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ANTI-OXIDATIVE EFFECT OF L-LYSINE ON POST THAW QUALITY OF NILI-RAVI BUFFALO BULL SEMEN (*BUBALUS BUBALIS*)

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ABSTRACT

This study was designed to investigate the effect of L-lysine on post-thaw quality of Nili-Ravi buffalo bull semen. Twenty eight ejaculates were collected from four Nili-Ravi buffalo bulls. Semen samples were pooled and split into five equal aliquots and diluted in Tris-citric acid extender containing different concentrations of L-lysine (0.25, 0.5, 1 and 2mM). As a control, semen was extended without addition of L-lysine. The semen samples were cryo-preserved in liquid nitrogen. Post-thaw semen assessment revealed that sperm motility, viability, plasma membrane integrity, acrosomal integrity, mitochondrial integrity and DNA integrity were significantly improved ($P \leq 0.05$) in 2mM L-lysine supplemented group as compared to control. For other groups supplemented with L-lysine; post-thaw motility, acrosomal integrity and mitochondrial integrity were also improved ($P \leq 0.05$) for 1mM treated group as compared to control. DNA integrity was improved significantly by all L-lysine treated groups as compared to control. Malondialdehyde concentration was higher ($P \leq 0.05$) in control group as compared to L-lysine treated groups. Significantly higher rate of catalase was recorded for 1mM and 2mM L-lysine treated groups as compared to control. In conclusion, the supplementation of 2mM L-lysine in semen extender can be used for cryopreservation of Nili-Ravi buffalo bull semen.

Keywords: Nili-Ravi, Semen, Cryopreservation, L-lysine, Antioxidant
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INTRODUCTION

Artificial insemination (AI) is the most extensive applied tool of reproductive biotechnologies for the rapid improvement in genetic potential of the livestock (Ciptadi *et al.,* 2014). Success of AI is linked with proper semen cryopreservation, heat detection and skills of the technician (Anzar and Graham, 1995). Cryopreservation is the most crucial part during semen processing technology. Cold shock, ice crystal formation, oxidative stress, lipid and protein oxidation are the major sequelae of the cryopreservation process (Vishwanath and Shannon, 2000). During the process of cryopreservation approximately 50% of viable spermatozoa are rendered immotile (Watson, 2000) .

Reactive oxygen species (ROS) are generated during cryopreservation process and deteriorates semen quality. Excessive generation of ROS reduce antioxidant enzyme activities, induce structural and biochemical alteration such as lipid peroxidation, depletion of ATP and DNA fragmentation and ultimately reduce motility, viability as well as fertility rate of the spermatozoa (Kim *et al.,* 2011).

Various efforts have been made to bridge this gap in various species and a number of cryo-protective and anti-oxidative properties of various amino acids, fatty acid, sugar and salt have been investigated in semen extender (Yildiz *et al.,* 2000; Gadea, 2003; Iqbal *et al.,* 2016 a, b) Amino acids are reputed well among antioxidants used in semen extender. Addition of cysteine in the semen extender of buffalo bull, improved post thaw semen quality, anti-oxidative enzyme activity and bull fertility (Iqbal *et al.* 2016 a). Similarly, supplementation of taurine, hypotaurine, proline, glutamine, glycine, histidine, and methionine in semen extenders also improved post thaw sperm quality, and reduced sperm damage and DNA fragmentation (Kutluyer *et al.,* 2016).

L-lysine is one of nine essential amino acids required for growth and tissue repair. The normal concentration of lysine in Human seminal plasma is 2.70µM/ml (Silvestroni *et al.,* 1979). The concentration of lysine in bovine rete testes fluid, epididymal plasma, seminal vesicle fluid and seminal plasma are 0·16±0·04, 0·38±0·06, 0.24±0.04 and 0·65±0·11mM, respectively (Brown-Woodman and White, 1974). The lysine contents of seminal plasma are 0.177µM/ml of Holstein bull (Al-Hakim *et al.,* 1970). Dietary supplementation of Lysine

to the animal feed improved semen quality and enhanced various anti oxidative enzymes. (Nizza *et al.,* 2010; Li *et al.,* 2014).The dietary use of lysine at the rate of 1.1g/kg body weight per day improved sperm concentration from 77.1 to 78.2% in the second ejaculate and decreased necessary interval between mating and ejaculation from 20.8 to 21.3sec and 22.6 to 23.1sec for 1st and 2nd ejaculates, respectively (Nizza *et al.,* 2000). L-arginine, L-lysine and L-histidine improved cryo-protection of buck spermatozoa obtained from epididymis by 0–5%(Kundu *et al.,* 2001). The addition of 3.25% (v/v) glycerol along with 0.5% (w/v) carboxylated poly L-Lysine (CPLL) in Japanese black bull semen improved sperm plasma membrane and fertility. The addition of CPLL in cryopreservation medium improves sperm cells survival due to its cryoprotective property and results in improved conception rates (Fujikawa *et al.,* 2017)

According to our knowledge, no work has been reported on addition of L-lysine in buffalo bull semen in terms of post-thaw quality. This study aimed to evaluate post-thaw semen quality parameters, antioxidant enzymatic activity and oxidative stress after addition of various concentrations of L-lysine in Nili-Ravi buffalo bull semen during cryopreservation.

MATERIALS AND METHODS

Experimental animals and Chemicals: Four mature Nili-Ravi buffalo bulls of 6 years age, housed at Semen Production Unit of Cattle Breeding and Dairy Farm Harich and, Charsadda Khyber Pakhtunkhwa (Latitude: 34°23'1.19"N and Longitude: 71° 48' 10.79" E) were used for semen collection. All the chemicals used were of Sigma– Aldrich elsewhere stated.

Semen collection, evaluation and cryopreservation: A total of 28 ejaculates from bulls (n=4) were collected (7 ejaculates per bull) by using pre-warmed artificial vagina (42°C). Semen was collected once a week with two consecutive ejaculates. First ejaculate was used for routine commercial purpose and second ejaculate was used for processing. Ejaculate with ≥ 65% visual motility and sperm concentration ≥ $500\times10^6$was selected for cryopreservation. Tris-citric acid-based extender was used for dilution and extension of semen. Semen samples were pooled and split into five aliquots of equal volume and finally diluted with different concentrations of L-lysine (0.25mM, 0.5 mM, 1.0mM, and 2.0mM). As a control, semen was extended without addition of L-lysine. Extended semen samples were cooled to 4°C in two hours and equilibrated at 4°C for 4 hours in a cold cabinet. Straws (0.5ml French) were filled and sealed at 4°C and exposed to liquid nitrogen vapors for 10 minutes before plunged in to liquid nitrogen (-196°C).

Post thaw semen evaluation: Post-thaw sperm quality parameters were evaluated at laboratories of the Department of Theriogenology and Department of Physiology, University of Veterinary and Animal Sciences Lahore.

Catalase and Lipid Peroxidation assessment: Spermatozoa were lysed by the sonication process (Akalin *et al.,* 2016) and samples were centrifuged (3000 g for 5 minutes) and 50µl of supernatant was aliquoted into two cuvettes for the evaluation of catalase activity as described previously with minor changes (Hadwan and Abed, 2016). The absorbance of sample and control was measured and catalase activity was calculated by given equation

$$\text{Semen catalase activity (KU)} = \frac{2.3}{t} * \{\log S°/S - M\} * VT/VS$$

Malondialdehyde concentration in frozen-thawed Nili-Ravi buffalo bull semen was assessed as described by Ahmed *et al*., (2018). Briefly semen was mixed with 8% (w/v) sodium dodecyl sulfate solution followed by addition of 20% (v/v) acetic acid. Later thiobarbituric acid solution and distilled water were added. Then, the whole solution was heated up to 95°C and left at room temperature to cool down and 5 ml butanol was supplemented and centrifuged at 4000 g for 15 minutes. Finally, supernatant layer of 100 µl was prudently removed from each treated and control tubes to measure the absorbance at 492 nm length using spectrophotometer (BMS; UV-2800; USA).

Post thaw motility, velocity distribution and CASA kinematics: Post-thaw sperm motility parameters, velocity distribution and kinematics were evaluated through computer-assisted sperm motion analysis (CASA, AndroVision system with Zeiss Axio Scopeoptic and automated scan stage Minitub, Germany; Frame Rate: 60HZ/sec, Number of Frames: 30, minimum cell contrast; 15, minimum cell size (pixel) 8).
Frozen thawed semen sample was added to pre-warmed (37 ^{0}C) 4 chamber slide (Leja products; Holland) and placed on automated scan stage and observed under 10 X at 37 ^{0}C.

Sperm Viability and Plasma Membrane Integrity (PI): Sperm viability was assessed by Propidium Iodide (PI) test as previously describe by (Aquila *et al.,* 2011) with minor changes. Two hundred sperm were counted from each sample for presence or absence of red fluorescence emitted by PI. Sperm emanated red fluorescence at head region under the fluorescence light were counted dead while sperm without fluorescence were counted as viable sperm.

Plasma membrane integrity was assessed using Hypo Osmotic Swilling test (HOST) describe by (Tartaglione and Ritta, 2004). One hundred spermatozoa per sample were counted. Spermatozoa with coiled tail were counted for the intact plasma membrane.

Sperm Acrosome Integrity, Sperm DNA Integrity and Mitochondrial Integrity: Assessment of post thaw semen for the acrosomal integrity was evaluated through Normal Apical Ridge (NAR) test. One hundred spermatozoa for each sample were counted under phase contrast microscope at 100X for normal apical ridge. Numbers of Spermatozoa with sharp crescent type appearance on apical ridge were counted as intact acrosomal membrane.

DNA integrity was assessed through Acridine Orange staining technique as described by (Mughal *et al.,* 2013). Spermatozoa with shining green at head region, enlisted for intact DNA, while shining yellow to red were counted as damage DNA.

For evaluation of mitochondria membrane potential, 50µl thawed semen sample was diluted with 50µl Tris citric acid fructose (TCF) and centrifuged (300 g) for 5 min. Supernatant was discarded and pellet was resuspended in TCF to make a total volume of 245µl, followed by adding 5µl Rhodamine (RH-123, Sigma Aldrich USA) and incubated at room temperature for 20 minutes. Finally, 5µl drop of sample was placed on glass slide, placed a cover slip and examined under fluorescence microscope (480/550 nm excitation/barrier filter CX41 Olympus, Japan). A total of 200 spermatozoa were viewed for each sample at different microscopic field, spermatozoa with green fluorescence at mid-piece region were considered to have intact mitochondria (Dodaran *et al.,* 2015).

Statistical Analysis: The present experiment was repeated seven times. Results were expressed as the mean ± standard error of means. Means were analyzed by one way analysis of variances (ANOVA) and differences among treatment groups P≤0.05 were established by post hoc Duncan's multiple range test using software Statistical Package for the Social Sciences (SPSS) (version 16.0).

RESULTS

The results of post-thawed sperm motility, viability, acrosomal and plasma membrane integrity, DNA damage and total abnormalities are presented in Table 1. Post-thaw semen assessment revealed that sperm motility (44.64±1.48%), viability (48.28±0.44%), plasma membrane integrity (47.28±0.64%), acrosomal integrity (50.71±2.12%), mitochondrial integrity (47.14±1.45%) and DNA integrity (98.00±O.78%) were significantly improved (P ≤ 0.05) in 2mM L-lysine supplemented group as compared to control. Post-thaw motility was also significantly improved (P ≤ 0.05) for 1mM L-lysine treated group as compared to control, whereas this improvement was non-significant(P > 0.05) for 0.25 and 0.5mM treated groups as compared to control. The percentage of mitochondrial integrity was higher (P ≤0.05) for 1mM treated group as compared to 0.25mM and control groups. However, the difference of mitochondrial integrity percentage between 0.25mM, 0.5mM and control was non-significant. Acrosomal integrity was also higher (P ≤ 0.05) for 1mM L-lysine treated group as compared to control. DNA integrity was improved significantly (P ≤ 0.05) by all L-lysine treated groups as compared to control. The difference for sperm abnormalities (%) for all groups was non-significant (P > 0.05).

The influence of L-lysine on post thaw enzymatic profile and lipid per oxidation is illustrated in Table 2. Malondialdehyde (MDA) concentration was significantly higher 6.76±0.38umol/l in control group as compared to L-lysine treated groups with minimum concentration3.77±0.60 µmol/l in 1mM treated group. The difference in MDA values among all L-lysine treated groups was non-significant. Significantly higher (P ≤ 0.05) rate of catalase (74.29±8.46 and 43.11±9.59Ku/l) were recorded for 1mM and 2mM L-lysine treated groups as compared to 0.25, 0.5mM and control groups. There was non-significant (P > 0.05) difference in catalase values among 0.25, 0.5mM treated L-lysine and control groups.

The influence of L-lysine in semen extender on post thaw CASA parameters of spermatozoa are illustrated in Table 3. Post-thaw semen analysis using Computer Assisted Semen Analyzer (CASA) revealed that total motility (49.30±1.01%), progressive motility (39.65±1.66%) and progressive fast motility (15.17±0.85%) and progressive slow motility (24.45±1.40%) were higher (P ≤ 0.05) for 2mM treated L-lysine group as compared control. Same trend was observed for 1mM L-Lysine treated group except progressive fast motility where the improvement was non-significant (P > 0.05) as compared to control group. There were non-significant (P > 0.05) differences for motility parameters among 0.25, 0.5mM L-lysine treated groups and control group.

The 2mM L-lysine treated group enhanced (P ≤ 0.05) average path velocity (34.30±1.24µm/s), straight linear velocity (27.57±1.24µm/s), curvilinear velocity (63.40±1.73µm/s), beat cross frequency (7.45±1.22Hz) and linear distance7.80±.94µm as compared to control group. Straight linear velocity, curvilinear velocity were also improved significantly (P ≤ 0.05) by 0.5 and 1mM L-lysine treated groups as compared to control. The difference in beat cross frequency and linear distance was non-significant (P > 0.05) among 0.25, 0.5, 1mM L-lysine treated and control groups. There were non-significant (P > 0.05) differences in amplitude of lateral head displacement, distance average path, linearity of forward progression and straightness values among all the treated groups as well as control (Table 4).

Table 1. Mean (±S.E.) percentages of motility, sperm viability, Plasma membrane integrity, mitochondrial integrity, acrosomal integrity, DNA integrity in and sperm abnormalities in Nili-Ravi Buffalo Bull semen frozen with different concentration of L. lysine. (n =4 bulls and n = 7 ejaculates / bull).

Parameters	L.lysine concentration				
	0 mM	0.25 mM	0.5 mM	1 mM	2 mM
Post thaw motility %	34.64±1.27 ^{c}	35.35±1.14^{c}	37.50±1.44^{bc}	40.35±1.38 ^{b}	44.64±1.48 ^{a}
Sperm viability%	41.28±1.86 ^{b}	41.57±1.42 ^{b}	43.50±1.16 ^{b}	43.50±1.85 ^{b}	48.28±0.44 ^{a}
Plasma membrane integrity%	40.50±1.17 ^{b}	41.64±1.80 ^{b}	43.21±0.95 ^{b}	44.00±1.38^{ab}	47.28±0.64 ^{a}
Mitochondrial integrity%	37.92±1.72 ^{b}	39.21±1.55^{b}	42.92±1.50^{ab}	44.78±2.11^{a}	47.14±1.45 ^{a}
Acrosomal integrity%	42.00±1.55 ^{c}	43.78±1.22^{bc}	45.21±1.96^{bc}	47.50±1.72 ^{ab}	50.71±2.12 ^{a}
DNA integrity%	92.64±1.85 ^{c}	95.57±1.09 ^{ab}	95.28±1.23^{ab}	97.42±O.44^{a}	98.00±O.78 ^{a}
Abnormalities%	9.14±0.85	9.00±01.30	8.71±1.32	8.57±1.13	8.71±0.96

$^{a-c}$: Denote differences (P≤0.05) within rows.
Data are mean±SE

Table 2. Effect of L-lysine on Post thaw enzymatic profile (Catalase) and lipid per oxidation in Nili-Ravi buffalo bull semen.

L.lysine concentration	0.0 mM	0.25 mM	0.5 Mm	1.0 mM	2.0 mM
MDA (µmol/l)	6.76±0.38 ^{a}	4.61±0.53^{b}	3.79±0.30 ^{b}	3.77±0.60 ^{b}	3.80±0.49 ^{b}
Catalase (Ku/l)	14.13±3.30^{c}	15.48±1.90^{c}	20.86±3.07^{c}	43.11±9.59^{b}	74.29±8.46 ^{a}

$^{a-c}$: Denote differences (P≤0.05) within rows.
Data are mean±SE (n =4 bulls and n = 7 ejaculates / bull).

Table 3. Effect of L-lysine in extender on post-thaw CASA motility parameters (Total motility, Progressive motility, Progressive fast motility and Progressive slow motility) of Nili-Ravi buffalo bull semen.

	Concentration of L-lysine (mM)				
Semen quality parameters	0	0.25	0.5	1	2
Total motility (%)	37.92±1.12c	38.28±2.39^{c}	41.06±1.32^{bc}	45.66±1.78^{ab}	49.30±1.01^{a}
Progressive motility (%)	29.92±1.73^{c}	29.01±2.6^{c}	30.91±1.67^{bc}	35.53±1.05^{ab}	39.65±1.66^{a}
Progressive fast motility (%)	10.99±1.23^{b}	11.01±0.75^{b}	14.61±1.46^{ab}	13.14±1.40^{ab}	15.17±0.85^{a}
Progressive slow motility (%)	17.26±1.19^{c}	17.91±2.48^{bc}	16.16±1.05^{c}	22.32±1.77^{ab}	24.45±1.40^{a}

$^{a-c}$: Denote differences (P≤0.05) within rows.
Data are mean±SE (n =4 bulls and n = 7 ejaculates / bull).

Table 4. Effect of L-lysine in extender on post-thaw CASA kinematics.

Semen quality parameters	Concentration of L.lysine (mM)				
	0	0.25	0.5	1	2
Average path velocity (µm s^{-1})	25.79±1.67^{c}	28.32±1.33^{bc}	30.06±.89^{bc}	30.70±.56^{b}	34.30±1.24^{a}
Straight linear velocity (µm s^{-1})	20.74±1.53 ^{c}	22.89±1.21^{bc}	24.55±.63^{ab}	24.74±.83^{ab}	27.57±1.24 ^{a}
Curvilinear velocity (µm s^{-1})	47.36±2.48 ^{c}	51.98±1.73^{bc}	55.61±2.77 ^{b}	53.51±1.45^{ab}	63.40±1.73 ^{a}
Beat cross frequency (Hz)	5.01±.61^{b}	6.32±1.95^{ab}	6.59±1.76^{ab}	5.90±.87^{ab}	7.45±1.22 ^{a}
Amplitude of lateral head displacement (µm)	0.68±0.02	0.59±0.03	0.63±0.02	0.69±0.02	0.62±0.05
Linear distance (µm)	5.51±24^{b}	6.45±.62^{ab}	6.60±.77^{ab}	6.47±.40^{ab}	7.80±.94^{a}
Distance average path (µm)	8.76±.34	8.46±.67	8.64±.71	8.32±.69	10.33±.86
LIN (VSL/VAP)	0.41±0.02	0.45±0.02	0.46±0.01	0.41±0.02	0.43±0.01
STR (VSL/VAP)	0.75±0.02	0.80±0.01	0.76±0.05	0.69±0.06	0.81±0.01

$^{a-c}$: Denote differences (P≤0.05) within rows.
Data are mean±SE (n =4 bulls and n = 7 ejaculates / bull).

DISCUSSION

This study is the first practical attempt for evaluation of role of amino acid L-lysine in improving post-thaw quality of Nili-Ravi buffalo bull semen. The results revealed significant ($P \leq 0.05$) improvement in post-thaw progressive motility of Nili-Ravi bull semen by supplementation of 1mM and 2mM L-lysine treated groups (table 3) as reported in other studies in which amino acids like taurine and Hypotaurine were used in sea bass (Martínez-Páramo *et al.,* 2013) and glutamine, proline and histidine in stallion semen extender (Trimeche *et al.,* 1999).

Damage to the sperm plasma membrane during the process of cryopreservation eventually a bridged the bull fertility (Andrabi *et al.,* 2008). Spermatozoa with intact plasma membrane are more capable to fertilize the oocyte. The findings of this study indicated that supplementation of 2mM L-lysine into Tris-citric acid-based egg yolk extender, significantly ($P \leq 0.05$) improved post-thaw number of viable spermatozoa with intact plasma membrane (table 1). The protective effect of L-Lysine remained unclear. However, this protective effect may be due to positive charge group present on amino acids such asproline, glutamine, glycine and L-lysine which offers the capability to form a protective layer over the sperm surface by binding to phosphate group of phospholipids in sperm plasma membrane (Kundu *et al.* 2001).

Acrosomal reaction plays a vital role in fertilizing capability of the spermatozoa. Successful acrosomal reaction is linked with presence of normal acrosome. In the present study 2mM concentration of L-Lysine added to semen extender, significantly ($P \leq 0.05$) improved acrosomal integrity (table 1). The protective effect of L-lysine on acrosomal integrity might be due to its ability as proteinase inhibitor as reported by (Sánchez *et al.,* 1995) that proteinase inhibitors N-L-p-tosyl-L-lysine-chloromethyl ketone and 4-nitrophenyl-4-guanidino benzoate reduced acrosomal damage.

Mitochondria generate ATPs for various functions of spermatozoa, most importantly for sperm motility. A negative correlation in between ROS and mitochondrial membrane activity is reported (Kadirvel *et al.,* 2009). Results of current study indicated that 2mM of Lysine added in semen extender significantly ($P \leq 0.05$) improved post thaw mitochondrial membrane integrity (table 1). The L-lysine enhances glutathione metabolism, resulting in increased glutathione concentrations, hence lowering reactive oxygen species and improves oxidant tolerance (Olin-Sandoval *et al.,* 2019).

Lipid per oxidation is accelerated with the overproduction of ROS, hence adversely affects the process of cryopreservation and finally reduced fertilizing capability of the spermatozoa (Soltanpour *et al.,* 2014).Level of lipid per oxidation is directly proportional to the concentration of Malondialdehyde. The result of this study revealed significant ($P \leq 0.05$) reduction in malondi aldehyde concentrations in all L-lysine treated groups as compared to control (table 2). This may be due to diffusion of lysine from extender to sperm cytoplasm, resulting in NADPH utilization for glutathione biosynthesis, decreased reactive oxygen species and improved oxidative tolerance (Olin-Sandoval *et al.* 2019). DNA integrity was found higher in 2mMgroup as compared to the control group (table 1). These results are in agreement with those of (Nizza *et al.* 2000) who reported that protamines like lysine, arginine and ornithine due to highly positive charge peptides result in DNA packing to high densities in sperm nuclei which ultimately helps in minimizing DNA damage by reactive oxygen species and mutagen.

The antioxidants enzymatic defense system of spermatozoa comprised of Superoxide dismutase(SOD), Glutathione peroxidase (GPx), Catalase and Glutathione enzymatic system (GSH) as reported by (Bucak *et al.,* 2010). In the present study, addition of L-lysine at 1mM and 2mM concentrations in cryopreservation extender significantly ($P \leq 0.05$) improved post thaw catalase profile as compared to the control group (Table 2) which may be due to increased glutathione contents (Ighodaro and Akinloye*,* 2018). Catalase enzyme play a vital role in spermatozoa antioxidant system by reducing oxidative stress through conversion of H_2O_2 in to H_2O and O_2 (Bansal and Bilaspuri*,* 2011).

The improvement in semen quality parameters of L-lysine treated groups in terms of sperm total motility, progressive motility, progressive fast motility, average path velocity, straight linear velocity and curvilinear velocity was observed (table 3 and 4).The improvement in these aforementioned parameters may be attributed towards anti-oxidative property of lysine (Li *et al.,* 2016)which ultimately results in decreased lipid per oxidation, lesser production of reactive oxygen species, increased glutathione peroxidase and catalase in L-lysine treated groups.

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