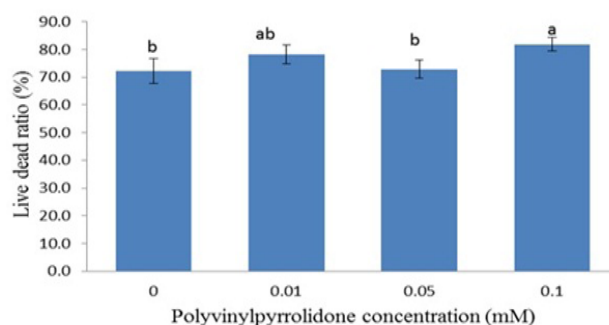


Fig. 9. Effect of polyvinylpyrrolidone addition in extender on post thaw sperm live dead ratio (Mean \pm SE) of Nili-Ravi buffalo bull (1) semen. Bars with dissimilar letters differ ($P<0.05$) significantly.

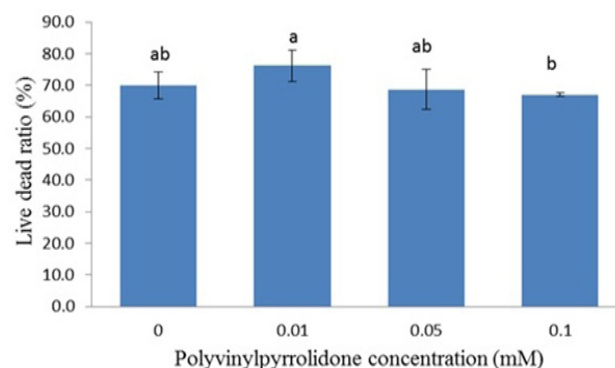


Fig. 10. Role of polyvinylpyrrolidone addition in extender on post thaw sperm live dead ratio (Mean \pm SE) of Nili-Ravi buffalo bull (2) semen. Bars with dissimilar letters differ ($P<0.05$) significantly.

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 0.1mM (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 0.01mM (78.1 ± 1.76), 0.05mM (74.0 ± 5.99), 0.1mM (73.2 ± 7.70) and control (69.6 ± 2.76). In this study live sperm percentage was not significantly improved by addition of PVP.

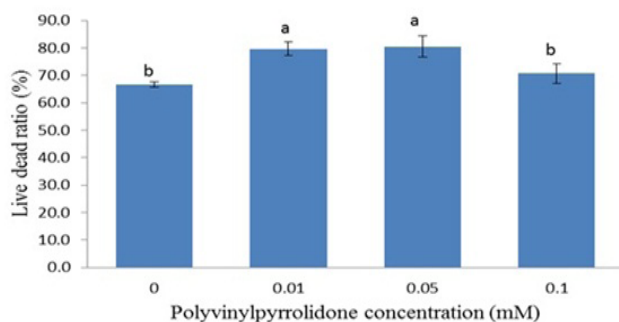


Fig. 11. Role of polyvinylpyrrolidone addition in extender on post thaw sperm live dead ratio (Mean±SE) of Nili-Ravi buffalo bull (3) semen. Bars with dissimilar letters differ ($P < 0.05$) significantly.

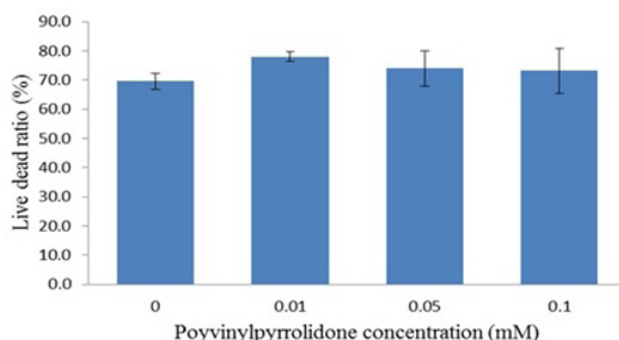


Fig. 12. Role of polyvinylpyrrolidone addition in extender on post thaw sperm live dead ratio (Mean±SE) of Nili-Ravi buffalo bull ($n=3$) semen.

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.

REFERENCES

- Andrabi, S.M., Ansari, M.S., Ullah, N. and Afzal, M., 2008. Effect of non-enzymatic antioxidants in extender on post-thaw quality of buffalo (*Bubalus bubalis*) bull spermatozoa. *Pakistan Vet. J.*, **28**: 159-162.
- Chan, P.J., Tredway, D.R., Corselli, J., Pang, S. and Su, B.C., 1991. Combined supravital staining and hypoosmotic swelling. *Hum. Reprod.*, **6**: 115-118. <https://doi.org/10.1093/oxfordjournals.humrep.a137495>
- Christensen, P., Stryhn, H. and Hansen, C., 2005. Discrepancies in the determination of sperm concentration using Burkert-Turk, Thoma and Makler counting chambers. *Theriogenology*, **63**: 992-1003. <https://doi.org/10.1016/j.theriogenology.2004.05.026>
- Das, D., Reddy, M., Upadhyaya, K. and Sopory, S., 2002. An efficient leaf-disc culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera* L.). *Pl. Cell Rep.*, **20**: 999-1005. <https://doi.org/10.1007/s00299-002-0441-4>
- Dozortsev, D., Rybouchkin, A., Sutter, P.D., Qian, C. and Dhont, M., 1995. Fertilization and early embryology: Human oocyte activation following intracytoplasmic injection: the role of the sperm cell. *Human Reprod.*, **10**: 403-407.
- Feichtinge, W., O.A. Bruca and M. Brunner. 1995. Sex chromosomal abnormalities and intracytoplasmic sperm injection. *Lancet*, **346**: 1566. [https://doi.org/10.1016/S0140-6736\(95\)92098-6](https://doi.org/10.1016/S0140-6736(95)92098-6)
- Foot, R.H., 1975. Estrus detection and estrus detection aids. *J. Dairy Sci.*, **58**: 248-220. [https://doi.org/10.3168/jds.S0022-0302\(75\)84555-3](https://doi.org/10.3168/jds.S0022-0302(75)84555-3)
- Graham, E.F. and Crabo, B.G., 1972. Some factors influencing the freezing of boar spermatozoa. *Anim. Reprod. Sci.*, **9**: 1627-1972.
- Holt, W.V. and Medrano, A., 1997. Assessment of boar sperm function in relation to freezing and storage. *J. Reprod. Fertil.*, **52**: 213-222.
- Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., Carbo, B.G. and Zaneveld, L.J.D., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J. Reprod. Fertil.*, **70**: 219-228. <https://doi.org/10.1530/jrf.0.0700219>

- Kumar, H.A. and Mahmood, S., 2001. The use of fast acting antioxidants for the reduction of cow placental retention and subsequent endometritis. *Indian J. Anim. Sci.*, **71**: 650-653.
- Leeuw, F.E., De Leeuw, A.M. and Den Daas, J.H., 1993. Effects of various cryoprotective agents and membrane-stabilizing compounds on bull sperm membrane integrity after cooling and freezing. *Cryobiology*, **30**: 32-44. <https://doi.org/10.1006/cryo.1993.1005>
- Kolm, M.K.G. and Aurich, J., 2005. Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5°C. *Theriogenology*, **63**: 1354-1365. <https://doi.org/10.1016/j.theriogenology.2004.07.005>
- Schmehl, M.K., Vazquez, I.A. and Graham, E.F., 1986. The effects of nonpenetrating cryoprotectants added to TEST-Yolk-glycerol extender on the post-thaw motility of ram spermatozoa. *Cryobiology*, **23**: 512-517. [https://doi.org/10.1016/0011-2240\(86\)90060-X](https://doi.org/10.1016/0011-2240(86)90060-X)
- Titterton, J.L. and Robinson, J., 1996. The protective action of polyvinylpyrrolidone-percoll during the cryopreservation of mouse 2-cell embryos and its effect on subsequent developmental potential post-thaw *in vitro* and *in vivo*. *Hum. Reprod.*, **11**: 2697-2702. <https://doi.org/10.1093/oxfordjournals.humrep.a019194>
- Tsai, M.Y., Huang, F.J., Kung, F.T., Lin, Y.C., Chang, S.Y., Wu, J.F. and Chang, H.W., 2000. Influence of polyvinylpyrrolidone on the outcome of intra cytoplasmic sperm injection. *J. Reprod. Med.*, **45**: 115-120.
- Vale, W.G., 1997. News on reproductive biotechnology in males. **1**: 103-123.
- Yinghui, D., 2008. Biological functions of antioxidants in plant transformation. *In Vitro Cell. Dev. Biol. Pl.*, **44**: 149-161. <https://doi.org/10.1007/s11627-008-9110-9>