EFFECT OF L-PENICILLAMINE, N-ACETYL CYSTEINE AND α-TOCOPHEROL ACETATE ON BUFFALO (BUBALUS BUBALIS) SPERM KINEMATICS IN VITRO

Sharanabasav Badami, Sudhir C. Roy*, Arindam Dhali

Molecular Biology Laboratory, ICAR-National Institute of Animal Nutrition and Physiology, Hosur Road, Adugodi, Bangalore-560030, India

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ABSTRACT

The post-thaw viability and motility of buffalo sperm are reduced significantly when they are cryopreserved. One of the etiologies of this reduced post-thaw viability and motility of buffalo sperm has been attributed to cryopreservation-associated generation of reactive oxygen species (ROS) in sperm. Hence, there is an urgent need to address this issue by incorporating some of the external additives which can reduce or scavenge the production of ROS. Sperm were separated from seminal plasma as it contains many identified and unidentified motility stimulating/inhibiting factors that may interfere in the interpretation of any action of external agent. Thus, in the present study, sperm were cultured in vitro in sp-TALP media, pH 7.4, at 38.5 °C for 1 hour under 5% CO2 in the absence and presence of L-penicillamine, n-acetyl cysteine and α-tocopherol acetate. The sperm kinematics was studied using computer-assisted semen analyzer (CASA). The results revealed that 0.25 mM L-penicillamine could increase the total motility, progressive motility, rapid motility, amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) of buffalo sperm as compared to the control group. Similarly, 600 µM α-tocopherol acetate could increase the total motility, rapid motility, curvilinear velocity, straight-line velocity, ALH and BCF of sperm. However, n-acetyl cysteine at the tested concentrations (0.125-1.0 mM) could not increase the sperm kinematics parameters. Thus, L-penicillamine and α-tocopherol acetate were found to be two promising additives for improving buffalo sperm motility. However, effect of pre-freeze addition of these additives to the semen extender on post-thaw motility of buffalo sperm warrants further investigation.

* Corresponding author
E-mail: scroy67@gmail.com; scroy67@yahoo.co.in (Dr. Sudhir C. Roy, Principal Scientist)

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1 Introduction

Reactive Oxygen Species (ROS), also referred to as free radicals, are formed as an essential byproduct of aerobic respiration in mitochondria and required to drive physiological inter and intracellular signaling. ROS are highly reactive species due to the presence of an unpaired electron in their outermost shell and depending on their concentration they can be beneficial or harmful to cells and tissues (Halliwell & Gutteridge, 1989). In mammalian spermatozoa, at physiological concentrations, ROS are produced normally and required to cater diverse physiological functions; however, when generated in excess, these species cause oxidative stress (Aitken & Curry, 2011). Mammalian spermatozoa are vulnerable to oxidative stress due to the presence of limited cytoplasmic antioxidant enzymes. Consequently, when the generation of ROS overwhelms the sperm’s antioxidant defense mechanism, it causes detrimental effects on the functional competence of sperm through the peroxidation of lipids (Alvarez et al., 1987; Alvarez & Storey 1992), DNA (Duru et al., 2000), and proteins (Alvarez et al., 1987; Aitken & Clarkson, 1988; de Lamirande & Gagnon, 1992) and affects the sperm motility. Hence, the above work indicated that antioxidant elements of sperm play a pivotal role in preserving both functional and structural integrity of spermatozoa.

Semen cryopreservation is an essential tool to preserve and propagate the elite germplasm in livestock. However, the process of cryopreservation has been reported to be associated with increased generation of ROS and induction of oxidative stress with the concomitant reduction in sperm viability, motility and fertilizing potential (Thomson et al., 2009). Further, during cryopreservation, a large amount of sperm cytoplasmic components including antioxidants are leaked out (Bilodeau et al., 2000; Kar et al., 2015). Thus, the sperm antioxidant capacity is not sufficient for long term storage and cryopreservation of semen. Hence, supplementation of any external antioxidants/additives that could reduce the impact of oxidative stress and thereby improve the post-thaw sperm viability and motility may be one of the solutions. Different agents having antioxidant property are being used in different species for improving the post-thaw sperm functions. Addition of L-penicillamine at 75 mM concentration increased the motility in vitro of ram sperm (El-Shahat et al., 2017). Similarly, addition of N-acetyl-L-cysteine at 15 mM increased significantly the progressive motility of chicken sperm during liquid storage at 5 °C (Partyka et al., 2015). Pre-freeze addition of Trolox, a vitamin E analogue, at 200 μM concentration increased significantly the post-thaw motility of rabbit spermatozoa (Zhu et al., 2015). However, the optimum concentration of the additive has to be determined for each species prior to incorporation in the freezing medium. Thus, the present study was undertaken to determine the optimum concentrations of three additives such as L-penicillamine, N-acetyl cysteine and α-tocopherol acetate. Further, seminal plasma is a mixture of varieties of biomolecules and other motility stimulating and inhibiting factors (de Lamirande et al., 1984; Robert & Gagnon, 1996; Juyna & Stelleta, 2012). It is possible that some of the factor of the seminal plasma may interact with the test agent and may interfere in our conclusion. Thus, to study the effect of a particular additive on sperm functions, the spermatozoa were separated from seminal plasma and cultured in the presence of the test additive in defined media.

The results of this study could provide definitive picture about the optimum concentration of each additive that could be non-lethal but motility enhancer, and can be incorporated into the freezing medium prior to the cryopreservation.

2 Materials and Methods

2.1 Animals

Four healthy Murrah buffalo (Bubalus bubalis) bulls, 3-5 years of age, weighing 450 to 740 kg, were maintained under uniform feeding and management regimen at Nandini Sperm Station, Hessarghatta, Bangalore. All the experimental protocols of the study were carried out following the Institutional Animal Ethics Committee guidelines.

2.2 Collection of neat semen and preparation of spermatozoa

Semen was collected twice a week from each bull using an artificial vagina (IMV Technologies, L’Aigle, France) maintained at 40 °C. In a day, two ejaculates were collected from each bull after an interval of approximately 30 minutes and both the ejaculates of the same bull were pooled and this constituted one semen sample. Freshly ejaculated semen samples were collected from buffalo and were assessed for their mass activities by light microscopy at 10 X magnifications. The semen samples having progressive motility ≥ 75% only were used in the study. Aliquots of the neat semen samples were collected in 15 mL centrifuge tubes and transported to the laboratory within 2 hours in thermosto flask maintained at 37 °C. The semen samples were diluted in 1:6 ratios with a modified Tyrode’s Hepes-buffered medium designated as sp-TALPH, pH 7.4 [composed of 100 mM NaCl, 3.1 mM KCl, 0.4 mM EDTA, 0.4 mM MgCl2 • 6 H2O; 0.3 mM NaH2PO4 • 2 H2O, 21.6 mM Na lactate, 2 mM CaCl2 • 2 H2O, 1 mM Na pyruvate, 40 mM Hepes, 10 mM NaHCO₃, and polyvinyl alcohol (PVA, 30-70 kDa;1mg/mL)] and subjected to two washes by centrifugation at 350 x g for 5 minutes at 25 °C in the same media. The final wash was carried out at 350 x g for 5 minutes at 25 °C to remove the sp-TALPH, pH 7.4 medium. The sperm pellets were resuspended in double the volume of sp-TALP, pH 7.4 [Modified Tyrode’s bicarbonate-buffered medium containing the same ingredients as stated above except it contained 10 mM...
Hepes instead of 40 mM, 25 mM NaHCO₃ instead of 10 mM and BSA (1 mg/mL) instead of PVA) and the sperm concentration was determined by haemocytometer. The concentration of sperm suspensions were adjusted to 80x10⁶ cells/mL with sp-TALP, pH 7.4.

2.3 Effect of various concentrations of additives on buffalo sperm kinematics in vitro

To study the effect of various concentrations of additives on buffalo sperm kinematics, buffalo sperm were cultured in vitro at a concentration of 40x10⁶ cells/mL in sp-TALP, pH 7.4 in the absence or presence of the various additives such as 0.25, 0.50 and 0.75 mM L-penicillamine, 0.125, 0.25 and 1.0 mM N-acetyl cysteine and 200, 400 and 600 µM α-tocopherol acetate at 38.5 ºC for 1 hour under 5% CO₂. The effect of each concentration of an additive was assessed by dispensing 250 µL aliquots of 80x10⁶ cells/mL sp-TALP, pH 7.4 to different 1.5 mL microcentrifuge tubes containing 250 µL of sp-TALP, pH 7.4 added with desired concentrations of additives to attain a final concentration of additives. These additives treated sperm suspensions were cultured in vitro in CO₂ incubator [NB-203, N-BIOTEK Inc. Lab System, South Korea] at 38.5 ºC under 5% CO₂ for 1 hour. After incubation, the sperm suspensions were diluted 5 times to attain final sperm concentration of 8x10⁶/mL with pre-warmed sp-TALP, pH 7.4 and immediately subjected for analysis of various sperm motility parameters such as total motility, progressive motility, rapid motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH) and beat/cross frequency (BCF) using computer-assisted semen analyzer (CASA, version 3.2.0; Microptic, Barcelona, Spain) according to the method of Dott & Foster (1979).

2.4 Statistical analysis

Data were analyzed by ANOVA using SPSS, version 10.0.1 software (SPSS Inc., Chicago, IL, USA). Data are presented as mean ± standard error of the mean (SEM). A difference with the value P < 0.05 was considered statistically significant.

3 Results and Discussion

Semen cryopreservation is an essential technique for propagation and storage of livestock semen. However, it is associated with low post-thaw motility and fertility of sperm leading to reduced conception rate. Several earlier reports have already established that the cryopreserved sperm undergo a significant degree of structural and functional changes due to damage and modification to its lipids, proteins and DNA (Watson, 2000; Andrabi, 2009; Thomson et al., 2009; Aitken, 2017).

Recently, these cryopreservation-associated biomolecular changes are being studied in human, boar, stallion, ram, canine and bull (Brouwers et al., 2005; Neild et al., 2005; Peris et al., 2007; Kim et al., 2010; Mostek et al., 2017). The principal causative factor for the above biomolecular changes have been attributed to the production of ROS during and after cryopreservation of semen. However, details of the above biomolecular changes in terms of lipid peroxidation (LPO), protein oxidation and nuclear damage are less studied for the semen of domestic animals including buffalo. Moreover, reports pertaining to any additives or additives that can reduce these biomolecular changes are also very limited (Partyka et al., 2015; Zhu et al., 2015; El-Shahat et al., 2017).

A mammalian spermatozoon demonstrates various types of motility parameters such as total motility, progressive motility, rapid motility, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH) and beat/cross frequency (BCF) under different physiological conditions (Mortimer & Mortimer, 1990). After ejaculation, a mammalian spermatozoon demonstrates progressive motility, which is characterized by highest VCL, VSL, VAP, ALH and high BCF (Cremades et al., 2005). After capacitation in the female reproductive tract or culture in vitro in a capacitating medium, a mammalian spermatozoon display hyperactive motility which is characterized by movement of tail in the form of figure of “8” with higher values of VCL and ALH together with moderate BCF (Muino et al., 2009).

In the present study, in 0.25 mM L-penicillamine treatment group, the total motility, progressive motility, rapid motility, amplitude of lateral head displacement and beat cross frequency of spermatozoa were increased (P=0.081, P=0.125, P=0.109, P=0.635 and P=0.431, respectively) as compared to the control group (without L-penicillamine treatment) (Table.1). There was a marginal decrease (P=0.199, P=0.151 and P=0.214) in VCL, VSL and VAP of spermatozoa in this treatment group as compared to control group (Table.1). In 0.50 mM and 0.75 mM L-penicillamine treatment group, the total motility, progressive motility, amplitude of lateral head displacement and beat cross frequency of spermatozoa were increased (P=0.071 & P=0.469; P=0.143 & P=0.175; P=0.184 & P=0.300; and P=0.317 & P=0.381, respectively) as compared to control group (H; Table.1). However, 0.50 and 0.75 mM L-penicillamine treatment groups tend to reduce sperm rapid motility, VCL, VSL and VAP (P=0.501 & P=0.754; P=0.103 & 0.091; P=0.108 & P=0.084 and P=0.102 & P=0.082) of spermatozoa as compared to control group spermatozoa (Table 1).

L-penicillamine, an amino acid derivative, also a metal chelating agent, when added in vitro at 0.25 mM concentration to the sperm suspension in sp-TALP, pH 7.4 media, could increase progressive motility and rapid motility. Thus, 0.25 mM L-penicillamine was found to be a promising semen extender additive for buffalo sperm. Earlier, L-penicillamine has been reported to increase
motility of equine, human and rat sperm (Aitken et al., 2012). L-penicillamine preserves the sperm motility by preventing the loss of critical sperm protein thiols from the mitochondrial ROS products (Aitken et al., 2012). Recently, L-penicillamine has been found to prevent ram sperm agglutination by reducing the disulphide bonds of a copper-binding protein present on the sperm plasma membrane (Leahy et al., 2016).

In 0.125 mM n-acetyl cysteine treatment group, the total motility, rapid motility, VCL, VAP, ALH and BCF of spermatozoa were increased but non-significant (P=0.323, P=0.827, P=0.740, P=0.848, P=0.182 and P=0.664, respectively, Table 2), whereas progressive motility and VSL of spermatozoa were decreased non-significantly (P=0.132 and P=0.576, respectively, Table 2) as compared to control group (without n-acetyl cysteine treatment).

In other groups such as 0.25 and 1.0 mM n-acetyl cysteine treatment groups, the VSL of spermatozoa was significantly decreased (P ≤ 0.05, Table 2), and there was a marginal decrease in progressive motility, VCL, VAP and BCF (P=0.145 & P=0.062; P=0.223 & P=0.392; P=0.165 & P=0.295; and P=0.262 & P=0.144, respectively, Table 2) as compared to the control group.

N-acetylcysteine, an another amino acid derivative and also a nucleophilic thiol, when added at different concentrations such as 0.125, 0.25 and 1 mM to buffalo sperm suspension cultured in vitro in sp-TALP, pH 7.4, could not improve any sperm motility parameters. However, in an earlier report, the same agent at 0.25 and 1.0 mM concentration could increase the progressive motility of human spermatozoa (Aitken et al., 2012). This may be attributed to the species-specific variation in sensitivity of a particular agent towards the sperm.

In 200 μM α-tocopherol acetate treatment group, progressive motility, VCL, VSL and VAP of spermatozoa was decreased non-significantly (P=0.149, P=0.434, P=0.263 and P=0.340,

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**Table 1 Motility parameters of buffalo sperm after treatment with L-penicillamine in sp-TALP, pH 7.4, at 38.5°C for 1 hour under 5% CO₂.**

| Parameter      | Control      | 0.25 mM L-penicillamine | 0.5 mM L-penicillamine | 0.75 mM L-penicillamine |
|----------------|--------------|-------------------------|------------------------|-------------------------|
| Total motility | 85.43 ± 0.42 | 90.97 ± 2.17            | 88.50 ± 0.67           | 87.67 ± 2.14            |
| Progressive motility | 76.20 ± 1.70 | 82.70 ± 0.05            | 78.23 ± 1.68           | 80.47 ± 2.54            |
| Rapid motility  | 78.13 ± 3.37 | 87.50 ± 0.12            | 74.47 ± 1.79           | 76.27 ± 1.84            |
| VCL            | 102.67 ± 4.09 | 97.10 ± 5.91           | 81.20 ± 11.38          | 79.93 ± 6.97            |
| VSL            | 89.37 ± 3.24 | 82.77 ± 5.22            | 67.00 ± 10.85          | 66.03 ± 6.90            |
| VAP            | 97.00 ± 4.26 | 90.73 ± 7.11            | 73.53 ± 12.23          | 71.43 ± 8.57            |
| ALH            | 1.87 ± 0.09  | 1.93 ± 0.15             | 1.93 ± 0.09            | 2.03 ± 0.15             |
| BCF            | 8.80 ± 0.35  | 9.33 ± 0.43             | 9.30 ± 0.50            | 9.70 ± 0.78             |

Data are mean±SEM of three replicates.

**Table 2 Motility parameters of buffalo sperm after treatment with n-acetyl cysteine in sp-TALP, pH 7.4, at 38.5°C for 1 hour under 5% CO₂.**

| Parameter      | Control      | 0.125 mM NAC | 0.25 mM NAC | 1 mM NAC   |
|----------------|--------------|--------------|-------------|------------|
| Total motility | 94.90±1.30   | 96.33±1.27   | 95.90±0.89  | 97.20±0.83 |
| Progressive motility | 85.25±1.45   | 80.08±2.76   | 78.35±4.16  | 79.00±1.20 |
| Rapid motility  | 93.50±1.30   | 94.00±1.65   | 93.03±1.81  | 94.05±1.43 |
| VCL            | 123.98±6.7   | 125.80±4.11  | 118.25±4.06 | 120.13±3.20|
| VSL            | 105.90±4.85a | 102.53±3.14a | 96.78±4.39a | 96.28±2.27a |
| VAP            | 118.38±5.6   | 119.33±3.92  | 113.10±4.34 | 113.95±2.92|
| ALH            | 1.93±0.08    | 2.03±0.03    | 1.88±0.08   | 1.95±0.06   |
| BCF            | 8.30±0.39    | 8.40±0.25    | 7.80±0.22   | 7.60±0.07   |

Data are mean±SEM of four replicates. Different letter above the figures indicate significant differences between the groups (P ≤ 0.05).
respectively, Table 3), with simultaneous marginal increase in total motility, rapid motility, ALH and BCF (P=0.438, P=0.435, P=0.638 and P=0.631, respectively, Table 3) of spermatozoa compared to the control group. In 400 µM α-tocopherol acetate treatment group, the spermatozoa exhibited a minor decrease in progressive motility, VCL, VSL, VAP, ALH and BCF (P=0.192, P=0.505, P=0.045, P=0.401, P=0.651 and P=0.625, respectively, Table 3), with simultaneous marginal increase in total motility and rapid motility (P=0.467 and P=0.755, respectively, Table 3) as compared to the control group. In 600 µM α-tocopherol acetate treatment group, the total motility, rapid motility, VCL, VSL, ALH and BCF of spermatozoa were increased (P=0.095, P=0.541, P=0.982, P=0.938, P=0.239 and P=0.238, respectively) as compared to spermatozoa of control group (Table 3).

α-tocopherol acetate is a small-molecule chain breaking antioxidant; this feature neutralizes lipid radicals and offers membrane protection. Addition in vitro of 600 µM concentration to the sperm suspension in sp-TALP, pH 7.4 media, increased the total motility, rapid motility, VCL, VSL, ALH and BCF of spermatozoa. Earlier studies in boar have demonstrated that α-tocopherol can prevent oxidative damage by breaking the ROS induced covalent links between the fatty acid side chains of membrane lipids and thereby improving post-thaw sperm viability and motility (Breininger et al., 2005; Jeong et al., 2009).

Conclusion

The present study demonstrated that in vitro treatment of buffalo spermatozoa in sp-TALP, pH 7.4 medium with 0.25 mM L-penicillamine, 600 µM α-tocopherol acetate improved the sperm kinematics as compared to the untreated control group. Hence, these two additives were found to be promising for incorporation in to the semen extender prior to the cryopreservation to improve post-thaw sperm motility; however, this can be confirmed by another set of experiments. N-acetyl cysteine could not improve sperm motility parameters. Thus, further investigation is required with other concentrations of n-acetyl cysteine.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this research paper.

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