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Deciphering dose and time dependent effect of supplementing SNP and L-NAME in extender on progressive motility of Murrah bull spermatozoa

V R UPADHYAY[1]✉, A K ROY[1], SUJATA PANDITA[1], DIANA KSHETRIMAYUM[1], PRIYANKA PATOLIYA[1], KATHAN RAVAL[1], RAJU KR DEWRY[1], SARITA[1] and SAPNA NATH[1]

ICAR-National Dairy Research Institute, Karnal, Haryana 132 001 India

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ABSTRACT

Male germ cells have the potential to generate reactive oxygen species (ROS) during different stages of differentiation, laboratory manipulation and during their interaction with dissolved oxygen in extender. These ROS and its subset reactive nitrogen species (RNS) perform a concentration specific bimodal role, favouring oxidative eustress at physiological concentrations. The present study was aimed to investigate the dose and time dependent effect of the most potent RNS, i.e. nitric oxide (NO) compounds, specifically SNP (Sodium nitroprusside) and L-NAME (N-Nitro-L-Arginine Methyl Ester) supplementation in extender on progressive motility. The semen samples were divided into 8 aliquots comprising of various concentrations of SNP (Control, 0.1, 1 and 10 µmol/L) and L-NAME (Control, 1, 10 and 100 µmol/L) and refrigerated for 0, 6, 24 and 48 h. The progressive motility (%) improved significantly particularly with the addition of 1 µM SNP and 10 µM L-NAME as compared to control during all time intervals. Subsequently semen sample was cryopreserved with optimum level of SNP (1 µmol/L) and L-NAME (10 µmol/L) from these results by adding it in extender and assessed for progressive motility before cryopreservation and after 24 h, 1 month and 2 months of cryopreservation. It is concluded that the addition of SNP and L NAME in cryopreservation media at 1 µM and 10 µM respectively improved the sperm motility significantly. On further evaluation a significant increase in progressive motility was observed for L-NAME treated extender at 24 h and SNP treated extender at 1 and 2 month interval in comparison to control.

Keywords: Cryopreservation, Extender, NO, Progressive motility, RNS, ROS

Spermatogenesis is a highly sophisticated and complex process in which functional spermatozoa are generated from differentiation and transformation of several types of germ cells (Lee and Cheng 2009). Thus to respond the whole process of differentiation, transformation and maturation there are involvement of intrinsic signaling mechanisms and profound changes in gene expression to modulate and regulate this development. Therefore to harness the above mentioned attributes there is an acquisition of free radicals to fulfill a wide range of essential seminal quality parameters (Dowling and Simmons 2008). ROS and RNS comprise a class of radical and non-radical oxygen and nitrogen derivatives that play a significant role in various reproductive processes (Doshi *et al.* 2012). RNS include peroxynitrite anion, nitroxyl ion, nitrosyl containing compounds and nitric oxide. RNS particularly NO, depending on magnitude and duration of synthesis can have both beneficial and detrimental effects on spermatozoa (Upadhyay *et al*. 2021a). Thus, a balance between the amount of RNS produced and scavenged will determine whether the motility will be promoted or downgraded. Out of the above mentioned RNS, NO is found to be very potent and being non-polar molecule can diffuse easily through cellular membranes (Dixit and Parvizi 2001). NO can affect the functionality of spermatozoa through signaling and free radical scavenging activity (de Andrade *et al.* 2018). These functions are highly dependent on the concentration of NO which is produced from L-arginine via Nitric Oxide Synthase (NOS) in the male reproductive system and accordingly in extender during the cryopreservation. SNP (Sodium Nitroprusside) and L-NAME (N-Nitro-L-Arginine Methyl Ester) are L-arginine analogue functions as NO donor and inhibitor respectively, serving as a potential regulator of numerous sperm functions. Incubation of semen with NO compounds, increases intracellular cyclic guanosine monophosphate (cGMP) levels of the spermatozoa of bulls (Zamir *et al.* 1995) and improves its functional attributes (Mohammed *et al.* 2020). The positive role played by NO in spermatozoa worth leads us to speculate that this paradoxical involvement in both pathological and physiological processes depends on the alternative redox state and relative concentration of NO (Balercia *et al.* 2004).

The supra-physiological concentration of these reactive species causes oxidative stress and may add to the

Present address: [1]ICAR-National Dairy Research Institute, Karnal, Haryana. ✉Corresponding author email: vishwaranjanhzb@gmail.com

breakdown of lipids and proteins, mitochondrial dysfunction and DNA fragmentation (Fraser *et al.* 2010, Cottet-Rousselle *et al.* 2011, Upadhyay *et al*. 2021b) which is ultimately linked to reduced sperm motility. Therefore, it is imperative that novel strategies should be developed to assess and treat oxidative stress. The motility evaluation is the first step being the most desirable spermatozoa characteristic to decide the fate of the sample for further processing (Hidalgo *et al.* 2006). The motility is the most certain way of determining the viability and it is also found to be the main limiting attribute which decides whether the spermatozoa reach the utero-tubal junction (Srivastava and Pande 2017). The motility of sperm is affected more abruptly after freezing (Raad *et al*. 2018) particularly when exposed to uneven concentration of free radicals in the semen extender during cryopreservation and storage period. While many studies have largely focussed on one aspect of oxidative stress and ROS, the purpose of this paper is to assess the optimum dose of NO compounds to be added in extender in order to acquire a deeper insight about the role of RNS and its correlation in the pathophysiology of sperm motility. Keeping these points in view the first part of study investigates the impact of bimodal action of nitric oxide on fresh and refrigerated semen while second part deals with its impact on the cryopreserved semen.

MATERIALS AND METHODS

The present research was carried out at Artificial Breeding Research Centre and Animal Physiology Division, ICAR-National Dairy Research Institute, Karnal. The animal experiments performed were acceptable to the ethical standards of the institute; vide order number 44-IAEC-19-10. The investigation was conducted on 8 Murrah breeding bulls maintained in good libido, healthy, sexually mature and clinically normal state. The semen was collected twice a week from each bull using Artificial Vagina method (IMV Technologies, France) with standard semen collection procedure. The samples were immediately classified on the basis of their volume, colour, mass-activity, individual motility and sperm concentration after the collection. Semen samples with mass motility ≥ 2+ and individual progressive motility ≥70% were selected for further experimentation. The progressive motility was recorded as a percentage of progressively motile spermatozoa after the extension of semen with TRIS-egg yolk extender which was always prepared fresh, generally an hour before collection of semen so that the medium gets stabilized. The motility was assessed by placing a drop of diluted semen (diluted with Tris-egg yolk extender) on a clean, grease-free glass slide mounted on a stage maintained at 37°C and observed under 20X objective after covering with a coverslip. The percentage of progressively motile sperm was estimated by observing five representative areas of the slide. The chemical compounds used were sodium nitroprusside dihydrate (SNP) having molecular weight of 297.95 g/mol and N-Nitro-L-Arginine Methyl Ester (L-NAME) having molecular weight of 235.228 g/mol. From these compounds three different concentrations of SNP, viz. 0.1 µmol/L, 1 µmol/L and 10 µmol/L and three different concentrations of L-NAME, viz. 1 µmol/L, 10 µmol/L and 100 µmol/L were prepared. Afterwards, a standardization procedure was followed, for optimizing the best concentration of SNP and L-NAME to be used for modifying semen extender. The semen samples were divided into 8 aliquots containing various concentrations of SNP (0, 0.1, 1 and 10 µmol/L) and L-NAME (0, 1, 10 and 100 µmol/L). These aliquots were cooled at 4°C and evaluated after 0, 6, 24 and 48 h.

The concentration giving highest motility was selected for further modification of the extender during cryopreservation. Again from the same 8 Murrah bulls, semen samples were collected at fortnightly intervals for 2 months and assessed for ejaculate volume, sperm concentration, mass motility and progressive motility. The semen samples were cryopreserved with the optimized concentration of SNP and L-NAME from the above results by adding it into the extender and assessed for quality before cryopreservation and at 24 h, 1 month and 2 months after cryopreservation.

The variations in motility at different treatment and time interval were quantified using both one-way and two-way ANOVA using SPSS software for statistical analysis. The P value ≤0.05 was considered statistically significant. Results were expressed as mean ± SE.

RESULTS AND DISCUSSION

The magnitude and duration of NO synthesis along with the redox state and composition of extender are critical determinant of physiological action of RNS. With this in observance, the first step followed in this research was to optimize the best possible concentration of SNP and L-NAME that has positive impact on motility of spermatozoa. Prior to this, routine evaluation of samples were done immediately after collection to ensure the minimum requirement for fertilization and further experimentation which is mentioned in Table 1. For optimization of best possible concentration, semen samples were divided into 8 aliquots containing various concentrations of SNP (Control, 0.1, 1 and 10 µmol/L) and L-NAME (Control, 1, 10 and 100 µmol/L). These aliquots were cooled at 4°C and evaluated at 0, 6, 24 and 48 h for motility which are presented in Table 2 and 3. It was observed that progressive motility was affected by SNP and L-NAME in both dose and time dependent manner. Per cent Motility improved significantly (P<0.05) with the addition of 1 µM SNP and

Table 1. Seminal attributes (Mean ±SE) immediately after collection

Seminal parameter	Mean±SE
Volume (ml)	4.03±0.28
Colour	Creamy white to yellow
Mass activity (0–5 scale)	2.53±0.11
Initial progressive motility (%)	73.12±1.57
Sperm concentration (10^6/ml)	1052.69±87.5

Table 2. Time and dose dependent effects of SNP on % progressive motility of spermatozoa

Treatment (SNP)	Control	0.1 µM	1 µM	10 µM
0 Hour	$70.00^{wa}\pm$ 2.53	$70.36^{wa}\pm$ 2.28	$72.86^{wa}\pm$ 2.71	$68.57^{wa}\pm$ 3.13
6 Hour	$50.42^{xa}\pm$ 2.08	$52.50^{xab}\pm$ 2.18	$56.67^{xb}\pm$ 2.07	$47.50^{xa}\pm$ 1.90
24 Hour	$40.83^{yab}\pm$ 1.35	$42.08^{yab}\pm$ 1.68	$44.58^{yb}\pm$ 1.30	$37.92^{ya}\pm$ 1.56
48 Hour	$31.67^{za}\pm$ 1.67	$34.17^{zab}\pm$ 1.49	$37.08^{zb}\pm$ 1.68	$31.25^{za}\pm$ 1.52

Values with different superscripts w, x, y and z within same rows and a and b within same columns differ significantly (P<0.05).

Table 3. Time and dose dependent effects of L-NAME on % progressive motility of spermatozoa

Treatment (L-NAME)	Control	1 µM	10 µM	100 µM
0 Hour	$69.64^{wa}\pm$ 2.34	$73.21^{wa}\pm$ 2.29	$75.00^{wa}\pm$ 2.26	$73.21^{wa}\pm$ 2.75
6 Hour	$50.00^{xa}\pm$ 2.30	$54.58^{xa}\pm$ 2.57	$57.92^{xa}\pm$ 2.57	$53.75^{xa}\pm$ 2.31
24 Hour	$41.25^{ya}\pm$ 1.64	$45.42^{yab}\pm$ 1.68	$48.33^{yb}\pm$ 1.42	$43.75^{yab}\pm$ 2.23
48 Hour	$31.67^{za}\pm$ 1.42	$34.58^{zab}\pm$ 2.08	$39.17^{zb}\pm$ 1.72	$34.58^{zab}\pm$ 1.68

Values with different superscripts w, x, y and z within same rows and a and b within same columns differ significantly (P<0.05).

10 µM L-NAME as compared to 0.1, 10 µM SNP and 1, 100 µM L-NAME concentration and there was significant (P<0.01) reduction in motility with increase in the duration of refrigeration (viz. 6 h, 24 h and 48 h). At 1 µM SNP concentration, sperm motility increased as compared to control, but at higher concentration (10 µM) there was reduction in sperm motility which was in consonance with the findings of Panth (2017) and Naskar (2018). Similar results were reported by Balercia *et al.* (2004) that NO at low concentrations (<1 µM) increased the sperm motility and higher concentrations (>1 µM) decreased sperm motility in a dose dependent manner. Wu *et al.* (2004) observed NO synthase blocker like L-NAME, did not affect sperm cell motility at 0, 1, 2 or 4 h, respectively, while higher concentration of SNP (10^{-4} M) significantly inhibited sperm cell motility and caused apoptosis. Khodaei *et al.* (2016) also found that concentration of 0.1 µM or above increased progressive motility significantly in comparison to low concentration. Rahman *et al.* (2014) reported that higher dose of SNP decreased the sperm motility and caused hyper-activation of mice spermatozoa; this significant decrease in sperm motility was observed particularly at the highest concentration of 100 µM. The results of the present investigation are also similar to those of Rodriguez *et al.* (2005) in bulls, who reported no effect of SNP on sperm motility at low concentration but it reduces sperm motility in a dose dependent manner at higher concentration. However, the motility improved significantly (P<0.05) with the addition of 10 µM L-NAME as compared to 1 and 100 µM concentration. Rosselli *et al.* (1995) reported a significantly higher percentage of forward progressive sperm motility in washed semen incubated with L-NAME (0.15 mM). However, Rodriguez *et al.* (2005) did not observe any change in bull seminal attributes after adding L-NAME (0.001, 0.01, 0.1 and 0.5 mM) after 45 min of culture. In contrast to our findings, Lewis *et al.* (1996) reported the reduction in the various motility indices after 30 min in the presence of (10^{-5} M) L-NAME. Kameshwari *et al.* (2003) observed a decrease in sperm motility after addition of L-NAME in hamster sperms. Francavilla *et al.* (2000) also found similar results in which there were no significant changes in human sperm motility at varying concentrations of L-NAME. The differences in these results could be due to the different *in vitro* conditions or due to the species related variations.

It is equally important to evaluate motility of spermatozoa in post-thaw semen to discriminate between a good or bad batch of semen at different time intervals during storage (Srivastava and Pande 2017). This part of the experiment dealt with *in vivo* effects of adding Sodium nitroprusside (SNP) and N-Nitro L-Arginine methyl ester (L-NAME) in extender to investigate its impact on motility before cryopreservation and at different time intervals after cryopreservation. Percent progressive motility (Mean±S.E.) in the 3 groups, viz. control (73.13±1.57), SNP (73.44±1.42) and L-NAME (75.31±1.61) treatment had insignificant effect in the fresh semen. After cryopreservation there is significant (P<0.01) reduction in progressive motility as compared to fresh semen in control, SNP and L-NAME treated groups at 24 h, 1 month and 2 months respectively (Table 4). The ANOVA revealed that there was a significant (P<0.05) increase in per cent progressive motility between control (40.94±1.89) and L-NAME treated (48.13±2.13) extender at 24 h and between control and SNP treated extender at 1 month (46.88±1.82) and 2 months (45.00±1.51) interval.

In fresh semen (within 15 min after incubation) there

Table 4. Effect of modified semen extender on the progressive motility of spermatozoa

Group	Fresh semen (0 h)	Cryopreserved semen		
		24 h	1 month	2 month
Control	$73.13^{xa}\pm$ 1.57	$40.94^{xb}\pm$ 1.89	$39.38^{xb}\pm$ 2.09	$39.69^{xb}\pm$ 1.33
SNP	$73.44^{xa}\pm$ 1.42	$46.25^{xyb}\pm$ 2.12	$46.88^{yb}\pm$ 1.82	$45.00^{yb}\pm$ 1.51
L-NAME	$75.31^{xa}\pm$ 1.61	$48.13^{yb}\pm$ 2.13	$42.50^{xyb}\pm$ 2.89	$41.56^{xyb}\pm$ 2.13

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

was no significant change in per cent progressive motility between control and treated semen samples which is in consonance with the finding of Naskar (2018) and other researchers. The limited work done in this field are related to the addition of such compounds after thawing which has given contrasting results. Role of NO is still in paradox suggesting that ROS and RNS are not only injurious by-products of cellular metabolism but also fundamental participants in cell signaling and regulation mechanism (Finkel 1998) which mostly appears to be related to the concentration of these species. The higher level of ROS and RNS generation during the process of freezing can damage normal spermatozoa by inducing lipid peroxidation and DNA damage (Ollero *et al.* 2001, Saleh *et al.* 2002 and Doshi *et al.* 2012) and also deteriorates its quality by compromising membrane integrity, chromatin integrity and blocking oxidative metabolism (Nash *et al.* 2012, Lone *et al.* 2016, Lone *et al.* 2018). These spermatozoa may become more sensitive to the free radicals due to the polyunsaturated fatty acids (PUFAs) rich sperm plasma membrane (Sarlos *et al.* 2002) and reduced seminal antioxidant levels. While, within physiological limit NOS/NO pathway provides an important antioxidant action that protects cells from lipid peroxidation resulting in less destruction of membrane (Srivastava *et al.* 2006 and Leal *et al.* 2009) and improves sperm motility. It was observed in our experiment that there was significant ($P<0.05$) increase in percent progressive motility for L-NAME treated extender after 24 h while after 1 and 2 month interval it was significantly higher in case of SNP treated extender in comparison to control. It is likely that these compounds exert a significant effect on sperm motility only when the level of intracellular cGMP is maintained for a longer time after the intervention of NO compounds (Miraglia *et al.* 2011). Similar findings were observed by Khodaei *et al.* (2016), they reported that different concentrations of SNP (10, 50 and 100 nmol/ml) cause alterations in membranes integrity in time and dose dependent manner. On the contrary, some investigators did not find any significant variation in motility parameters after addition of these compounds while Digamber *et al.* (2016) concluded that less number of normal spermatozoa with reduced viability and motility were found in SNP treated group as compared to control. This difference could be due to the different breeds, extender, climatic conditions, NO compound used; and the different experimental procedure might have been followed to measure sperm motility. Faster metabolism augment free radicals production but during the period of storage there is a slower metabolism which may subside the essential physiological functions of these free radicals. Thus, optimized addition of NO compounds may help to balance the levels of reactive nitrogen species and potentiate the sperm motility and its fertilizing ability.

Thus it can be concluded that the addition of 1 µM SNP and 10 µM L-NAME in the extender during refrigeration was found to be most optimum for spermatozoa at all time periods, giving significant increase in motility than control. Later during freezing, addition of SNP and L-NAME in extender can serve as a biggest determinant to counteract oxidative stress and better responses were observed with SNP over L-NAME after 1 month of storage.

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