Research Article

DOI: 10.48165/ijar.2022.43.1.4

The Indian Journal of Animal Reproduction

The official journal of the Indian Society for Study of Animal Reproduction

Year 2022, Volume-43, Issue-1 (June)

ACS Publisher
www.acspublisher.com

ISSN 0970-2997 (Print)

Is Mitochondrial Uncoupler Dinitrophenol Effective in Counterbalancing the Dilution Effect in Buffalo Semen?

Venkateshappa Arjun[1], Pradeep Kumar[2], Ravi Dutt[1*], Dharmendra Kumar[2], Amit Kumar[3], Renu Bala[2], Nisha Verma[2], Andonissamy Jerome[2], Meenakshi Virmani[4], Chandrashekhar Santosh Patil[5], Shivani Bhardwaj[2], Prem Singh Yadav[2]

[1]Department of Gynaecology and Obstetrics, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125001, Haryana, India.
[2]Division of Animal Physiology and Reproduction, ICAR- Central Institute for Research on Buffaloes, Hisar-125001, Haryana, India.
[3]Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141012, Punjab, India.
[4]Department of Veterinary Physiology and Biochemistry, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125001, Haryana, India.
[5]Department of Animal Genetics and Breeding, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar-125001, Haryana, India.

ABSTRACT

Semen ejaculates were collected from Murrah buffalo (*Bubalus bubalis*) bulls and were diluted with low density lipoprotein (LDL) based extender into 20 million sperm/0.25 mL and 2 million sperm/0.25 mL. Dinitrophenol (DNP) was added (@ 0, 1, 10 and 50 µM) to 20 million and 2 million sperm concentration which was followed by cryopreservation. . After thawing, the parameters studied were plasma membrane integrity by HOST, sperm motility and kinetics by CASA, the thermal resistance of sperm by incubation test, mitochondrial superoxide status by MitoSOX through flow cytometry, mitochondrial membrane potential (MMP) evaluation by JC-1 through flow cytometry. There was no significant (P>0.05) change in plasma membrane integrity, sperm motility and kinematics; thermal resistance of sperm, mitochondrial superoxide status and hMMP in comparison to control within the 2 and 20 million sperm doses. Two million sperm doses resulted in low plasma membrane integrity, low thermal resistance of sperm, high mitochondrial superoxide status and decreased high MMP and no significant (P>0.05) change in sperm motility and kinematics, but reduced total motility, beat cross frequency and no change in progressive motility, straight linear velocity, average path velocity, curvilinear velocity, amplitude of lateral head displacement, straightness, linearity, and wobble in comparison to 20 million sperm doses. Supplementation of DNP (0, 1, 10 and 50 µM) in extender failed to improve semen quality in both 2 and 20 million sperm doses in buffalo.

Key words: Buffalo, CASA, Semen, Dinitrophenol, HOST, Mitochondrial membrane potential

How to cite: Arjun, V., Kumar, P., Dutt, R., Kumar, D., Kumar, A., Bala, R., Verma, N., Jerome, A., Virmani, M., Patil, C., Bhardwaj, S., & Yadav, P.S. (2022). Is Mitochondrial Uncoupler Dinitrophenol Effective in Counterbalancing the Dilution Effect in Buffalo Semen?
The Indian Journal of Animal Reproduction, 43(1), 22–29. 10.48165/ijar.2022.43.1.4

*Corresponding author.
E-mail address: raviduttvets@yahoo.co.in (Ravi Dutt)
Received 06-06-2022; Accepted 03-04-2023

INTRODUCTION

Artificial insemination (AI) is a widely employed technique that uses frozen-thawed sperm to accelerate the rate of genetic improvement by inseminating females (Masoudi *et al.,* 2016). In AI industry, the genetic impact of sires is limited due to the production efficiencies and decreased sperm functions during cryopreservation (Arjun *et al.,* 2022). Currently, packaging of 20 million sperm in French mini straws has been used as a standard for AI in the dairy industry. But, due to increased demand for superior germplasm, there is an urgent need to implement packaging of low sperm numbers. However, AI with low-sperm numbers results in reduced post-thaw viability (Maxwell and Johnson, 1999). The effect of semen dilution on sperm viability has not been adequately examined. A potential decrease in fertility could arise not only because of the reduced sperm numbers, but also due to the proportional decrease in some essential seminal plasma (SP) components of the suspending medium. The exact cause of the dilution effect is still unknown. Some researchers suggested a loss of protective seminal plasma components (Ashworth *et al.,* 1994) and/or intracellular and/or cell surface components from the sperm resulting in a capacitation-like state and ultimately, premature acrosome reaction (Centurion *et al.,* 2003). The 'dilution effect' adversely affects the sperm mitochondria resulting in poor sperm motility after cryopreservation (Kurland and Andersson, 2000). The sperm mitochondria are one of the most damaged organelles during cryopreservation (Gonzalez-Fernandez *et al.,* 2012). The use of mitochondrial targeted antioxidants and uncoupler may be a good strategy to reduce the dilution effect.

Mitochondria are normally responsible for 80-90% of cellular oxygen consumption and the majority of adenosine triphosphate (ATP) production. Approximately 0.1–2% of the oxygen taken up by cells is converted to reactive oxygen species (ROS) by mitochondria, mainly through the production of superoxide anion (Hansford *et al.,* 1997; Kumar *et al.,* 2021; 2022). In the proton-pumping mechanism of Complex-I, ROS generation is more dependent on changes in the transmembrane pH gradient (ΔpH) than on the membrane potential (ΔΨ) (Lambert and Brand, 2004). As mild uncoupling decreases proton motive force by lowering both ΔpH and ΔΨ, it is one of the effective means to lower mitochondrial superoxide production at the cost of efficient ATP synthesis (Brand *et al*., 2004). Thus, mild mitochondrial uncoupling probably can be regarded as the reduction of the efficiency of energy conversion without compromising intracellular high energy phosphate levels (Cunha *et al*., 2011).

Uncoupling of mitochondria may be induced by exogenous chemical protonophores such as "classical" protonophore 2,4-dinitrophenol (DNP) and by activating endogenous innate mitochondrial uncoupling pathways involving, for instance, uncoupling proteins (Gruber *et al*., 2013). DNP was necessary for the beneficial effect to occur on low post-thaw survival of *Rhesus macaque* sperm (Dong *et al*., 2010), but the addition of DNP did not exert any action on swine spermatozoa and did not affect variables evaluated regardless of storage time at 17°C (Silva *et al*., 2016). Further, culturing bovine post-compaction embryos in the presence of high glucose (>2.5 mM) or supplementation with DNP preferentially favour the survival of embryos (Green *et al.,* 2016). The effects of DNP on sperm are debatable. So, this study was carried out to assess the effect of DNP in counteracting the dilution effect in cryopreserved buffalo semen.

MATERIALS AND METHODS

The present study was conducted at the Semen Freezing Laboratory (SFL), Division of Animal Physiology and Reproduction, Central Institute for Research on Buffaloes (ICAR), Hisar, Haryana, India. All reagents were purchased from Sigma-Aldrich (St. Louis, MO, United States) unless otherwise stated.

Preparation of stock solutions

DNP (PESTANAL®, Analytical standard; Cat No: 34334) was weighed and 50 μM stock solution was prepared in Tris buffer.

Semen collection, evaluation and cryopreservation

A total of 11 semen ejaculates were collected using an artificial vagina from five Murrah buffalo bulls. Samples were assessed for volume, colour, mass motility and sperm concentration by a photometer (Accucell bovine photometer (IMV), L'Aigla, France). Semen ejaculates were diluted with the help of low density lipoproteins (LDL) based extender as described by Dalal *et al.* (2020) [Tris (3.02 % w/v), citric acid (1.67 % w/v), fructose (1 % w/v), LDL (12 % g/v), penicillin (10 lakhs units/L) and streptomycin (1.25 g/L) plus glycerol (6.4 % v/v, Thermo Fisher Scientific, Mumbai, India)] into 20 million and 2 million sperm/0.25 mL straw. DNP was added (@ 0, 1, 10 and 50 μM) to 20 million and 2 million sperm/0.25 mL straw. Sperm motility was assessed and ejaculates having ≥70% sperm motility were

loaded into French mini straws (0.25 mL) (IMV, France) and equilibrated at 4ºC for 4 h and cryopreserved (Patil *et al.,* 2020).

Estimation of sperm kinetics and motility

Sperm kinetics and motility were analyzed using the computer-assisted sperm analyser (CASA) (IVOS 12.1, Hamilton S. Thorne Biosciences, Beverly, MA, USA) as described by Kumar *et al.* (2015). The sperm motion characteristics recorded were: total motility - TM (%), progressive motility - PM (%), straight linear velocity - VSL (µm/s), average path velocity - VAP (µm/s), curvilinear velocity - VCL (µm/s), amplitude of lateral head displacement - ALH (µm), beat cross frequency - BCF (Hz), straightness - STR (%), linearity - LIN (%) and wobble - WOB (%).

Evaluation of thermal resistance and sperm plasma membrane integrity

Sperm thermal resistance was evaluated through the incubation test; and sperm plasma membrane functional integrity was evaluated by the Hypo-Osmotic Swelling Test (HOST) as described by Kumar *et al.* (2015) and Arjun *et al.* (2021). The motility of sperm was evaluated at different time intervals (0, 30, 60, 90 and 120 min) by using a phase-contrast microscope. The number of tail curled spermatozoa percentage in PBS was deducted from the HOS test to get the true HOST-reactive sperm.

Flow cytometry analysis

Flow cytometry analyses were conducted using a CytoFLEX (Beckman Coulter-Life Sciences) equipped with laser light 488 nm for excitation. Samples were analysed at a sample flow rate of 30 µL/min and 10000 events were recorded. Fluorescent channels FITC (525/40 BP) and PE (585/42 BP) were used for evaluation.

Detection of mitochondrial superoxide anion status

The mitochondrial superoxide anion status (MSAS) in sperm cells was detected through flow cytometry using MitoSOX™ Red mitochondrial superoxide indicator (Cat No. M36008; Invitrogen) with blue laser excitation (488 nm) and detection with a 585/42 BP (FL2 channel/PE) as described by Patil *et al.* (2020) and Arjun *et al.* (2022). The sample flow rate at 30 µL/min and 10000 events were recorded for each sample. The P1 population was obtained under FSC-A *vs* FSC-H dot plot, where doublets and debris were excluded from the main population using a polygon gate and the obtained P1 population was gated under the FSC-A *vs* SCC-A dot plot to obtain the P2 population. P2 population was gated under the histogram and classified into two types, positive for MitoSOX and negative for MitoSOX (Fig. 1a,b). The data were analyzed in the CytExpert software (v.2.3).

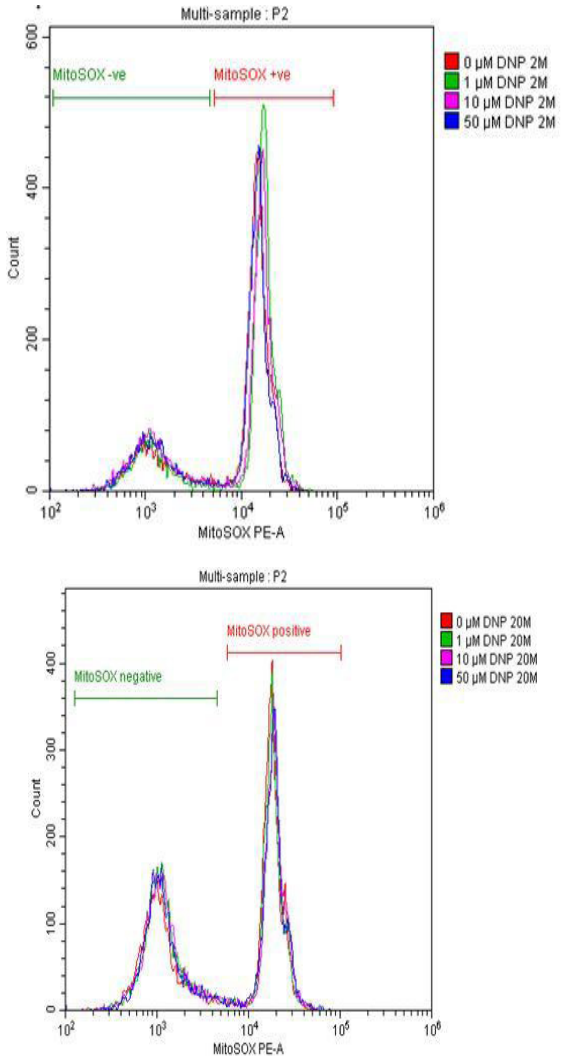

Fig. 1: Histogram overlay showing effect of DNP on MitoSOX positive (%) in 2 million sperm/dose (a) and 20 million sperm/dose (b)

Evaluation of mitochondrial membrane potential

The mitochondrial membrane potential was evaluated using JC-1 dye (Cat No. T3168; Invitrogen) as described by Ghaleno *et al.* (2014) and Arjun *et al.* (2022) with slight modification and with the help of polygon gate.

Spermatozoa were classified into two types viz., JC-1 aggregates or high mitochondrial membrane potential (hMMP) and JC-1 monomers or low mitochondrial membrane potential (lMMP). The data were analysed in the CytExpert software (v.2.3).

Statistical analysis

All statistical analyses were carried out using IBM SPSS Statistics software (IBM Corporation, USA) for windows. Data were analyzed using a two-way ANOVA and means comparisons were done with the Duncan's multiple range test (DMRT). Statistical significance was set at a 0.05 probability level. Results were expressed as mean ± standard error (SE).

RESULTS AND DISCUSSION

The effect of DNP on sperm motility and kinetic parameters is depicted in Table 1. The TM was found significantly (P<0.05) higher in all groups of 20 million sperm as compared to 2 million sperm. Whereas, no statistical difference (P>0.05) was observed in PM between 20 and 2 million sperm extended at different concentrations of DNP. Supplementation of DNP up to 50 µM showed no adverse effect on TM and PM in both 2 and 20 million sperm groups and their motility was comparable with the control group in respective sperm concentration. The supplementation of DNP in semen extender maintained sperm kinetics such as VCL, VSL and VAP in diluted semen (2 million) which is comparable with 20 million sperm kinetics. The BCF was found significantly (P<0.05) higher in 20 million sperm in comparison to 2 million in all study groups, while ALH was higher in 2 million sperm in comparison to 20 million in all, except 0 µM. Within the treatment groups of 2 million and 20 million, no difference (P>0.05) was observed in the BCF and ALH. The DNP treatment @ 1 µM reduced the sperm LIN significantly (P<0.05) in 2 million than 20 million sperm, but, other parameters were comparable, which indicated the effectiveness of DNP to maintain the STR, LIN and WOB of the sperm even in diluted semen.

Table 1: Effect of DNP on sperm motility and kinetic parameters (n=11)

Parameters	Sperm concentration	DNP			
		0 µM	1 µM	10 µM	50 µM
TM (%)	20M	45.19±2.26^{A}	44.11±3.45^{A}	41.57±2.66^{A}	37.81±2.83^{A}
	2M	25.91±3.17^{B}	27.64±3.47^{B}	28.38±3.80^{B}	25.73±2.99^{B}
PM (%)	20M	37.27±3.66	33.92±3.35	33.64±3.82	30.98±3.75
	2M	22.78±4.38	22.19±3.99	23.78±3.47	22.46±3.93
VCL (µm/sec)	20M	200.27±8.29	194.39±7.10	195.27±7.27	189.29±10.20
	2M	209.39±13.95	215.09±15.34	223.79±16.07	211.33±11.95
VSL (µm/sec)	20M	102.05±5.47	99.54±4.91	96.84±4.70	90.79±6.16
	2M	96.34±7.06	95.51±7.97	99.65±7.45	94.62±5.45
VAP (µm/sec)	20M	118.33±5.59	115.12±4.93	113.77±4.77	108.93±6.10
	2M	116.55±7.52	116.05±8.82	121.38±8.91	115.50±5.51
BCF (Hz)	20M	28.20±0.71^{A}	28.57±0.67^{A}	27.85±0.76^{A}	26.95±0.63^{A}
	2M	24.68±0.81^{B}	24.75±0.97^{B}	24.05±0.61^{B}	24.21±0.86^{B}
ALH (µm)	20M	8.27±0.30	7.97±0.24^{B}	8.17±0.31^{B}	8.22±0.35^{B}
	2M	9.00±0.44	9.67±0.43^{A}	9.64±0.53^{A}	9.30±0.44^{A}
STR (%)	20M	85.17±0.79	85.28±0.82	83.85±1.10	82.28±1.24
	2M	83.35±1.61	82.55±1.05	82.32±0.76	82.36±1.34
LIN (%)	20M	52.47±1.53	52.32±1.13^{A}	50.92±1.44	49.29±1.23
	2M	48.91±1.72	47.28±1.21^{B}	46.69±1.39	47.24±1.68
WOB (%)	20M	60.77±1.40	60.49±0.82	59.75±1.13	59.05±1.23
	2M	58.25±1.54	56.58±0.90	56.02±1.33	57.45±1.61

$^{A-B}$values (Mean ± SEM) with a different superscript in column differ significantly (P<0.05) between groups of 2M and 20M concentration (N=11).20M: 20 million sperm/dose; 2M: 2 million sperm/dose.

TM: Total motility; PM: Progressive motility; VCL: Curvilinear Velocity; VSL: Straight Line Velocity; VAP: Average Path Velocity; BCF: Beat Cross Frequency; ALH: Amplitude of Lateral Head Displacement; STR: Straightness; LIN: Linearity and WOB: Wobble

CASA measures the proportion of motile spermatozoa and other sperm motion parameters derived from single spermatozoa cells and it has a more predictive value of fertility potential of semen ejaculates (Mortimer and Swan, 1995). In bovine species, specific motion parameters have been positively correlated with fertility (Perumal *et al.*, 2014). Addition of 0.5 nM of DNP to ram semen significantly (P<0.05) increased the motility parameters and decreased abnormal sperm (Nazari *et al.*, 2020). In the present study, the addition of DNP did not result in any change in the CASA based sperm parameters. However, DNP activity may depend on morphological and physiological characteristics of spermatozoa because ejaculates with low post-thaw survival are usually benefited more than those with high post-thaw motility from treatment with DNP (Dong *et al.*, 2010).

The effects of DNP on HOST, MSAS and MMP are elucidated in Table 2. The sperm plasma membrane integrity as assessed by HOST was significantly higher in the 20 million than 2 million sperm concentration in all concentrations of DNP used in this study. There was no significant (P>0.05) difference between the group of 20 million and 2 million (Fig. 2a, b). MSAS by spermatozoa was found higher in 2 million concentrations in comparison with 20 million concentrations, which significantly (P<0.05) differed in all treated groups. There was no significant difference (P>0.05) within the groups of 20 million and 2 million sperm concentrations. The hMMP was significantly reduced in 2 million sperm as compared to 20 million sperm in all study groups of DNPs whereas, no significant difference was observed within the groups of 20 million and 2 million sperm at different concentrations of DNP. The addition of DNP at three different concentrations, *viz.*, 1, 10 and 50 µM did not have any adverse effect on sperm membrane integrity as compared to the control group, but failed to yield better results than the control. Similar results were seen in 2 million, but in comparison to 20 million, the sperm plasma membrane integrity was recorded to be decreased in 2 million groups. Silva *et al.* (2016) found that incubation with 0.1, 1 and 10 µM concentrations of DNP in boar ejaculates for 96 h did not cause damage to the sperm plasma membrane. However, the addition of DNP in the semen extender increased the post-thaw motility in *Rhesus* monkey semen (Dong *et al.*, 2010).

In the present study, the addition of DNP at 1, 10 and 50 µM resulted in no significant reduction of superox-

Table 2: Effect of DNP on HOST, MSAS and MMP levels (n=11)

Parameters	Sperm concentration	DNP			
		0 µM	1 µM	10 µM	50 µM
HOST positive (%)	20M	40.09±2.01^{A}	41.73±2.41^{A}	39.59±2.77^{A}	36.45±2.21^{A}
	2M	23.91±2.50^{B}	23.64±1.35^{B}	24.27±1.95^{B}	26.00±2.04^{B}
MitoSOX positive (%)	20M	52.65±2.44^{B}	53.90±2.40^{B}	50.62±3.22^{B}	54.50±3.77^{B}
	2M	66.34±2.97^{A}	69.31±1.44^{A}	69.51±1.56^{A}	66.21±2.41^{A}
hMMP (%)	20M	45.58±1.88^{A}	45.75±1.78^{A}	49.11±2.53^{A}	45.03±2.23^{A}
	2M	34.69±2.38^{B}	34.54±1.30^{B}	33.43±1.04^{B}	37.06±1.71^{B}

$^{A-B}$ values (Mean ± SEM) with a different superscript in column differ significantly (P<0.05) between groups of 2M and 20M concentration (n=11)., 20M: 20 million sperm/dose; 2M: 2 million sperm/dose.

HOST: Hypo osmotic swelling test; hMMP: high Mitochondrial Membrane Potential

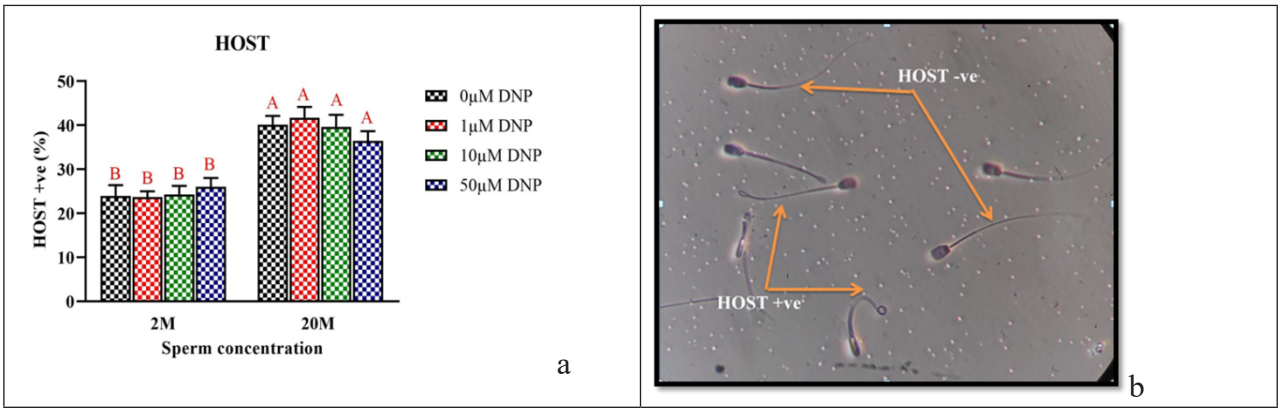

Fig. 2: (a) HOST positive (%) in 2 (2M) and 20 (20M) million sperm/dose. (b) arrow mark indicating HOST positive and HOST negative sperm (100 X Objective)

Table 3: Comparison of buffalo sperm thermal resistance in DNP treated samples (Mean ±SEM)

	Incubation Time and sperm concentration									
	0 min		30 min		60 min		90 min		120 min	
DNP	2 M	20 M	2 M	20 M	2 M	20 M	2 M	20 M	2 M	20 M
0 µM	41.82± 2.05^{Bx}	55.00± 0.95^{Ax}	34.09± 2.92^{Bx}	49.09± 1.63^{Axy}	26.36±3.10^{Bx}	42.27± 2.17^{Axy}	20.00± 2.94^{Bx}	37.27± 3.26^{Axy}	13.64± 2.34^{Bx}	33.18± 3.04^{Ax}
1 µM	40.91± 2.41^{Bxy}	54.55± 1.84^{Ax}	31.36± 1.80^{Bxy}	51.36± 1.80^{Ax}	22.73±2.17^{Bxy}	44.55± 2.18^{Ax}	17.27± 1.83^{Bx}	40.45± 2.28^{Ax}	11.14± 1.32^{Bxy}	35.00± 3.09^{Ax}
10 µM	40.00± 2.70^{Bxy}	54.55± 2.28^{Ax}	31.82± 2.05^{Bxy}	48.64± 2.70^{Axy}	23.18±1.69^{Bxy}	42.27± 2.81^{Axy}	16.82± 2.26^{Bx}	38.18± 2.88^{Axy}	11.36± 1.63^{Bxy}	34.09± 3.08^{Ax}
50 µM	33.18± 3.46^{By}	49.09± 3.08^{Ax}	25.91± 3.29^{By}	42.27± 3.19^{Ay}	18.18±2.46^{By}	34.55± 3.05^{Ay}	12.95± 2.54^{Bx}	30.45± 3.05^{Ay}	7.36± 1.73^{BX}	26.82± 2.63^{Ax}

$^{x-y}$values (Mean ± SEM) with a different superscript in column differ significantly (P<0.05) within the groups of 2M and 20M concentration.

$^{A-B}$values (Mean ± SEM) with a different superscript in row differ significantly (P<0.05) between the groups of 2M and 20M concentration. (N=11)., 20M: 20 million sperm/dose, 2M: 2 million sperm/dose.

ide levels in frozen-thawed semen in both 2 million and 20 million groups. Similar to our results, no significant reduction of ROS levels in boar fresh semen ejaculates incubated with the 0.1, 1 and 10 µM DNP concentrations (Silva *et al*., 2016) and fresh yellow catfish semen (Fang *et al*., 2014) was reported. But in contrast to our findings, Fang *et al*. (2014) found a significant reduction of ROS levels in frozen-thawed yellow catfish sperm samples supplied with DNP at 1 µM concentration. Similarly, Nazari *et al*. (2020) reported that DNP at 0.5, 0.75 nM concentration significantly (P<0.05) increased sperm viability and membrane integrity in ram semen. Since, DNP is mitochondrial uncoupler in ETC, used to prevent intracellular oxidative stress, and the use of DNP in *Rhesus* monkeys with low cryo-resistant ejaculates, reduced ROS through mild mitochondrial uncoupling (Dong *et al*., 2010). But in our experiment, DNP failed to combat the oxidative stress occurred during the freeze-thaw cycle.

In the present study, there was no significant change in MMP with the addition of DNP at 0, 1, 10 and 50 µM in 2 million and 20 million groups, which concurred with the findings of Silva *et al*. (2016) in fresh boar ejaculates on 96 hours of incubation. Fang *et al*. (2014) reported a significant decrease of MMP in 0.1, 1.0 and 10 µM DNP groups as compared to the control, where fresh sperm samples were equilibrated with DNP at 37 °C for 30 min. However, Davila *et al*. (2016) reported that incubation of DNP at 1-400 µM for 1 and 3 h did not affect MMP in stallion sperm.

A comparison of buffalo sperm thermal resistance in DNP treated samples is shown in Table 3. Sperm thermal resistance by incubation test indicated that the longevity of the sperm in 20 million concentrations was high up to 120 min than 2 million sperm. The longevity of the sperm in 2 million and 20 million was comparable with control when cryopreserved with the supplementation of DNP @ 1 and 10 µM up to 120 min of incubation. In the 20 million group, the use of DNP at 50 µM concentration resulted in a slight decrease in the motility than the control at 1 h incubation. Silva *et al*. (2016) in fresh boar ejaculates did not find any change in the motility between the 0, 0.1, 1 and 10 µM groups after incubation with DNP for 96 h.

CONCLUSIONS

In conclusion, incorporation of DNP at the 1, 10 and 50 µM concentration and dilution does not have a protective effect on buffalo spermatozoa during cryopreservation of 20 and 2 million sperm concentrations.

ACKNOWLEDGEMENTS

The authors are thankful to Head, Department of Veterinary Gynaecology, LUVAS, Hisar, and Director, ICAR-CIRB for providing all the necessary facilities for the study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Arjun, V, Kumar, P., Dutt, R., Kumar, A., Bala, R., Verma, N., Jerome A., Virmani, M., Patil, C., Singh, S. and Kumar, D. (2021). «Is addition or removal of seminal plasma able

to compensate for the dilution effect of buffalo semen?» *Andrologia.* e14123.

Arjun, V., Kumar, P., Dutt, R., Kumar, A., Bala, R., Verma, N., Jerome, A., Virmani, M., Patil, C. S., Bhardwaj, S., Kumar, D. and Yadav, P. S. (2022). Effect of mitochondria-targeted antioxidant on the regulation of the mitochondrial function of sperm during cryopreservation. *Andrologia*, e14431.

Ashworth, P.J.C., Harrison, R.A.P., Miller, N.G., Aplummer, J.M. and Watson, P.F. (1994). Survival of ram spermatozoa at high dilution: protective effect of simple constituents of culture media as compared with seminal plasma. *Reprod. Fert. Dev.,* **6**: 173-180.

Brand, M.D., Affourtit, C., Esteves, T.C., Green, K., Lambert, A.J., Miwa, S. and Parker, N. (2004). Mitochondrial superoxide: production, biological effects, and activation of uncoupling proteins. *Free Radic. Biol. Med*., **37**(6): 755-767.

Centurion, F., Vazquez, J.M., Calvete, J.J., Roca, J., Sanz, L., Parrilla, I. and Martinez, E.A. (2003). Influence of porcine sperm adhesins on the susceptibility of boar spermatozoa to high dilution. *Biol. Reprod.,* **69**(2): 640-646.

Cunha, F.M., Caldeira da Silva, C.C., Cerqueira, F.M. and Kowaltowski, A.J. (2011). Mild mitochondrial uncoupling as a therapeutic strategy. *Curr. Drug Targets*., **12**(6): 783-789.

Dalal, J., Chandolia, R.K., Pawaria, S., Kumar., A., Kumar, D., Selokar, N.L., Jerome A., . Yadav, P.S. and Kumar, P. (2020). Low-density lipoproteins protect sperm during cryopreservation in buffalo: Unravelling mechanism of action. *Mol. Reprod. Dev.,* **87**(12):1231-1244.

Davila, M.P., Muñoz, P.M., Bolanos, J.M., Stout, T.A., Gadella, B.M., Tapia, J.A. and Pena, F.J. (2016). Mitochondrial ATP is required for the maintenance of membrane integrity in stallion spermatozoa, whereas motility requires both glycolysis and oxidative phosphorylation. *Reproduction.*, **152**(6): 683-694.

Dong, Q., Tollner, T.L., Rodenburg, S.E., Hill, D.L. and Vande Voort, C.A. (2010). Antioxidants, Oxyrase, and mitochondrial uncoupler 2, 4-dinitrophenol improved post-thaw survival of *Rhesus* monkey sperm from ejaculates with low cryosurvival. *Fertil. Steril.*, **94**(6): 2359-2361.

Fang, L., Bai, C., Chen, Y., Dai, J., Xiang, Y., Ji, X. and Dong, Q. (2014). Inhibition of ROS production through mitochondria-targeted antioxidant and mitochondrial uncoupling increases post-thaw sperm viability in yellow catfish. *Cryobiology.*, **69**(3): 386-393.

Ghaleno, L.R., Valojerdi, M.R., Janzamin, E., Chehrazi, M., Sharbatoghli, M. and Yazdi, R.S. (2014). Evaluation of conventional semen parameters, intracellular reactive oxygen species, DNA fragmentation and dysfunction of mitochondrial membrane potential after semen preparation techniques: a flow cytometric study. *Arch. Gynecol. Obstet*., **289**(1): 173-180.

Gonzalez-Fernandez, L., Morrell, J.M., Pena, F.J. and Macias-Garcia, B. (2012). Osmotic shock induces structural damage on equine spermatozoa plasmalemma and mitochondria. *Theriogenology.,* **78**: 415–422.

Green, M. P., Harvey, A. J., Spate, L. D., Kimura, K., Thompson, J. G., Roberts, R. M. (2016). The effects of 2, 4-dinitrophenol and d-glucose concentration on the development, sex ratio, and interferon-tau (IFNT) production of bovine blastocysts. *Molecul. Reprod. Dev.*, **83**(1): 50-60.

Gruber, J., Fong, S., Chen, C.B., Yoong, S., Pastorin, G., Schaffer, S. and Halliwell, B. (2013). Mitochondria-targeted antioxidants and metabolic modulators as pharmacological interventions to slow ageing. *Biotechnol. Adv.*, **31**(5): 563-592.

Hansford, R. G., Hogue, B. A. and Mildaziene, V. (1997) Dependence of H_2O_2 formation by rat heart mitochondria on substrate availability and donor age. *J. Bioenerg. Biomembr.,* **29**: 89-95.

Kumar, A., Ghosh, S. K., Katiyar, R., Gemeda, A.E., Rautela, R., Bisla, A., Srivastava, N., Bhure, S. K., Devi, H.L. and Chandra, V. (2022). Supplementation of Mito TEMPO and acetovanillone in semen extender improves freezability of buffalo spermatozoa. *Andrology*, **10**(4): 775-788.

Kumar, A., Ghosh, S.K., Katiyar, R., Rautela, R., Bisla, A., Ngou, A.A., Pande, M., Srivastava, N. and Bhure, S.K. (2021). Effect of Mito-TEMPO incorporated semen extender on physico-morphological attributes and functional membrane integrity of frozen thawed buffalo spermatozoa. Cryoletters, **42**(2): 111-119.

Kumar, P., Saini, M., Kumar, D., Balhara, A.K., Yadav, S.P., Singh, P. and Yadav, P.S. (2015). Liposome-based semen extender is suitable alternative to egg yolk-based extender for cryopreservation of buffalo (*Bubalus bubalis*) semen. *Anim. Reprod. Sci.,* **159**: 38-45.

Kurland, C. and Andersson, S. (2000). Origin and evolution of the mitochondrial proteome. *Microbiol. Molecul. Biol. Reviews.,* **64**: 786-820.

Lambert, A.J. and Brand, M.D. (2004). Superoxide production by NADH: ubiquinone oxidoreductase (complex I) depends on the pH gradient across the mitochondrial inner membrane. *Biochem. J.*, **382**(2): 511-517.

Masoudi, R., Sharafi, M., Shahneh, A.Z., Towhidi, A., Kohram, H., Esmaeili, V., Shahverdi, A. and Davachi, N.D. (2016). Fertility and flow cytometry study of frozen-thawed sperm in cryopreservation medium supplemented with soybean lecithin. *Cryobiology.,* **73**(1): 69-72.

Maxwell, W.M.C. and Johnson, L.A. (1999). Physiology of spermatozoa at high dilution rates: The influence of seminal plasma. *Theriogenology.,* **52**(8): 1353-1362.

Mortimer, S. T. and Swan, M. A. (1995). Andrology: Kinematics of capacitating human spermatozoa analysed at 60 Hz. *Human Reprod.*, **10**(4): 873-879.

Nazari, M., Daghigh Kia, H., Ebrahimi, M., Najafi, A. and Mahdipour, M. (2020). Effect of targeted antioxidant 2, 4 dinitrophenol on improving qualitative and quantitative parameters of Ghezel ram sperm after freeze-thawing process during non-breeding season. *Anim. Sci. J. (Pajouhesh & Sazandegi).,* **130:** 181-190.

Patil, S., Kumar, P., Singh, G., Bala, R., Jerome, A., Patil, C.S. and Sharma, R.K. (2020). 'Semen dilution effect' on sperm variables and conception rate in buffalo. *Anim. Reprod. Sci.*, **214**: 106304.

Perumal, P., Srivastava, S. K., Ghosh, S. K. and Baruah, K. K. (2014). Computer-assisted sperm analysis of freezable and non-freezable Mithun (*Bos frontalis*) semen. *J. Anim.,* 675031: 1-6.

Silva, E.F., Junior, A.S.V., Cardoso, T.F., Stefanello, F.M., Kalb, A.C., Martínez, P.E. and Corcini, C.D. (2016). Reproductive toxicology of 2, 4 dinitrophenol in boar sperm. *Toxicol. In Vitro*., **35**: 31-35.