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Original article

Effect of nitric oxide on boar sperm motility, membrane integrity, and acrosomal status during semen storage

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Abstract

Nitric oxide (NO) is a major gasotransmitter involved in several physiological processes of male reproduction. There is, nevertheless, little information concerning the role of NO during semen storage. The aim of this study was to evaluate the effect of NO on boar semen stored at 17°C for 72 h. For this purpose, sperm samples were treated with 0.625, 1.25, 2.5, 5, and 10 mM aminoguanidine (AG) or Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), a selective and non-selective NO synthase (NOS) inhibitor, respectively. Moreover, sodium nitroprusside (SNP), a NO donor, was used at the dose of 18.75, 37.5, 75, and 150 μM. Sperm motility, membrane integrity, and acrosomal status were evaluated at 0, 4, 24, 48, and 72 h of semen storage. A significant increase of the amplitude of lateral sperm head displacement (ALH), and both curvilinear and straight-line velocity (VCL and VSL, respectively) was observed at 72 h of semen storage in samples treated with 0.625 mM AG, probably because of the antioxidant properties of this NOS inhibitor. Contrarily, 0.625 mM L-NAME showed no effect on boar sperm parameters during the entire period of semen storage. Moreover, AG and L-NAME at 10 mM negatively affected sperm kinetics and acrosome integrity, which may provide further support to the notion that low NO levels are necessary for a normal sperm function. The concentrations of SNP used in this study had mostly no or negative effects on boar sperm parameters during semen storage. In conclusion, the results from this study increase the understanding of the role of NO on boar sperm physiology.

Key words: boar spermatozoa, nitric oxide, nitric oxide synthases, NO donor, NOS inhibitor

Introduction

Nitric oxide (NO) is one of the signalling molecules responsible for the regulation of sperm function (Herrero and Gagnon 2001). Intracellular NO is formed from guanidine by nitric oxide synthase-catalysed reaction. There are three nitric oxide synthase (NOS) isoforms: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). The nNOS and eNOS isoforms are constitutive and produce small amounts of NO, whereas the iNOS isoform produces large amounts of NO (Dixit and Parvizi 2001, Herrero and Gagnon 2001). The NOS isoforms have been described in human (Herrero et al. 1996), mouse

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(Herrero et al. 1996), and boar (Aquila et al. 2011) spermatozoa. There is a lot of evidence that NO/NOS pathways are involved in the regulation of sperm motility, viability, capacitation, hyperactivation, acrosome reaction, and fertilizing ability (e.g. Herrero and Gagnon 2001, Jeseta et al. 2017).

Although intracellular NO is essential for proper sperm function, NO has dose-dependent dual effect on sperm motility. While low concentrations of sodium nitroprusside (SNP), a NO donor, increase sperm motility (Hellstrom et al. 1994, Zhang and Zheng 1996), high concentrations of this compound exert negative effects on this sperm parameter (Rosselli et al. 1995, Weinberg et al. 1995, Hassanpour et al. 2007, Rahman et al. 2014). The high levels of reactive oxygen and nitrogen species (ROS and RNS, respectively) are considered to be main components of oxidative stress and one major cause of poor sperm quality in humans (Balercia et al. 2004, Uribe et al. 2015). The stress produced by high ROS and RNS levels decrease total and progressive sperm motility, kinetics, and mitochondrial membrane potential (Uribe et al. 2015, Jeseta et al. 2017). Likewise, the inhibition of NOS by Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME) inhibits sperm motility (Hassanpour et al. 2007) and acrosome reaction (Hou et al. 2008). On the other hand, aminoguanidine (AG) acts as a competitive specific inhibitor of the iNOS isoform (Misko et al. 1993) and has the capacity to decrease ROS formation, lipid peroxidation, and cell apoptosis (Giardino et al. 1998). In this way, recent studies show that AG improves sperm parameters in varicocelized rats that exhibit high NO and ROS levels (Abbasi et al. 2011a, 2011b, Alizadeh et al. 2010, 2016), but little is still known on the effect of this iNOS selective inhibitor on boar sperm parameters during semen storage.

The aim of this study was to evaluate the effects of NO on boar sperm motility, membrane integrity, and acrosomal status during 72 h of semen storage at 17°C. For this purpose, we used SNP as a NO donor, and L-NAME or AG as non-selective and selective NOS inhibitors, respectively. The sperm analyses were performed at 0 (control only), 4, 24, 48, and 72 h of semen storage.

Materials and Methods

Preparation of NOS inhibitors and NO donor

Stock solutions of L-NAME and AG were prepared by dilution of chemicals in physiological solution (NaCl 0.9%, w/v) at concentrations of 6.25, 12.5, 25, 50, and 100 mM. Stock solutions of SNP were prepared by dilution of compound in physiological solution (NaCl 0.9%, w/v) at concentrations of 187.5, 375, 750, and 1,500 µM. Stock solutions were stored at -20°C till usage.

Sperm samples collection and processing

Commercial sperm doses from boars (age: 2.7 ± 1.1 years old, mean ± SD, N = 11) of different breeds (e.g. Pietrain, Duroc, Czech Landrace, Přeštice Black-Pied) were purchased from a breeding company (Chovservis, Hradec Králové, Czech Republic). Boars were fed standard mixtures of cereals and proteins in the form of dry complete feed mixtures or liquid feeds. Sperm-rich fractions were collected every week (once per week) by gloved-hand method, diluted with Solusem® extender (pH ≈ 7, osmolality ≈ 300 mOsm/kg; AIM Worldwide, Vught, Netherlands), and transported to the laboratory at 17°C. Only sperm samples with at least 75% motile spermatozoa and less than 25% sperm abnormalities were used for the experiments. To reduce the effect of male variability, equal volume of semen samples from different boars was mixed. Then, sperm concentration was ckecked using a Bürker chamber and samples were further diluted with Solusem® to get a final concentration of 20×10^6 spermatozoa/ml. Then, 15 aliquots were made. Equal volume of L-NAME, AG or SNP stock solutions were added to the sperm samples at a tenfold dilution. Therefore, the final concentrations of NOS inhibitors (both L-NAME and AG) on sperm samples were: 0.625, 1.25, 2.5, 5, and 10 mM. The final concentrations of NO donor (SNP) on sperm samples were: 18.75, 37.5, 75, and 150 µM. For the control samples, equal volume of physiological solution was used. All sperm samples were stored at 17°C. Sperm motility, membrane integrity and acrosomal status were analysed at 0 (control only), 4, 24, 48, and 72 h of storage after incubating sperm samples at 38°C for 15 minutes. The experiment was replicated 5 times.

Assessment of sperm motility

A sperm aliquot (5 µl) was loaded into a pre-warmed (38°C) Makler chamber (Sefi-Medical instruments, Haifa, Israel; chamber depth: 10 µm). Sperm motility was evaluated subjectively by estimating the percentage of motile spermatozoa to the nearest 5% and the quality of movement (QM) using a scale from 0 (lowest: no motility) to 5 (highest: progressive and vigorous movements). The sperm motility index (SMI) was calculated according to the

Table 1. Effect of selective iNOS inhibitor on boar sperm motility during semen storage at 17°C.

Parameters	Treatments	Time				
		0h	4h	24h	48h	72h
SMI (%)	CTR	63.00 ± 4.47	62.00 ± 4.47	58.00 ± 4.47	53.50 ± 2.24	49.00 ± 2.24
	AG 0.625 mM		61.50 ± 4.87	61.00 ± 5.76	59.50 ± 5.42	54.00 ± 8.77
	AG 1.25 mM		63.50 ± 6.02	65.00 ± 10.16	**63.50 ± 6.98***	**59.50 ± 14.62***
	AG 2.5 mM		67.00 ± 8.37	65.00 ± 5.00	**66.50 ± 2.24****	**67.00 ± 7.79*****
	AG 5 mM		62.00 ± 4.47	65.00 ± 5.00	**62.00 ± 6.47***	**60.50 ± 9.91****
	AG 10 mM		62.00 ± 4.47	59.50 ± 1.12	57.50 ± 3.95	55.00 ± 9.52
ALH (µm)	CTR	2.91 ± 0.19	3.15 ± 0.34	3.12 ± 0.43	2.88 ± 0.14	2.64 ± 0.22
	AG 0.625 mM		3.24 ± 0.27	3.15 ± 0.19	3.17 ± 0.43	**3.36 ± 0.62*****
	AG 1.25 mM		3.03 ± 0.31	3.25 ± 0.16	3.02 ± 0.44	**3.12 ± 0.45***
	AG 2.5 mM		3.03 ± 0.17	2.95 ± 0.32	2.89 ± 0.31	2.76 ± 0.60
	AG 5 mM		3.04 ± 0.24	**2.70 ± 0.40***	**2.46 ± 0.53***	2.47 ± 0.50
	AG 10 mM		2.76 ± 0.37	**2.16 ± 0.23*****	**2.33 ± 0.38****	**2.15 ± 0.57***
VAP (µm/s)	CTR	35.17 ± 3.62	37.33 ± 6.04	37.03 ± 6.99	32.90 ± 3.47	30.31 ± 3.70
	AG 0.625 mM		33.30 ± 4.42	32.21 ± 2.52	32.51 ± 4.38	33.20 ± 4.78
	AG 1.25 mM		34.35 ± 5.21	32.06 ± 5.88	32.78 ± 4.21	30.28 ± 5.14
	AG 2.5 mM		33.20 ± 5.79	**30.90 ± 1.55***	31.68 ± 1.82	28.39 ± 3.34
	AG 5 mM		34.21 ± 2.57	**26.75 ± 2.49*****	**26.62 ± 3.16***	26.53 ± 3.73
	AG 10 mM		**28.23 ± 1.31*****	**22.98 ± 2.46*****	**23.36 ± 1.67*****	**20.61 ± 0.76*****
VCL (µm/s)	CTR	83.00 ± 2.32	89.38 ± 10.44	94.55 ± 12.71	82.37 ± 10.57	75.44 ± 8.73
	AG 0.625 mM		85.06 ± 13.05	85.29 ± 9.35	84.03 ± 13.51	**86.33 ± 11.71***
	AG 1.25 mM		88.52 ± 2.77	87.69 ± 3.54	78.61 ± 11.01	71.87 ± 14.07
	AG 2.5 mM		87.95 ± 5.85	**79.96 ± 5.65****	73.45 ± 8.84	69.93 ± 19.85
	AG 5 mM		92.83 ± 4.71	**66.77 ± 5.96*****	**64.86 ± 11.20*****	**63.04 ± 10.74***
	AG 10 mM		80.18 ± 9.48	**58.27 ± 7.40*****	**61.43 ± 9.44*****	**53.70 ± 11.82*****
VSL (µm/s)	CTR	25.64 ± 3.84	23.32 ± 1.83	23.43 ± 3.73	21.83 ± 3.07	20.40 ± 1.38
	AG 0.625 mM		24.76 ± 3.42	23.49 ± 4.08	23.17 ± 3.76	**25.06 ± 5.93***
	AG 1.25 mM		23.29 ± 4.59	22.24 ± 5.26	24.54 ± 4.81	23.88 ± 4.42
	AG 2.5 mM		21.57 ± 3.17	20.71 ± 1.36	22.96 ± 2.26	20.79 ± 3.32
	AG 5 mM		**18.29 ± 2.47***	**17.23 ± 2.03****	18.32 ± 1.97	18.94 ± 3.33
	AG 10 mM		**14.45 ± 1.05*****	**14.93 ± 1.52*****	**14.38 ± 1.66*****	**14.47 ± 9.21****

Asterisks indicate significant differences between the treatment and the control within each given time (*$p<0.05$; **$p\leq0.01$; ***$p\leq0.001$). SMI: sperm motility index; ALH: amplitude of lateral head displacement; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; CTR: control; AG: aminoguanidine. Data are shown as mean ± SD.

formula: [% individual motility + (QM × 20)]/2 (Comizzoli et al. 2001). Sperm kinetics was assessed by a Computer Assisted Sperm Analysis (CASA) (NIS-Elements, Nikon, Tokyo, Japan and Laboratory Imaging, Prague, Czech Republic), which consists of an Eclipse E600 tri-ocular phase contrast microscope (Nikon, Tokyo, Japan), equipped with a 10× negative phase-contrast objective (Nikon, Tokyo, Japan), a warming stage set at 38°C (Tokai Hit, Shizuoka, Japan) and a DMK 23UM021 digital camera (The Imaging Source, Bremen, Germany). A total of four descriptors of sperm kinetics were recorded analyzing 6 randomly selected fields and a minimum of 200 sperm cells per sample: average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight-line velocity (VSL, µm/s), amplitude of lateral head displacement (ALH, µm). The standard parameter settings were as follows: frames per second: 60; minimum of frames acquired: 21; VAP ≥ 10 µm/s to classify a spermatozoon as motile.

Assessment of sperm head membrane integrity

The assessment was performed as previously described (Grieblová et al. 2017). Briefly, sperm samples were incubated with carboxyfluorescein diacetate (stock solution: 0.46 mg/ml in dimethyl sulfoxide), propidium iodide (stock solution: 0.5 mg/ml in PBS), and formaldehyde solution (0.3%) for 10 minutes at 37°C in the dark. Then, 200 spermatozoa were evaluated in each sample using epi-fluorescence microscopy (40× objective) and the sperm cells showing complete green fluorescence of the head were considered to have an intact head membrane.

Assessment of sperm tail membrane integrity

The assessment was performed as previously described (Grieblová et al. 2017), using a hypo-osmotic solution consisting of 7.35 g/l sodium citrate and

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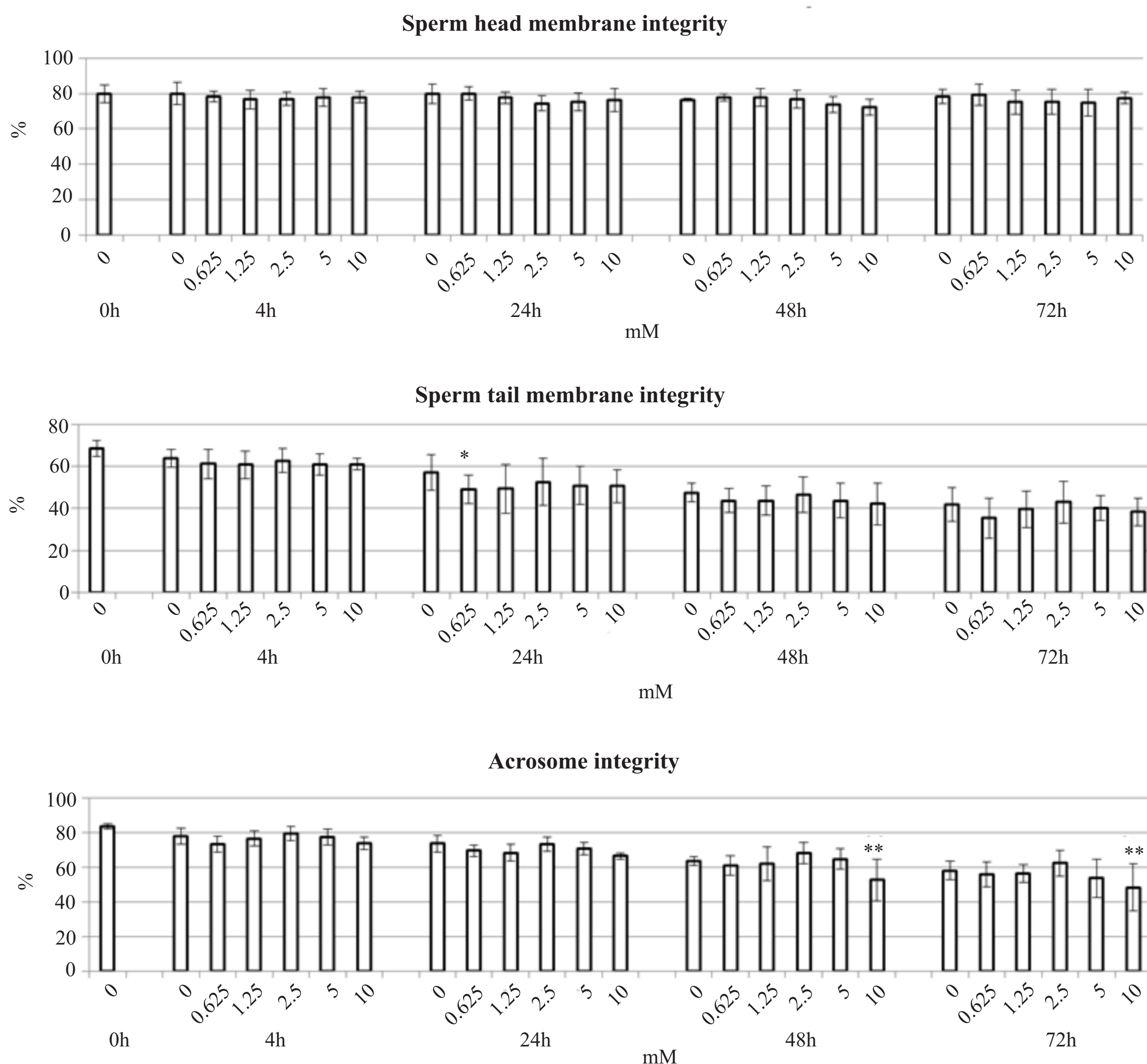

Fig. 1. Effect of selective iNOS inhibitor (aminoguanidine) on boar sperm membrane and acrosome integrity during semen storage at 17ºC.
Asterisks indicate significant differences between the treatment and the control within each given time (*$p<0.05$; **$p\leq0.01$). Data are shown as mean ± SD.

13.51 g/l fructose. Briefly, sperm samples were diluted into pre-warmed HOST solution and incubated for 30 minutes at 38ºC. At the end of the incubation, samples were fixed using a formaldehyde solution (3%). In each sample 200 spermatozoa were evaluated using phase-contrast microscopy (40× objective) and the sperm cells showing swollen tails were considered to have an intact tail membrane.

Assessment of acrosomal status

The sperm samples were fixed in 2% glutaraldehyde solution and examined under phase contrast microscopy (40× objective). In each sample 200 sperm cells were evaluated and the percentage of sperm cells with a normal apical ridge (NAR; Pursel et al. 1972) was determined.

Statistical analysis

All statistical analyses were performed using the SPSS 20.0 statistical software package (IBM Inc, Chicago, IL, USA). We used a generalized linear model (GZLM) to analyze the effects of the treatments and storage times on sperm variables. Data are shown as mean ± SD. Statistical significance was set at $p<0.05$.

Results

Effect of selective iNOS inhibitor on boar sperm parameters

Our results show that AG significantly improved the SMI at 48 h and 72 h of semen storage in all experimental groups (p<0.05), except those treated with the lowest (0.625 mM) and the highest (10 mM) concentrations (p>0.05; Table 1). Concerning sperm kinetic parameters, at 72 h of semen storage we found that 0.625 mM AG increased ALH, VCL, and VSL (p<0.001, p=0.042, and p=0.036, respectively; Table 1). In contrast, at 72 h of storage, the highest concentration of AG (10 mM) significantly decreased all kinetic parameters (p<0.05). The AG has no effect on the sperm head membrane integrity at any concentration and storage time in comparison with the control group (p>0.05; Fig. 1). Conversely, the lowest concentration of AG (0.625 mM) significantly decreased the percentage of sperm with intact tail membrane at 24 h of storage (p=0.046; Fig. 1), whereas the highest concentration of AG (10 mM) gave a significantly lower percentage of sperm with intact acrosome at 48 h and 72 h of storage (p=0.003 and p=0.008, respectively; Fig. 1). No effect was found on the sperm tail membrane and acrosome integrity in samples treated with the remaining AG concentrations (p>0.05).

Effect of non-selective NOS inhibitor on boar sperm parameters

Overall, we found that L-NAME had no effect on boar sperm motility and kinetics at the lowest concentration (0.625 mM; p>0.05), whereas negative effects on ALH and VCL were observed in samples treated with 5 and 10 mM concentrations during whole period of semen storage (p<0.05; Table 2). However, because less than 5% motile sperm were observed at 72 h of semen storage at 5 and 10 mM L-NAME concentrations, sperm kinetics could not be evaluated. In the same way, sperm membrane and acrosome integrity were not affected by L-NAME at the lowest concentration (0.625 mM) at any time of storage (p>0.05), whereas the highest concentration of this NOS inhibitor (10 mM) negatively affected all parameters starting from 24 h of semen storage (p<0.001, Fig. 2).

Effect of NO donor on boar sperm parameters

As shown in Table 3, we found that SNP at concentrations ranging from 18.75 to 150 μM showed mostly no or negative effects on boar sperm motility and kinetics. Concerning the SMI, for example, SNP did not have any effect at the lowest concentration (18.75 μM) during the entire period of storage (p>0.05), whereas the highest concentration (150 μM) of this NO donor significantly decreased the SMI at 48 h and 72 h of storage (p=0.001 and p<0.001, respectively). A significant increase of the SMI was observed only at 24 h of semen storage in samples treated with 37.5 μM SNP (p=0.021). Concerning sperm kinetics, ALH was significantly decreased at 4 h of semen storage at SNP concentrations of 18.75, 75, and 150 μM (p=0.026, p=0.043, and p=0.026, respectively), but at 24 h this parameter was significantly decreased at concentrations of 37.5, 75, and 150 μM (p=0.006, p=0.001, and p=0.002, respectively). Moreover, SNP significantly decreased VCL at any concentration and storage time (p<0.01), although differences were not significant at 72 h in samples treated with 18.75 μM SNP (p>0.05). Moreover, while the lowest SNP concentration (18.75 μM) did not show any effect on the sperm membrane and acrosome integrity during whole period of semen storage (p>0.05; Fig. 3), we found that 37.5 μM SNP increased the percentage of sperm with intact sperm tail membrane at 48 h and 72 h of semen storage (p=0.007 and p=0.001, respectively; Fig. 3). Conversely, the highest concentration of this NO donor (150 μM) significantly decreased the percentage of sperm with intact head membrane at 72 h of semen storage (p<0.001).

Discussion

The results of this study indicate that the inhibition of NO by selective or non-selective NOS inhibitors shows different effects on boar sperm motility, membrane integrity and acrosomal stutus during sperm storage. At 72 h of sperm storage, for instance, the selective iNOS inhibitor AG at concentration of 0.625 mM increased sperm kinetic parameters like ALH, VCL, and VSL, which are related to boar fertility (Broekhujise et al. 2012). The positive effects of AG on boar sperm motility might be due to its antioxidant properties and scavenger activity against free radicals like ROS and RNS (Yildiz et al. 1998). In this way, Abbasi et al. (2011a, 2011b) and Alizadeh et al. (2010, 2016) have shown that AG improves sperm parameters (i.e. concentration, motility, viability, normal morphology, mitochondrial membrane potential, and DNA integrity) in varicocelized rats, where the upregulated iNOS expression leads to high oxidative stress on sperm cells. We therefore hypothesize that the antioxidant properties of AG may protect boar sperm cells against ROS during semen storage. On the contrary, the negative effects of high AG concen-

Table 2. Effect of non-selective NOS inhibitor on boar sperm motility during semen storage at 17°C.

Parameters	Treatments	Time				
		0h	4h	24h	48h	72h
	CTR	63.00 ± 4.47	62.00 ± 4.47	58.00 ± 4.47	53.50 ± 2.24	49.00 ± 2.24
	L-NAME 0.625 mM		63.00 ± 4.47	59.00 ± 2.24	53.00 ± 5.70	52.00 ± 4.47
SMI (%)	L-NAME 1.25 mM		62.00 ± 4.47	56.00 ± 2.24	47.50 ± 3.95	43.00 ± 5.70
	L-NAME 2.5 mM		62.00 ± 4.47	52.00 ± 3.26	**44.50 ± 5.12***	**36.00 ± 4.18****
	L-NAME 5 mM		59.50 ± 3.71	**47.00 ± 2.09****	**33.00 ± 6.71*****	**27.00 ± 6.94*****
	L-NAME 10 mM		**52.50 ± 3.54***	**39.00 ± 6.75*****	**23.00 ± 5.70*****	**19.00 ± 4.18*****
	CTR	2.91 ± 0.19	3.15 ± 0.34	3.12 ± 0.43	2.88 ± 0.14	2.64 ± 0.22
	L-NAME 0.625 mM		3.15 ± 0.21	3.32 ± 0.26	3.26 ± 0.39	2.87 ± 0.24
ALH (μm)	L-NAME 1.25 m		M3.01 ± 0.28	3.01 ± 0.34	2.99 ± 0.48	2.38 ± 0.46
	L-NAME 2.5 mM		2.93 ± 0.22	2.73 ± 0.44	**2.36 ± 0.19****	1.73 ± 0.24
	L-NAME 5 mM		**2.58 ± 0.36****	**2.30 ± 0.31*****	**1.86 ± 0.18*****	N/A
	L-NAME 10 mM		**2.36 ± 0.35*****	**2.19 ± 0.34*****	**1.20 ± 0.53*****	N/A
	CTR	35.17 ± 3.62	37.33 ± 6.04	37.03 ± 6.99	32.90 ± 3.47	30.31 ± 3.70
	L-NAME 0.625 mM		38.91 ± 5.82	35.53 ± 5.38	30.50 ± 6.42	30.19 ± 3.71
VAP (μm/s)	L-NAME 1.25 mM		37.71 ± 6.32	34.07 ± 6.02	31.69 ± 9.19	**23.84 ± 1.81***
	L-NAME 2.5 mM		36.89 ± 3.76	**30.69 ± 4.46***	**26.27 ± 6.33***	**18.33 ± 4.24*****
	L-NAME 5 mM		32.87 ± 5.32	**25.69 ± 1.91*****	**16.79 ± 1.40*****	N/A
	L-NAME 10 mM		**28.61 ± 5.37*****	**19.39 ± 2.20*****	**14.85 ± 1.41*****	N/A
	CTR	83.00 ± 2.32	89.38 ± 10.44	94.55 ± 12.71	82.37 ± 10.57	75.44 ± 8.73
	L-NAME 0.625 mM		94.96 ± 9.64	92.79 ± 10.56	86.09 ± 11.11	82.93 ± 6.51
VCL (μm/s)	L-NAME 1.25 mM		86.68 ± 6.21	87.47 ± 13.47	83.00 ± 15.71	67.23 ± 6.89
	L-NAME 2.5 mM		85.63 ± 3.69	**81.21 ± 13.00***	**69.08 ± 10.88***	**52.79 ± 4.01*****
	L-NAME 5 mM		**77.59 ± 7.75***	**66.08 ± 10.12*****	**53.27 ± 3.99*****	N/A
	L-NAME 10 mM		**65.56 ± 10.96*****	**59.14 ± 14.9*****	**38.31 ± 21.23*****	N/A
	CTR	25.64 ± 3.84	23.32 ± 1.83	23.43 ± 3.73	21.83 ± 3.07	20.40 ± 1.38
	L-NAME 0.625 mM		25.04 ± 2.37	25.47 ± 3.49	21.63 ± 6.32	22.94 ± 4.62
VSL (μm/s)	L-NAME 1.25 mM		25.92 ± 3.75	25.79 ± 4.12	24.42 ± 8.44	19.62 ± 2.44
	L-NAME 2.5 m		M26.09 ± 2.24	24.07 ± 3.47	21.90 ± 6.18	15.94 ± 4.33
	L-NAME 5 mM		22.84 ± 3.06	21.01 ± 1.25	**12.91 ± 3.49*****	N/A
	L-NAME 10 mM		21.16 ± 2.41	**15.09 ± 3.62*****	**13.60 ± 0.03****	N/A

Asterisks indicate significant differences between the treatment and the control within each given time (*$p<0.05$; **$p\leq0.01$; ***$p\leq0.001$). SMI: sperm motility index; ALH: amplitude of lateral head displacement; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; CTR: control; L-NAME: Nω-Nitro-L-arginine methyl ester hydrochloride; N/A: not available. Data are shown as mean ± SD.

trations (10 mM) might be explained by the inhibition of catalase activity leading to a reduced hydrogen peroxide (H_2O_2) removal (Ou and Wolff 1993). Given that the catalase content in boar semen is low (Foote 1962), the consequently increase of H_2O_2 levels may promote cell membrane damage by lipid peroxidation, which in turn may decrease sperm motility and acrosome integrity. Nevertheless, further studies have to be performed in order to evaluate the effects of AG treatment on boar sperm parameters under induced oxidative stress.

Our findings concerning the effect of L-NAME, a non-selective NOS inhibitor, are overall in agreement with previous studies showing that L-NAME negatively affects sperm motility by decreasing the percentage of sperm cells showing rapid progressive motiliy as well as by increasing immotile spermatozoa (human: Rosselli et al. 1995, ram: Hassanpur et al. 2007). In human spermatozoa, Lewis et al. (1996) found that L-NAME decreases VAP, VCL, and VSL, which were also negatively affected in our study. Because of its non-selective inhibitory activity against NOS isoforms, the negative effects of L-NAME might be the consequence of extremely low NO levels. Under no capacitating conditions, as in our study, boar spermatozoa produce low and constant levels of NO

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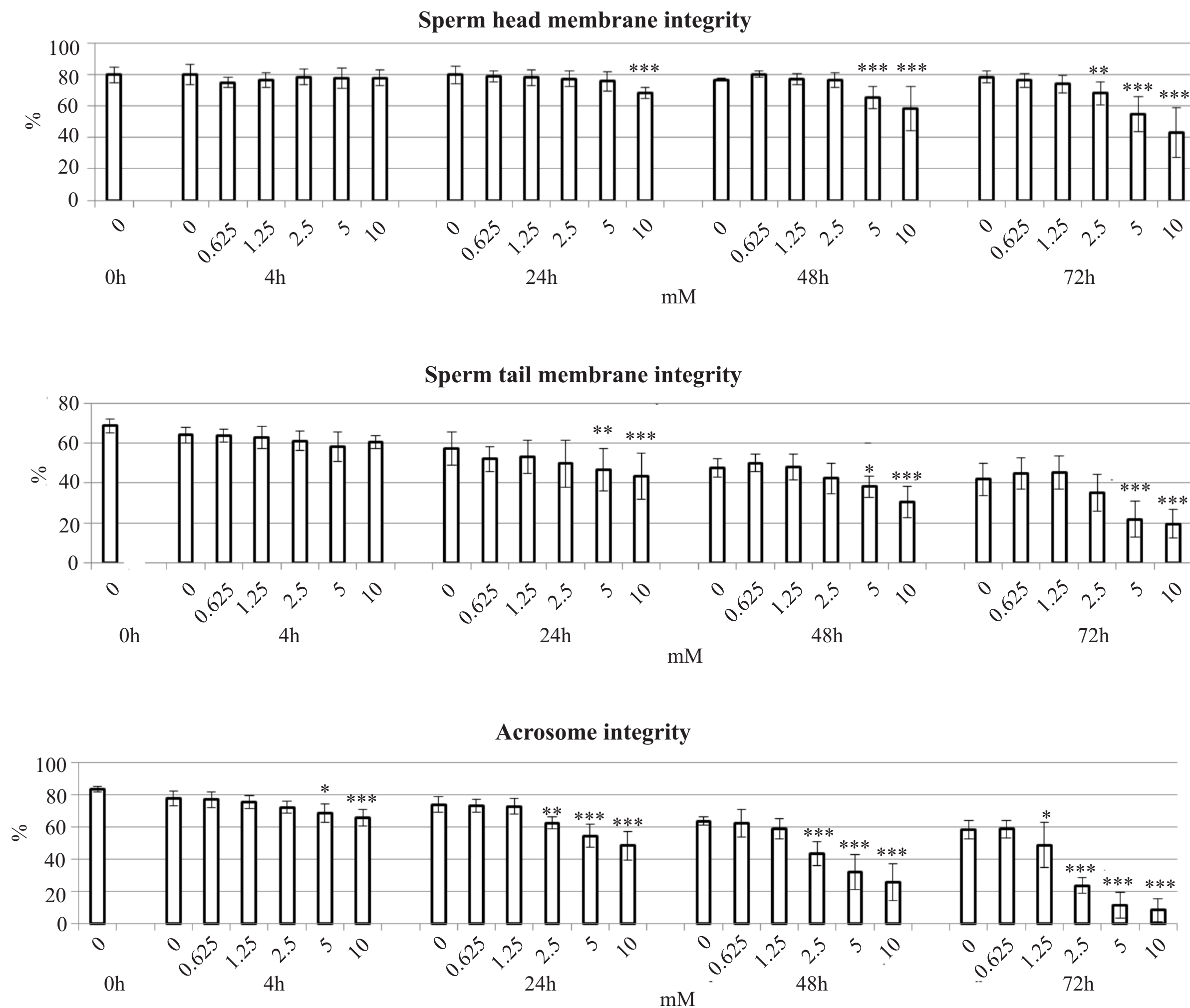

Fig. 2. Effect of non-selective NOS inhibitor (L-NAME) on boar sperm membrane and acrosome integrity during semen storage at 17°C.
Asterisks indicate significant differences between the treatment and the control within each given time (*p≤0.05; **p≤0.01; ***p≤0.001). Data are shown as mean ± SD.

(Hou et al. 2008), which are important for sperm function (Awda et al. 2009). In human, for instance, NO stimulates sperm motility via activation of the soluble guanylate cyclase/cyclic guanosine monophosphate (sGC/GMP) pathway (Miraglia et al. 2011). The inhibition of the sGC/GMP pathway by NOS inhibitors may represent another plausible mechanism responsible for the decreased boar sperm motility in presence of high concentrations of L-NAME or AG. However, in pathological conditions like varicocele, L-NAME exerts positive effects on sperm concentration and morphology, but not on motility (Bahmanzadeh et al. 2008).

Similarly to our findings, previous studies have shown that concentrations of the NO donor SNP ranging from 0.1 to 2,500 μM have no or only negative effects on sperm motility (Rosselli et al. 1995, Weinberg et al. 1995, Rodriguez et al. 2005, Hassanpour et al. 2007, Rahman et al. 2014). In this way, Balercia et al. (2004) have found that astenoozoospermic men exhibit higher levels of NO than those of normozoospermic men. In the same study, authors also found that NO levels were negatively related to the sperm motility, VCL, and VSL, providing further support to our findings. On the other hand, positive effects of SNP on sperm motility and viability were observed at much lower concentrations (i.e. 25-100 nM) (Hellstrom et al. 1994, Zhang and Zheng 1996). The negative effects of SNP might be related to the caspase activation that promotes cell apoptosis (Moran et al. 2008). In this way, Zhang and Zheng (1996) found that concentrations of SNP higher than 100 nM show detrimental effects on sperm viability both in fertile and asthenozoospermic infertile men. In addition to this mechanism, more recently Rahman et al. (2014) found that SNP decreases sperm kinetic parameters

Table 3. Effect of NO donor on boar sperm motility during semen storage at 17°C.

Parameters	Treatments	Time				
		0h	4h	24h	48h	72h
SMI (%)	CTR	63.00 ± 4.47	62.00 ± 4.47	58.00 ± 4.47	53.50 ± 2.24	49.00 ± 2.24
	SNP 18.75 µM		60.50 ± 4.47	58.50 ± 10.55	55.50 ± 6.22	43.50 ± 14.96
	SNP 37.5 µM		62.50 ± 4.33	**68.00 ± 3.71***	54.50 ± 10.95	45.50 ± 16.24
	SNP 75 µM		60.50 ± 4.47	59.00 ± 7.83	48.00 ± 11.65	**40.00** ± 19.96*
	SNP 150 µM		60.00 ± 6.37	50.50 ± 10.52	**39.00 ± 8.94*****	**31.00 ± 17.82*****
ALH (µm)	CTR	2.91± 0.19	3.15 ± 0.34	3.12 ± 0.43	2.88 ± 0.14	2.64 ± 0.22
	SNP 18.75 µM		**2.72 ± 0.12***	2.95 ± 0.52	2.92 ± 0.47	2.74 ± 0.27
	SNP 37.5 µM		2.78 ± 0.21	**2.54 ± 0.21****	2.60 ± 0.53	2.58 ± 0.22
	SNP 75 µM		**2.76 ± 0.20***	**2.46 ± 0.15*****	2.57 ± 0.16	2.68 ± 0.21
	SNP 150 µM		**2.72 ± 0.14***	**2.48 ± 0.71****	2.87 ± 0.53	2.39 ± 0.00
VAP (µm/s)	CTR	35.17 ± 3.62	37.33 ± 6.04	37.03 ± 6.99	32.90 ± 3.47	30.31 ± 3.70
	SNP 18.75 µM		32.54 ± 3.47	**27.97 ± 6.41*****	28.38 ± 4.73	26.13 ± 4.05
	SNP 37.5 µM		**30.98 ± 4.53***	**28.32 ± 3.98*****	**25.09 ± 4.17****	29.68 ± 2.86
	SNP 75 µM		32.57 ± 3.26	**27.31 ± 5.08*****	**26.50 ± 2.87***	**23.20 ± 5.04****
	SNP 150 µM		**29.90 ± 7.70****	**25.87 ± 4.47*****	**24.86 ± 4.10****	30.17 ± 1.48
VCL (µm/s)	CTR	83.00 ± 2.32	89.38 ± 10.44	94.55 ± 12.71	82.37 ± 10.57	75.44 ± 8.73
	SNP 18.75 µM		**74.45 ± 1.54****	**66.67 ± 5.14*****	**66.17 ± 12.84****	64.46 ± 9.23
	SNP 37.5 µM		**68.79 ± 7.39*****	**62.32 ± 5.40*****	**55.80 ± 8.93*****	**59.23 ± 1.96****
	SNP 75 µM		**69.14 ± 5.66*****	**59.47 ± 5.95*****	**58.53 ± 3.02*****	**51.38 ± 2.42*****
	SNP 150 µM		**68.55 ± 9.57*****	**57.72 ± 1.93*****	**54.59 ± 7.96*****	**57.07 ± 3.41****
VSL (µm/s)	CTR	25.64 ± 3.84	23.32 ± 1.83	23.43 ± 3.73	21.83 ± 3.07	20.40 ± 1.38
	SNP 18.75 µM		26.29 ± 3.38	22.04 ± 7.08	22.68 ± 6.48	21.12 ± 5.00
	SNP 37.5 µM		25.06 ± 3.74	23.62 ± 4.57	21.30 ± 4.21	26.59 ± 3.16
	SNP 75 µM		26.71 ± 3.99	22.43 ± 5.73	22.51 ± 3.21	**18.71 ± 7.03***
	SNP 150 µM		23.61 ± 6.23	21.03 ± 5.08	19.61 ± 5.23	26.55 ± 0.80

Asterisks indicate significant differences between the treatment and the control within each given time (*$p<0.05$; **$p \le 0.01$; ***$p \le 0.001$). SMI: sperm motility index; ALH: amplitude of lateral head displacement; VAP: average path velocity; VCL: curvilinear velocity; VSL: straight-line velocity; CTR: control; SNP: sodium nitroprusside. Data are shown as mean ± SD.

by increasing intracellular Fe^{2+} and ROS levels and by decreasing Ca^{2+} and adenosine triphosphate (ATP) levels. Although several studies show that SNP induces the acrosome reaction in capacitated spermatozoa (human: Revelli et al. 2001, bull: Rodriguez et al. 2005, boar: Hou et al. 2008, mouse: Rahman et al. 2014), as expected, we found no effect on the acrosome integrity given that in our experimental design the semen was evaluated under no capacitating conditions.

Conclusion

Our results show that low concentration of AG increases sperm kinetics and may indicate the potential use of this selective iNOS inhibitor to palliate the effects oxidative stress during semen storage. Moreover, high concentrations of both selective and non-selective NOS inhibitors negatively affect sperm kinetics and acrosome integrity, which suggests that low NO levels are necessary for boar sperm physiology. Concerning NO donor, we found that SNP concentrations from 18.75 till 150 µM had mostly no or only negative effects on boar sperm parameters during semen storage. In conclusion, the results from this study increase the understanding of the role of NO on boar sperm physiology.

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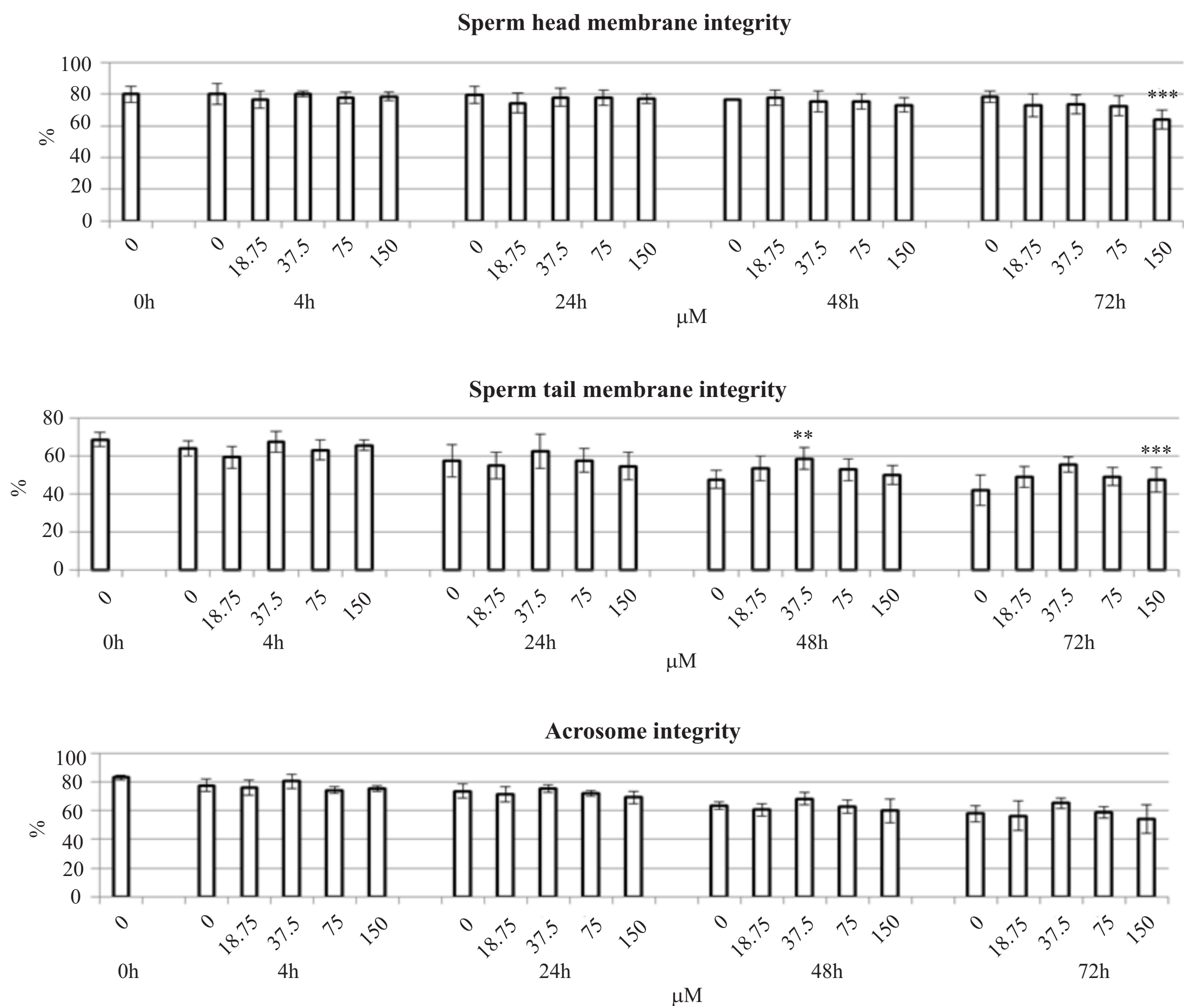

Fig. 3. Effect of NO donor (SNP) on on boar sperm membrane and acrosome integrity during semen storage at 17°C. Asterisks indicate significant differences between the treatment and the control within each given time (**$p\leq0.01$; ***$p\leq0.001$). Data are shown as mean ± SD.

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