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REGULAR ARTICLES

Optimized addition of nitric oxide compounds in semen extender improves post-thaw seminal attributes of Murrah buffaloes

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Abstract

Semen dilution and cryopreservation alter the homogeneity of seminal plasma, resulting in a non-physiological redox milieu and consequently poor sperm functionality. Considering the concentration-specific bimodal action of nitric oxide (NO) in the regulation of sperm functions, cryopreservation media supplemented with optimized concentrations can improve the semen attributes. The present study aimed to evaluate the effect of adding an optimized concentration of sodium nitroprusside (SNP) and N-nitro-L-arginine methyl ester (L-NAME) in an extender on in vitro semen quality. An aliquot of semen samples ($n = 32$) from Murrah buffalo bulls ($n = 8$) was divided into control (C) and treatment (T-I: SNP in extender at 1 µmol/L; T-II: L-NAME in extender at 10 µmol/L). Fresh semen quality parameters showed no significant difference at 0 h except for the structural integrity in the T-II group. Post-thaw semen quality parameters and sperm kinematics using computer-aided sperm analysis (CASA) revealed significantly higher ($p < 0.05$) cryoresistance in the treatment groups. Viability, acrosome integrity, and membrane integrity were significantly higher ($p < 0.05$) in both treatment groups; however, the results were pervasive in T-II. Lower abnormal spermatozoa were observed in both T-I and T-II. SNP supplementation led to a significant rise ($p < 0.05$) in NO, whereas L-NAME reduced the NO concentration in post-thawed samples, which was directly correlated with different sperm functionality and associated biomarkers viz. total antioxidant capacity (TAC) and thiobarbituric acid reactive substance (TBARS). It was concluded that the cryopreservation media supplemented with SNP and L-NAME at 1 µmol/L and 10 µmol/L, respectively, lower the cryo-damage and improve post-thaw seminal attributes.

Keywords Semen · N-nitro-L-arginine methyl ester (L-NAME) · Sodium nitroprusside (SNP) · Cryo-injury

Introduction

Although buffalo has paramount importance as the chief dairy animal in several countries, their full production potential has not been exploited, due to low semen freezability, high susceptibility to cryopreservation-associated damages, and poor conception rates following artificial insemination (Andrabi 2009; Mughal et al. 2017). It is well established that only about half of spermatozoa survive through the cryopreservation process (Santos et al. 2019; Ugur et al. 2019; Upadhyay et al. 2021a), and cells that survive exhibit some degree of sub-lethal damage, which decreases their lifespan inside the female genital tract (Agarwal et al. 2014). The generation of reactive oxidants especially at the higher concentration before freezing and during freezing and thawing has been reported as a severe factor inflicting oxidative damage (Lone et al. 2016; Kumar et al. 2019) which affects the entire cellular functions and causes genome instability (Len et al. 2019). Reactive oxidants such as reactive oxygen species (ROS) and their subset reactive nitrogen species (RNS) are a double edge sword that performs a bimodal role depending upon their concentration (Nash et al. 2012; Upadhyay et al. 2022). RNS such as nitric oxide (NO) is a diffusible free radical synthesized via nitric oxide synthase from L-arginine (Hancock and Neill 2019). NO acts as an intracellular messenger and has been implicated in numerous physiological and pathological

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conditions (Pacher et al. 2007) and integrates with biological and physiological events prompting reproductive processes. At low/physiological concentrations, NO has been reported as a novel mediator of sperm functions (Kothari et al. 2010; Doshi et al. 2012; Ozer Kaya et al. 2020) through free radical scavenging, deactivating, and inhibiting the production of superoxide anions (O_2^-) (de Andrade et al. 2018; Upadhyay et al. 2022). Therefore, the maintenance of a suitable RNS environment is essential for adequate sperm functionality. The sodium nitroprusside (SNP) and N-nitro-L-arginine methyl ester (L-NAME) are L-arginine analogues that function as NO donor and inhibitor, respectively. There exists a dose- and time-dependent variation in sperm motility, viability, membrane integrity, acrosome integrity, and total abnormality as evidenced by various researchers in bovine spermatozoa after in vitro addition of SNP and L-NAME (Panth 2017; Naskar 2018; Upadhyay et al. 2021b). The SNP-treated semen samples (up to 100 nM) in humans and the Holstein bull (Khodaei et al. 2016) maintain motility, viability, and acrosome integrity of spermatozoa at pre- and post-thawing stages while at higher concentration reported to damage the spermatozoa through nitration and oxidation process. In cattle and buffalo, the addition of L-NAME to sperm capacitation media decreased sperm membrane integrity (Leal et al. 2009), inhibited premature capacitation (Rodriguez et al. 2005), and induced acrosome reaction 6 h after capacitation (Roy and Atreja 2008). The higher concentration of L-NAME (10 mM) significantly ($p < 0.05$) inhibited NO synthesis and functional seminal parameters, suggesting the positive correlation of NO and seminal attributes (Leal et al. 2009).

The common approach used during the last few decades to counter cryo-injury has been a defensive one, based on the use of reagents that contain antioxidant properties as well as cryoprotectants (Forouzanfar et al. 2010; Emamverdi et al. 2013) which do not address the source of the free radicals (Bisla et al. 2020). The use of oxidative cum nitrosative sub-lethal stress can serve to induce a temporary resistance to different future stresses (Sharafi et al. 2015) like freezing and thawing. We hypothesized that cryopreservation media supplemented with optimum levels of SNP and L-NAME lower the cryo-damage and improve post-thaw seminal attributes. Therefore, we aimed to investigate the role of NO donor and inhibitor compounds on Murrah bull sperm attributes and seminal biomarkers.

Materials and methods

Experimental animals and location of study

The study was conducted at the Artificial Breeding Research Centre, ICAR-National Dairy Research Institute, Karnal, during the winter and early spring seasons. The 8 experimental Murrah breeding bulls of 6–7 years of age, body weight between 450 and 550 kg, and body condition score of 5–6 out of 10 (classified as good) were selected. The bulls were maintained under a loose housing system and were clinically normal and sexually mature and maintained good libido. The bulls were fed according to ICAR 2013 standards with an average of 2.5 kg of concentrate feed (70% total digestible nutrients and 20% crude protein), dry fodder (wheat straw), and seasonal green fodders such as berseem, maize, and jowar on daily basis. Animals were offered ad libitum drinking water.

Semen collection

Single ejaculate per day was collected twice weekly over a month from different bulls (8 bulls × 4 ejaculates: $n = 32$) during the morning hours by artificial vagina technique (IMV Technologies, France). Immediately after collection, ejaculates were placed in a water bath (35 °C), and samples with ≥ 70% progressive motility, ≥ 2 mass motility, and ≥ 500 million/mL concentration were selected for further processing.

Nitric oxide additives

Sodium nitroprusside dihydrate (SIGMA-ALDRICH71778-25G; Bangalore, India) with a molecular weight of 297.95 g/mol and N-nitro-L-arginine methyl ester (CAYMAN chemical company, cat# 80,210) with a molecular weight of 235.228 g/mol were used for the study. SNP is a nitric oxide generator, which mimics the effect of L-arginine (Srivastava et al. 2006), while L-NAME is a specific inhibitor of constituent and inducible nitric oxide synthase (NOS) forms (Joly et al. 1994).

Dose optimization of SNP and L-NAME for modifying semen extender

For the optimization process, three different concentrations of SNP viz. 0.1 µmol/L, 1 µmol/L, and 10 µmol/L and L-NAME viz. 1 µmol/L, 10 µmol/L, and 100 µmol/L were prepared and subsequently added to the semen extender. The concentration that showed the highest progressive motility at all different time intervals, i.e., 1 µmol/L SNP and 10 µmol/L L-NAME, was selected for further experimentation (Fig. 1). The extender was supplemented with the optimized concentration of NO additives and was subsequently used for semen cryopreservation.

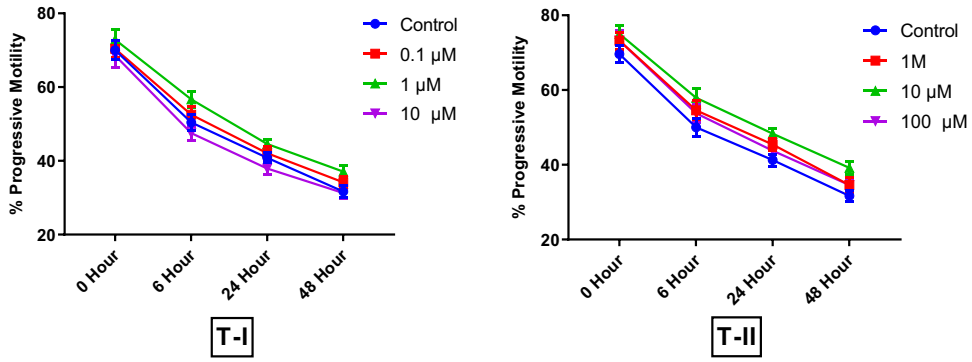

Fig.1 Time-dependent responses of different doses of SNP (T-I) and L-NAME (T-II) in extender on progressive motility of spermatozoa in comparison with control

Semen processing and freezing

An aliquot of semen samples was divided into control and treatment (T-I: sodium nitroprusside (SNP) at 1 µmol/L; T-II: N-nitro-L-arginine methyl ester (L-NAME) at 10 µmol/L in TRIS egg yolk extender) and semen samples further diluted with TRIS egg yolk extender (Table S1) to obtain a final concentration of 80 million spermatozoa/mL. After dilution and filling 20 million spermatozoa in French mini straws (0.25 mL), straws were equilibrated at 4 °C for 4 h in a cold cabinet. Subsequently, semen straws were exposed to liquid nitrogen (LN_2) vapor at a height of 4 cm above the level of LN_2 for 10 min and plunged the straws into LN_2 (− 196 °C).

Fresh and frozen-thawed semen evaluation

Semen volume was quantified using graduated tubes having 0.1-mL calibration. A photometer (IMV, L'Aigle, France) was used to determine spermatozoa concentration (million/mL) in the fresh semen. For mass activity (ranked 0 to 5), a drop of neat semen was placed on a clean grease-free glass slide mounted on a thermal stage maintained at 37 °C and examined directly under a phase-contrast microscope (Nikon Eclipse E600, Tokyo, Japan) at 10×. Individual motility of spermatozoa was recorded as a percentage of progressively motile spermatozoa in the extended semen at 200× objective after covering with a glass slip. To determine viability, acrosome integrity, membrane integrity, and total abnormality, 200 spermatozoa were examined in the smear. Sperm viability was assessed as per the method described by Blom (1950) and Hancock (1951). The same smear was used for enumerating sperm abnormalities. The functional membrane integrity of spermatozoa was evaluated by the hypo-osmotic swelling (HOS) test. The HOST was performed as previously described (Jeyendran et al. 1984) with slight modifications to assess the functional plasma membrane integrity of the spermatozoa. Acrosome integrity was assessed by the Giemsa staining technique as described by Hancock (1952). The motility and kinematic parameters were evaluated by a Hamilton Thorne Analyzer (HTM-IVOS, version IVOS 12.1, Beverly, USA). The total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head (ALH), beat cross frequency (BCF), straightness (STR), bent tail (BT), and coiled tail (CT) were recorded. In addition, the frozen-thawed straw samples were centrifuged at 10,000 g at 4 °C for 15 min in a cooling centrifuge. The diluted seminal plasma was stored at − 20 °C until analyzed for NO, TAC, and TBARS levels.

Nitrosative biomarkers (NO, TAC, and TBARS)

Nitric oxide was estimated in frozen-thawed samples using the "Bovine Nitric Oxide ELISA Kit" (Bioassay Technology, Cat. No E0249Bo) as per the manufacturer's instructions. Total antioxidant capacity was estimated using the "Bovine TAC-ELISA Kit" (Bioassay Technology, Cat. No E0384Bo). The optical density (OD) value of each well was determined using an ELISA reader set to 450 nm within 30 min after adding the stop solution, and concentration was analyzed using GraphPad Prism software. TBARS, an indicator of lipid peroxidation status, was estimated in the seminal plasma of frozen-thawed samples using QuantiChrom TMT-BARS Assay Kit (DTBA-100, Lot: BI04A18). The amount of malondialdehyde (MDA), as an index of lipid peroxidation, was measured with the thiobarbituric acid reaction. The diluted seminal plasma was deproteinized followed by standard preparation as per the manufacturer's instructions. Before the start of the assay, all reagents were equilibrated to room temperature. Briefly, 200 µL of each of the standard and the sample was pipetted followed by the addition of 200 µL TBA reagent to all the tubes. The contents were mixed thoroughly by vortexing the tubes and incubated at 100 °C for 60 min. After incubation, the tubes were cooled down to room temperature. The contents of the tubes (100 µL in duplicate) were vortexed, centrifuged, and loaded into the wells of the ELISA plate. Optical density was recorded at 535 nm using a Tecan® Nano Quant ELISA reader (Infinite M200 PRO, Bio Screen Instruments Pvt. Ltd.)

Statistical analysis

GraphPad Prism (version 7) and SPSS software were used for the statistical analysis of the data. Pairwise comparisons using the Bonferroni adjustment for multiple comparisons were used to evaluate semen attributes and sperm kinematics (before and after freezing) and levels of NO, TAC, and TBARS in control and treatment. The results are presented as the mean values ± SEM and considered statistically significant when $p < 0.05$. Pearson's correlation was used to correlate the seminal attributes with nitrosative biomarkers (NO, TAC, and TBARS). The semen parameter percent data were arcsine transformed before analysis.

Results

The progressive motility (%) improved significantly ($p < 0.05$) with the addition of 1 µmol/L SNP (T-I) and 10 µmol/L L-NAME (T-II) as compared to other concentrations at different time intervals (Fig. 1). Table 1 represents the effect of NO compounds on fresh and frozen seminal attributes between control and treatments. There was no significant difference in semen attributes of fresh semen between control and treatments (T-I and T-II), except the membrane and acrosome integrity of T-II samples; however, there was a significant ($p < 0.05$) difference in all seminal attributes of frozen-thawed semen (Table 1). The viability, acrosome integrity, and membrane integrity were significantly higher ($p < 0.05$) in both the treatment groups, though the results were more overt in T-II. Lower ($p < 0.05$) abnormal spermatozoa were observed in both T-I and T-II. The frozen-thawed sperm velocity and motility parameters between control and treatments are presented in Table 2. Post-thaw sperm kinematics has shown significantly higher ($p < 0.05$) cryoresistance in the treatment groups. BT and CT % in T-II were found significantly ($p < 0.05$) lower in comparison to the control, and the straight-line velocity of the T-II group was significantly ($p < 0.05$) higher than control and T-I.

In T-I, the NO levels (2.75 µmol/L) were significantly ($p < 0.05$) higher, TAC remained unaltered, and TBARS levels (0.53 µmol/L) were significantly ($p < 0.05$) reduced compared to control (Fig. 2). NO levels were positively correlated to progressive motility ($r = 0.40$, $p < 0.05$) and viability ($r = 0.36$, $p < 0.05$) in T-I (Table 3). TBARS impacted the motility and viability ($r = -0.059$, $p < 0.01$) and sperm abnormality ($r = 0.40$ $p < 0.05$) significantly. Other sperm attributes were not impacted by the addition of SNP in diluent (Table 3). In T-II, the NO levels (1.58 µmol/L) were significantly ($p < 0.05$) lower, and the TAC level (0.72U/mL) was significantly ($p < 0.05$) higher with no changes in TBARS levels compared to the control

Table 2 Effect of NO compounds on motility, velocity parameters, and microabnormalities measured by CASA in frozen-thawed semen of control and treatment (T-I and T-II)

CASA parameters	Control $n=32$	T-I $n=32$	T-II $n=32$
TM (%)	$49.7^{a} \pm 0.98$	$55.80^{b} \pm 1.29$	$55.90^{b} \pm 1.06$
PM (%)	$35.10^{a} \pm 0.85$	$40.02^{b} \pm 0.95$	$42.96^{b} \pm 0.75$
VAP (µm/s)	$103.38^{a} \pm 1.73$	$114.67^{b} \pm 2.51$	$117.83^{b} \pm 2.02$
VSL (µm/s)	$95.85^{a} \pm 1.55$	$104.76^{b} \pm 1.91$	$112.30^{c} \pm 1.82$
VCL (µm/s)	$171.32^{a} \pm 5.05$	$193.81^{b} \pm 5.13$	$193.31^{b} \pm 4.58$
ALH (µm)	$6.14^{a} \pm 0.20$	$7.13^{b} \pm 0.24$	$7.45^{b} \pm 0.18$
BCF (Hz)	$21.36^{a} \pm 0.76$	$26.46^{b} \pm 0.82$	$26.56^{b} \pm 0.79$
STR (%)	$77.48^{a} \pm 1.40$	$82.05^{b} \pm 1.23$	$83.38^{b} \pm 0.88$
Microabnormalities			
BT (%)	$3.83^{a} \pm 0.18$	$3.23^{ab} \pm 0.14$	$3.12^{b} \pm 0.15$
CT (%)	$0.62^{a} \pm 0.05$	$0.61^{ab} \pm 0.05$	$0.50^{b} \pm 0.03$

TM, total motility; *PM*, progressive motility; *VAP*, average path velocity; *VSL*, straight-line velocity; *VCL*, curvilinear velocity; *ALH*, amplitude of lateral head; *BCF*, beat cross frequency; *STR*, straightness; *BT*, bent tail; *CT*, coiled tail. Values with different superscripts a, b, and c within the same rows differ significantly ($p < 0.05$)

Table 1 Effect of NO compounds on seminal attributes in fresh and frozen-thawed semen of control and treatment (T-I: SNP in extender at 1 µmol/L; T-II: L-NAME in extender at 10 µmol/L)

Semen attributes		Control $n=32$	T-I $n=32$	T-II $n=32$
Viability (%)	Fresh	$90.56^{a} \pm 0.65$	$90.56^{a} \pm 1.19$	$92.69^{a} \pm 0.64$
	Frozen	$50.00^{xb} \pm 2.39$	$55.69^{yb} \pm 2.57$	$57.75^{yb} \pm 1.96$
Membrane integrity (%)	Fresh	$56.25^{xa} \pm 2.26$	$58.44^{xya} \pm 1.88$	$59.37^{ya} \pm 2.07$
	Frozen	$41.12^{xb} \pm 1.13$	$45.00^{yb} \pm 1.03$	$45.50^{yb} \pm 1.04$
Acrosome integrity (%)	Fresh	$83.69^{xa} \pm 1.34$	$85.19^{xa} \pm 1.62$	$87.75^{ya} \pm 1.15$
	Frozen	$56.63^{xb} \pm 2.16$	$61.19^{yb} \pm 1.60$	$62.88^{yb} \pm 1.46$
Total abnormality (%)	Fresh	$11.19^{a} \pm 0.48$	$11.19^{a} \pm 0.48$	$10.94^{a} \pm 0.45$
	Frozen	$18.38^{xb} \pm 0.62$	$16.69^{yb} \pm 0.60$	$16.38^{yb} \pm 0.63$

Values with different superscripts x and y within the same rows and a and b within the same columns differ significantly ($p < 0.05$)

(Fig. 2). The NO and TAC levels were positively correlated to progressive motility and viability (NO: $r=0.64$ and 0.54; TAC: $r=0.57$ and 0.52, respectively, $p<0.01$). The higher TAC level was positively correlated with AI ($r=0.39$, $p<0.05$). TBARS were negatively correlated to progressive motility and viability ($r=-0.61$ and -0.62, $p<0.01$) and to NO ($r=-0.48$, $p<0.01$). Total abnormality was negatively correlated to NO ($r=-0.56$, $p<0.01$) and TAC ($r=-0.46$, $p<0.01$); however, it was positively correlated ($r=0.59$, $p<0.01$) to TBARS (Table 4).

Discussion

The above results corroborate the findings of different researchers who resolved that the primary reason for reduced quality and altered levels of reactive oxidants in

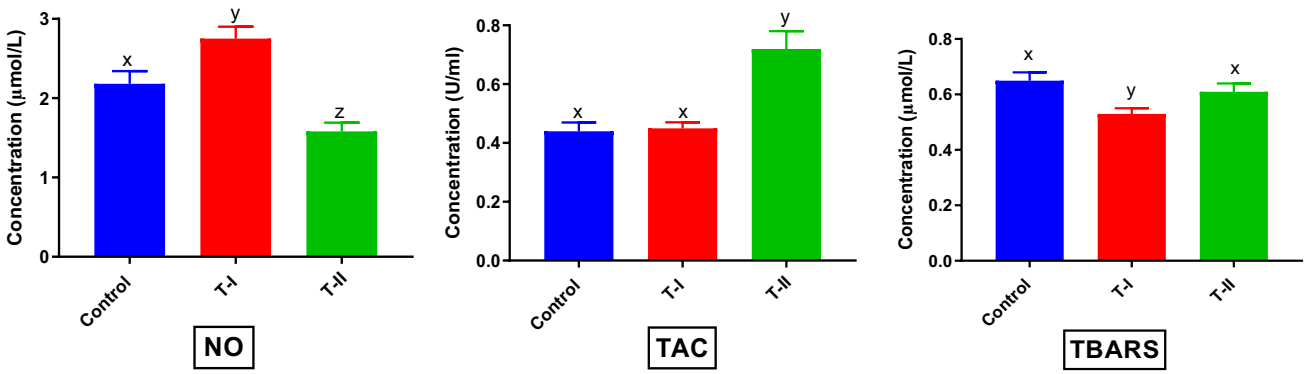

Fig. 2 Concentrations of nitrosative biomarkers (NO, TAC, and TBARS) in seminal plasma of frozen-thawed control and SNP (T-I)- and L-NAME (T-II)-treated semen. Values with different superscripts *x*, *y*, *z* differ significantly ($p<0.05$)

Table 3 Correlation between frozen-thawed seminal attributes and biomarkers in T-I (SNP in extender at 1 µmol/L) ($n=32$)

	PM	VIB	HOS	AI	AB	NO	TAC
VIB	0.94**						
HOS	0.04	0.11					
AI	0.16	0.19	0.37*				
AB	−0.76**	−0.69**	−0.11	−0.19			
NO	0.40*	0.36*	0.23	0.07	−0.12		
TAC	0.28	0.35	0.22	0.06	−0.22	0.33	
TBARS	−0.59**	−0.59**	−0.09	−0.13	0.40*	−0.11	−0.11

VIB, viability; *HOS*, hypo-osmotic swelling; *AI*, acrosome integrity; *AB*, abnormality; *NO*, nitric oxide; *TAC*, total antioxidant capacity; *TBARS*, thiobarituric acid reactive substance. **Correlation is significant at the .01 level (2 tailed). *Correlation is significant at the .05 level (2 tailed)

Table 4 Correlation between frozen-thawed seminal attributes and biomarkers in T-II (L-NAME in extender at 10 µmol/L) ($n=32$)

	PM	VIB	HOS	AI	AB	NO	TAC
VIB	0.95**						
HOS	0.61**	0.61**					
AI	0.46**	0.47**	0.19				
AB	−0.84**	−0.80**	−0.62**	−0.23			
NO	0.64**	0.54**	0.19	0.03	−0.56**		
TAC	0.57**	0.52**	0.34	0.39*	−0.46**	0.36*	
TBARS	−0.61**	−0.62**	−0.22	−0.11	0.59**	−0.48**	−0.13

VIB, viability; *HOS*, hypo-osmotic swelling; *AI*, acrosome integrity; *AB*, abnormality; *NO*, nitric oxide; *TAC*, total antioxidant capacity; *TBARS*, thiobarbituric acid reactive substance. **Correlation is significant at the .01 level (2 tailed).*Correlation is significant at the .05 level (2 tailed)

cryopreserved semen is the low anti-oxidative capacity of extended semen (Castro et al. 2016), the presence of dead and damaged spermatozoa (Martinez-Alborcia et al. 2012; Roca et al. 2013), and the absence of adequate nitric oxide release inflicting cell damage by other noxious free radicals. It was reported that the production of NO is compromised in egg yolk-containing extenders (Ortega Ferrusola et al. 2009). Other investigations in this direction equally reported the associated alterations (higher production and accumulation) in the level of reactive oxidants during the oxidative phosphorylation pathway for the generation of ATP (Gibb et al. 2014) and oxidative deamination of the aromatic amino acids of egg yolk by the sperm enzyme L-amino acid oxidase in bull spermatozoa (Aitken et al. 2015; Gibb et al. 2020). Since it is equally important to maintain an ideal level of reactive oxidants, particularly NO, and minimize the supra physiological level of noxious oxidants like superoxide and peroxynitrite anion, the addition of optimal concentration of NO donor and inhibitor compounds becomes inevitable. Moreover, the basis for the "failure" of antioxidant supplementation in lowering the cryo-damage is that ROS and RNS in small, controlled, physiological concentrations are essential for normal sperm functioning (Buzadzic et al. 2015) which was in agreement with the present study where supplementation of SNP or L-NAME in semen extender minimized the nitrosative/oxidative stress and improved post-thaw functionality of buffalo spermatozoa.

The addition of L-NAME at a concentration of 10 µmol/L resulted in the modulation of seminal plasma oxidant levels and concomitant reduction in nitrosative stress inflicting cryo-damage. In the present study, SNP addition in extender increased nitric oxide levels which are in agreement with the previous report on L-arginine addition (Ozer Kaya et al. 2020), a nitric oxide donor compound. The semen quality attributes were affected by SNP and L-NAME addition in different doses in a time-dependent manner, suggesting that optimized addition of these compounds (1 µmol/L SNP and 10 µmol/L L-NAME) had a positive effect. Significantly less reduction in motility was observed with the addition of 1 µmol/L of SNP and 10 µmol/L of L-NAME while at higher concentrations; there was a reduction in sperm motility which agreed with the in vitro findings of Panth (2017) in crossbreed and Naskar (2018) in buffalo. The other sperm kinematics parameters like VCL, VAP, and LIN which were reportedly related to fertility were higher in the treatment groups, suggesting the attuning role of NO compounds. Moreover, VSL which is an indicator of sperm flagellar motion and thrust has been positively correlated with fertilization rates and was significantly higher in both treatment groups. The results of the present investigation are in agreement with Balercia et al. (2004), who reported that NO at low concentrations (< 1 µmol/L) increased sperm motility and higher concentrations (> 1 µmol/L) decreased sperm motility in a dose-dependent manner in humans. Khodaei et al. (2016) also found that a concentration of 0.1 µmol/L or above increased progressive motility significantly in comparison to a low concentration in Holstein bulls. Rahman et al. (2014) reported that a higher dose of SNP decreased sperm motility and caused hyperactivation of mice spermatozoa; particularly at the highest concentration of 100 µmol/L. The NO-associated increased motility might be due to increased phosphorylation of flagella proteins after the exogenous addition of NO compounds (Thundathil et al. 2003; Harrison 2004). The NO causes an increase in motility with stimulation of cGMP synthesis and an increment in Ca^{2+} levels in the mitochondria, generating a higher ATP level (Srivastava et al. 2006; Miraglia et al. 2011). In contrast, Francavilla et al. (2000) and Rodriguez et al. (2005) did not observe any change in seminal attributes of human and bull semen respectively after adding varying concentrations of L-NAME.

Sperm viability was improved significantly by the addition of SNP and L-NAME in cryopreservation media. In humans, a low concentration of NO improved post-thaw sperm viability (Rosselli et al., 1995; Balercia et al. 2004). Stochmal (2000) reported that exogenous NO released by donors, such as SNP, is known to play an important role in post-thaw sperm viability. At the concentration of 10, 50, and 100 nmol/mL SNP, the viability of spermatozoa significantly increased after 1, 2, and 3 h post-thawing, but there was no significant difference evidenced before freezing and immediately after thawing (Khodaei et al., 2016). In post-thaw buffalo semen, Naskar (2018) observed that the percent viability increased significantly ($p < 0.05$) at 1 µmol/L SNP concentration within 15 min of incubation followed by a modest decline. However, few researchers have cited that SNP decreased sperm viability at higher concentrations of 10^{-4} M (Tomlinson et al. 1992) and 0.25–2.5 mM (Bolaños et al. 2008). A similar finding was made by Bahmanzadeh et al. (2008), indicating improved live sperm count and reduced morphological defects in L-NAME, which was in agreement with present findings where significantly less BT and CT sperms were observed in T-II. Khodaei et al. (2016) reported that different concentrations of SNP (10, 50, and 100 nmol/mL) reduced the membrane lipid peroxidation level of sperm and increased acrosome integrity in a time- and dose-dependent manner, especially at 50 and 100 nmol/mL concentrations. In contrast to our results, Rosselli et al. (1995) found a decrease in sperm membrane integrity after the addition of NO inhibitors in human semen. Ambrosini et al. (2006) and Vidya et al. (2011) reported a negative correlation between NO concentrations and sperm morphology. Ramya et al. (2011) and Wu et al. (2004) also reported similar results that higher spermatozoa abnormalities are associated with a high level of NO. However, Miraglia et al. (2007) could not find any significant difference between

NO production and sperm morphology since no association was found between poor semen quality and elevated levels of NO. The present research reported that in comparison to fresh semen, post-thaw samples have 58% more abnormal spermatozoa in the control group compared to < 50% in treatment groups. We can hypothesize imbalances in nitrogen-free radicals inflicted injuries to the spermatozoal membrane, resulting in a decrease in sperm motility and viability. In the present study, lower deteriorations observed in seminal attributes might be due to the scavenging property of nitric oxide after SNP supplementation, which protects against oxidative damage, which in turn elongates sperm life and decreases sperm abnormalities during storage (Al-Ebady 2012). Some researchers have reported that NO is strongly associated with the functional status of mitochondria by regulating its biogenesis, remodeling, respiration, and energy production (Vucetic et al. 2011; Buzadzic et al. 2015) implicating its role in sperm function (Otasevic et al. 2013). NO has a bimodal role, and in the present study, L-NAME was also observed to potentiate seminal parameters indicating its physiological role against peroxidative damage which arises during nitrosative stress. L-NAME being the NOS inhibitor may be involved in potentiating the seminal attributes by preventing the excess formation of NO and consequent reaction with superoxide-free radical forming toxic peroxynitrite anion (Upadhyay et al. 2022).

SNP supplementation led to a significant rise in NO, whereas L-NAME supplementation reduced the NO concentration of seminal plasma over the control. TBARS exhibited a decline in SNP-supplemented media. Since sperm cell membranes are specifically rich in PUFA, these cells are quite susceptible to the action of RNS (Semenova et al. 2005; Makker et al. 2009). However, in consonance with our findings, Al-Ebady (2012) reported that arginine at low concentration through increasing nitric oxide production acts as an antioxidant and protects spermatozoa against lipid peroxidation thus holds the same for the similar compound like SNP. However, the addition of L-NAME indicates its physiological role against peroxidative damage which arises during nitrosative stress, through the enhanced antioxidant status of seminal plasma which positively impacted several sperm functions. Thus, the NOS/NO pathway had an essential antioxidant action that protected cells from lipid peroxidation, by free radical damage to cell membranes (Srivastava et al. 2006).

It may be concluded that SNP, a nitric oxide generator, and L-NAME, a NO/NOS inhibitor, might reduce the freezing associated damages of sperms in a dose-dependent manner by modulating the NO production. The addition of both SNP and L-NAME in cryopreservation media at an optimized dose of 1 µmol/L and 10 µmol/L concentrations, respectively, improved seminal attributes significantly with L-NAME, giving better results over SNP, both before and after freezing. The effects of L-NAME were better pronounced by limiting the excess formation of NO which may culminate in the production of noxious peroxynitrite anions and by enhancing the anti-oxidative capacity of semen. These experiments also suggest that SNP provided long-term protection against membrane peroxidation through reduced production of TBARS and by maintaining the anti-oxidant milieu. Thus, fortification of these compounds in the extender before freezing not only lowers cryo-injury but can also be easily adopted during processing and packaging. However, there is a need to validate the results with field fertility trials to facilitate their extensive use in semen processing. From a future perspective, these results could contribute to the design of a new protocol that would help to enhance sperm quality.

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Data availability Data will be made available on reasonable request.

Code availability Not applicable.

Declarations

Ethics approval The animal experiments performed were acceptable to the ethical standards of the National Dairy Research Institute, Karnal, India (44-IAEC-19–10).

Consent to participate Informed consent was obtained from all individual participants included in the study.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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