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Nitric Oxide and Superoxide Anion Production During Heparin-Induced Capacitation in Cryopreserved Bovine Spermatozoa

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Contents

The aim of this work was to quantify NO, O_2^- and $ONOO^-$ production during heparin-induced capacitation of cryopreserved bovine spermatozoa. A time dependent hyperbolic increase was observed for heparin-dependent capacitation, O_2 uptake, and NO production. Conversely, O_2^- production was increased during the first 15 min of incubation, showing a decrease from this time until 45 min. At 15 min of heparin incubation, a threefold increase in O_2 consumption (5.9 ± 0.6 nmol/min $\times 10^7$ cells), an enhancement in NO release (1.1 ± 0.2 nmol/min $\times 10^7$ cells), and a five-fold increase in O_2^- production (1.3 ± 0.07 nmol/min $\times 10^7$ cells), were observed. Peroxynitrite production rate was estimated taking into account NO and O_2^- generation and the second-order rate constant of the reaction between these species. To conclude, heparin-induced capacitation of cryopreserved bovine spermatozoa activates (i) mitochondrial O_2 uptake by high ADP levels due to increased energy requirements, (ii) NO production by a constitutive NOS and (iii) O_2^- production by a membrane-bound NAD(P)H oxidase. The products of both enzymes are released to the extracellular space and could be involved in the process of sperm capacitation.

Introduction

Mammalian spermatozoa require a preparation period denominated capacitation to acquire the ability to fertilize mature oocytes. During this process, changes in plasma membrane fluidity, O_2 uptake and metabolism, intracellular ionic concentration and the activity of several enzymes, take place. Capacitation is followed by an exocytotic event termed acrosome reaction that is an absolute requisite for fertilization (Yanagimachi 1994). The molecular mechanism that underlies sperm capacitation has not been completely clarified but the process is regulated by a number of intracellular signals involving protein kinase A (PKA) (Leclerc et al. 1996), protein kinase C (PKC) (Thundathil et al. 2002) and protein tyrosine kinase (PTK) (Leclerc et al. 1997).

Mitochondrial oxidative phosphorylation is essential for the energy production required for sperm motility (Dreanno et al. 1999). It has been reported that mitochondria from frozen–thawed bovine spermatozoa retain an adequate respiratory chain function (Beconi et al. 1990; Beorlegui et al. 1997), and that the presence of heparin greatly increases flagellar beat cross frequency (Chamberland et al. 2001) and O_2 consumption (Córdoba et al. 2006). There is evidence that sperm capacitation involves an oxidative process and that superoxide anion (O_2^-) production is associated with capacitation in human (De Lamirande and Gagnon 1993) and bovine spermatozoa (O'Flaherty et al. 1997, 1999). It has been suggested that there is a NAD(P)H oxidase, responsible for O_2^- production during capacitation (De Lamirande et al. 1997). A NAD(P)H oxidase-like activity that produces O_2^- to the extracellular medium has been reported in the spermatozoa of all mammalian species examined to date (Aitken 1997; Fisher and Aitken 1997), i.e. rat (Vernet et al. 2001), bull (O'Flaherty et al. 1999), horse (Sabeur and Ball 2006; Burnaugh et al. 2007), buffalo (Roy and Atreja 2007) or human (De Iuliis et al. 2006). In addition to the above-mentioned NAD(P)H oxidase, the leakage of electrons from the mitochondrial electron transport chain has been considered as a O_2^- source (Aitken 1997; Vernet et al. 2001).

In addition, nitric oxide (NO) may be generated from the oxidation of L-arginine to L-citrulline by sperm nitric oxide synthases (NOS) during heparin-induced capacitation. Previous results from our laboratory showed that exogenous NO acts as a capacitation inducer, in a process that involves the participation of PKA, PKC and PTK as part of the intracellular mechanisms that lead to capacitation in cryopreserved bovine spermatozoa (Rodriguez et al. 2005). Experiments performed with antibodies raised against NOS showed that this enzyme is associated with the acrosome and the tail of mouse spermatozoa (Herrero et al. 1996) and appears to be involved in the fertilization process, including sperm motility and acrosome reaction (Herrero et al. 1997). Furthermore, indirect immunofluorescence assays showed that human spermatozoa express a constitutive NOS in the post-acrosomal and equatorial segments (Lewis et al. 1996), and spin trapping experiments confirmed that NO is synthesized by the human male gamete (Herrero et al. 2001). Previous results revealed that mammalian spermatozoa exhibit NOS activity for the biosynthesis of NO in several species like boar (Hou et al. 2008; Moran et al. 2008), human (Zhang et al, 2007) and bull (Meiser and Schulz 2003; Reyes et al. 2004) and that NO is involved in sperm capacitation (Hou et al. 2008).

Taking into account that exogenous NO and O_2^- induce capacitation in bovine and human spermatozoa, and that NOS inhibitors, NO and O_2^- scavengers prevent capacitation induced by heparin in bovine spermatozoa, the aim of this work was to quantify the endogenous NO and O_2^- generation rates during heparin-induced capacitation and their involvement in sperm motility, capacitation-like changes and O_2 uptake in cryopreserved bovine spermatozoa.

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Materials and methods

Semen freezing

Semen was collected by means of an artificial vagina from five pedigree Holstein bulls (4–5 years old) of proven fertility. The bulls belong to a controlled program of artificial insemination and were maintained under uniform nutritional and management conditions during the period of research. Bulls were kept in individual pens and received alfalfa hay and grass (10–12 kg/day), concentrate (2 kg/day) and water (*ad libitum*). For all ejaculates, progressive motility was greater than 70% and the percentage of abnormal spermatozoa was lower than 20%. Two ejaculates from each bull were obtained once a week during 12 weeks; they were pooled and diluted in a buffer containing 0.20 mmol/l Tris, 0.06 mmol/l citrate, 0.12 mmol/l glycine, 0.06 mmol/l fructose, 20% egg yolk and 7% glycerol at a 2 : 1 ratio. Final concentration was 3.0–4.5×10^7 spermatozoa/ml. A slow cooling curve to 5°C (1°C per min) was performed, and the semen was then equilibrated at 5°C for a further 90-min period. It was frozen at –76°C on dry ice, and pellets were preserved at –196°C in liquid nitrogen (O'Flaherty et al. 1999).

Evaluation of sperm concentration, motility and viability

Assessment of sperm concentration in semen or in sperm suspensions was conducted in a Neubauer chamber and expressed as spermatozoa/ml. Progressive motility was evaluated by light microscopy, 400× magnification (Globe microscope HKS-12, China) with a thermal stage (37°C) three times by the same observer; immediately post-thaw, at zero time and after each time of each treatment in order to assess the quality of the samples used for the determinations. The percentage of live spermatozoa was determined by the supravital eosin/nigrosin technique (Pintado et al. 2000). At least 200 spermatozoa were counted in each sample.

Preparation of the sperm suspension

Samples of frozen semen were thawed for 10 min in Tyrode's (99 mmol/l NaCl, 3.1 mmol/l KCl, 0.35 mmol/l NaH_2PO_4, 10 mmol/l Hepes, 1.1 mmol/l $MgCl_2$, 25 mmol/l $NaHCO_3$, 1 mmol/l sodium pyruvate, 21.6 mmol/l sodium lactate) albumin lactate pyruvate medium, pH 7.4 (TALP), at 36°C, in a 1 : 3 ratio (Parrish et al. 1998). Samples were washed by centrifugation (Centrifuge 5415 R-Eppendorf, China) at $600 \times g$ for 5 min at 38°C to separate the seminal plasma and the freezing buffer. The pellets were resuspended in TALP added with 2 mmol/l $CaCl_2$ and 6 mg/ml BSA, to a final concentration of about of $2–6 \times 10^7$ spermatozoa/ml. This buffer was used as incubation medium for all the experiments (Rodriguez et al. 2005). In those conditions, 1×10^7 spermatozoa/ml resulted equivalent to 1 mg protein/ml (Dong et al. 2000).

Sperm suspensions corresponding to each experience were incubated for 45 min at 38°C under 5% CO_2 in humidified air in the presence or absence of heparin as capacitation inducer (Fukui et al. 1990; O'Flaherty et al. 1997). Initial time was considered at the moment of addition of heparin. Aliquots of sperm suspension were obtained at several incubation times, and they were used to evaluate capacitation-like changes, O_2 consumption, NO release and O_2^- production. Experiments adding 500 μmol/l N^G-nitro-L-arginine methyl-ester hydrochloride (L-NAME), as inhibitor of NOS, and 2 μmol/l diphenyleneiodonium chloride (DPI), as inhibitor of NAD(P)H oxidase, were performed.

Determination of capacitation state

The chlortetracycline (CTC) fluorescent technique was used to detect changes in the plasma membrane of the bovine spermatozoon (Fraser et al. 1995). Three patterns were observed: F (fluorescent), intact non-capacitated spermatozoa displaying fluorescence throughout their surface; C (capacitated), intact capacitated spermatozoa that had lost fluorescence in the post-acrosomal region; AR (acrosome reacted), spermatozoa with a reacted acrosome that had lost fluorescence in the post-acrosomal and acrosomal regions, expressing fluorescence only in the mid piece. Chlortetracycline (500 μmol/l) was added to an equal volume of the medium containing the spermatozoa. Glutaraldehyde was then added to the mixture reaching a final concentration of 0.1%. Slides were examined at 400× magnification under fluorescence excitation at 410 nm using a Carl Zeiss Jena Jenamed 2 epifluorescence microscope. The percentage of capacitated spermatozoa determined at initial time was subtracted from the values obtained in control and capacitated samples to rule out cells damaged during freezing–thawing, 200 spermatozoa were counted in every experiment.

Oxygen consumption

Spermatozoa respiration was measured polarographically at 38°C with a Clark-type O_2 electrode (Oroboros oxygraph, Innsbruck, Austria). Spermatozoa were placed in a 1.5 ml electrode chamber suspended in incubation medium at 6×10^7 spermatozoa/ml. The evaluation of spermatozoa respiration was carried out during 45 min of heparin induced capacitation. Experiments adding 500 μmol/l L-NAME and 2 μmol/l DPI were performed. Oxygen uptake was expressed as nmol O_2/min $\times 10^7$ cells.

Nitric oxide production

Nitric oxide production was measured by following the oxidation of oxyhaemoglobin (HbO_2) to methaemoglobin at 38°C, of the sperm suspension (1.5×10^7 spermatozoa/ml) in the presence of 4 μmol/l SOD and 15 μmol/l HbO_2 (Murphy and Noack 1994; Valdez and Boveris 2001). The NO assay was performed using a Beckman DU 7400 diode array spectrophotometer in which the active wavelength was set at 577 nm and the reference wavelength at the isosbestic point at 591 nm (ϵ = 11.2/mM/cm). Experiments adding 500 μmol/l L-NAME to the sperm suspension were performed. The oxidation of HbO_2 inhibited by L-NAME was used to calculate the amount of NO released, this latter expressed as nmol NO/min $\times 10^7$ spermatozoa.

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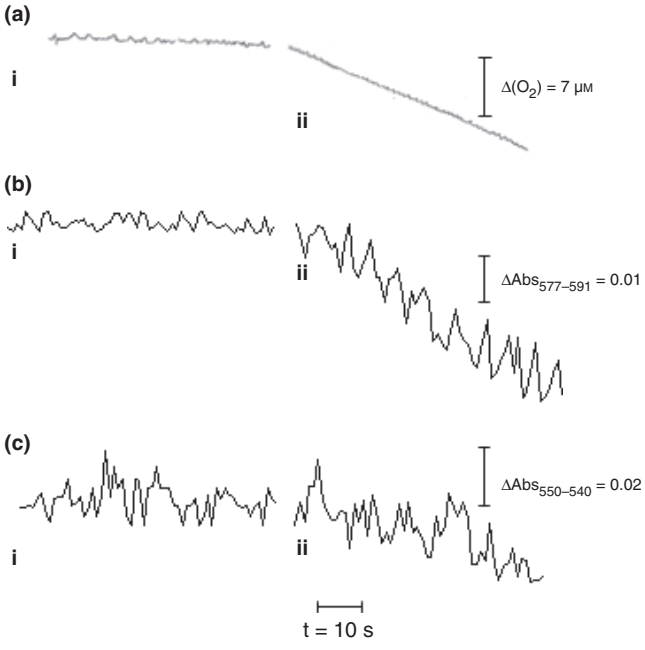

Fig. 1. Representative traces of determination of O_2 consumption (a), NO production (b) and O_2^- production (c) at 15 min of sperm incubation in the absence (i) or in the presence of 10 IU/ml heparin (ii)

Superoxide anion production

Superoxide anion production was measured spectrophotometrically by following the reduction of 40 μmol/l partially acetylated ferricytochrome *c*, by the sperm suspension (1.5×10^7 spermatozoa/ml) at 38°C (Boveris et al. 2002). The O_2^- assay was performed using a Beckman DU 7400 diode array spectrophotometer in which the active wavelength was set at 550 nm and the reference wavelength at the isosbestic point at 540 nm (ϵ = 19/mM/cm), in the absence or in the presence of 4 μmol/l SOD. The reduction of ferricytochrome *c* inhibited by SOD was used to calculate the amount of O_2^- released (McCord and Fridovich 1969; Boveris and Cadenas 1975), this latter expressed as nmol O_2^-/min × 10^7 spermatozoa. Experiments adding 500 μmol/l L-NAME and 2 μmol/l DPI were carried out.

Statistical analysis

The data in figures and tables are reported as mean values ± SEM of four to six independent experiments. For the analysis of different experimental conditions, the Analysis of variances (ANOVA) and the *post hoc* Bonferroni test were used. A value of $p < 0.05$ was considered as statistically significant.

Results

Oxygen consumption

Figure 1a illustrates a representative trace of O_2 uptake, measured polarographically at 15 min of sperm incubation. The addition of heparin produced a threefold increase in O_2 consumption at 15 min of incubation (5.9 ± 0.6 nmol O_2/min × 10^7 cells) respect to the control (1.9 ± 0.2 nmol O_2/min × 10^7 cells) (Figs 1a and 2). The difference between O_2 uptake from capacitated and non-capacitated spermatozoa was observed until the end of incubation (45 min). In order to study the possible role of NO in the regulation of mitochondrial respiratory chain, L-NAME was added to the capacitation medium. Although the O_2 uptake of heparin plus L-NAME treated spermatozoa was slightly higher than the O_2 consumption of spermatozoa treated only with heparin in the first 10 min of incubation, this difference was not significant (Fig. 2). O_2 uptake was not detectable in spermatozoa incubated with heparin plus DPI in the studied conditions (Table 1).

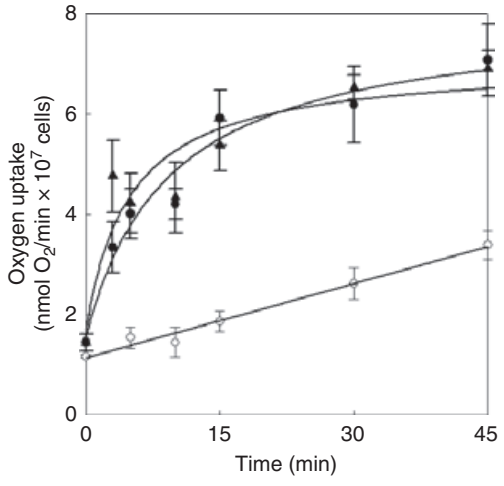

Fig. 2. Oxygen consumption during 45 min of sperm incubation (6×10^7 sp/ml). Control (white circles), heparin (black circles), heparin + L-NAME (black triangles)

Nitric oxide production

Figure 1b illustrates the rate of HbO_2 oxidation during 1 min of reaction of 15 min heparin capacitated spermatozoa suspension in comparison with a non-capacitated spermatozoa sample. Although sperm viability and progressive motility were not affected by the different treatments (data not shown), sperm hyperactivation characterized by a specific motility pattern acquired during capacitation, was evidenced in the NO production assay traces, in spermatozoa capacitated with heparin (Figs 1b and 3a). The rate of HbO_2

Table 1. Oxygen uptake and nitric oxide and superoxide anion release from bovine spermatozoa during capacitation (15 min of incubation)

	O_2 uptake (nmol/min × 10^7 cells)	NO production (nmol/min × 10^7 cells)	O_2^- production (nmol/min × 10^7 cells)
No addition (control)	1.86 ± 0.20	0.04 ± 0.003	0.23 ± 0.02
Heparin	5.92 ± 0.55*	1.1 ± 0.2*	1.27 ± 0.07*
Heparin + L-NAME	5.37 ± 0.50*	–	1.03 ± 0.10*
Heparin + DPI	n.d.	–	0.23 ± 0.03†

Additions: 10 IU/ml heparin, and 0.5 mM L-NAME or 2 μM DPI.
*$p < 0.01$ vs. control. †$p < 0.01$ vs. heparin. n.d.: non-detectable amounts.

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oxidation from capacitated spermatozoa decreased more than 50% in the presence of L-NAME (Fig. 3a). As the presence of heparin greatly increases flagellar beat cross frequency, the variability in the absorbance signal was more marked for heparin-added samples than for control and L-NAME-added samples. Figure 3b shows the time course of the HbO_2 oxidation produced by spermatozoa. The addition of L-NAME significantly decreased oxyhemoglobin oxidation ($p \leq 0.05$). Considering the HbO_2 oxidation rates in the absence and in the presence of L-NAME in the incubation medium, NO production was calculated. Samples added with heparin showed a sustained increase in NO production (Fig. 3b, dashed line) reaching a plateau between 15 and 45 min of incubation. At 15 min of incubation, capacitated spermatozoa produced 1.1 ± 0.2 nmol NO/min × 10^7 cells (Table 1). Control samples did not show NO release, indicating that non-capacitated spermatozoa do not produce detectable amounts of NO.

Capacitation-like changes

The fraction of capacitated spermatozoa was determined at different times of incubation. During the first 15 min of incubation with heparin, a time-dependent increase in the percentage of capacitated spermatozoa was observed, reaching a plateau at about 40 min (Fig. 4a), when the fraction of capacitation was significantly higher in spermatozoa incubated with heparin (37.3 ± 1.1%) than in control samples (6.3 ± 0.3%). Capacitation-like changes were significantly reduced by the addition of L-NAME (8.3 ± 0.3%; Fig. 4a), indicating that NO is involved in the capacitation process. Interestingly, similar patterns were observed for heparin-dependent capacitation (Fig. 4a), O_2 uptake (Fig. 2), and NO production (Fig. 3b). These parameters showed a hyperbolic increase as a function of time. There was a significant correlation ($r^2 = 0.98$; $p < 0.05$) between the fraction of heparin capacitated spermatozoa and NO production (Fig. 4b).

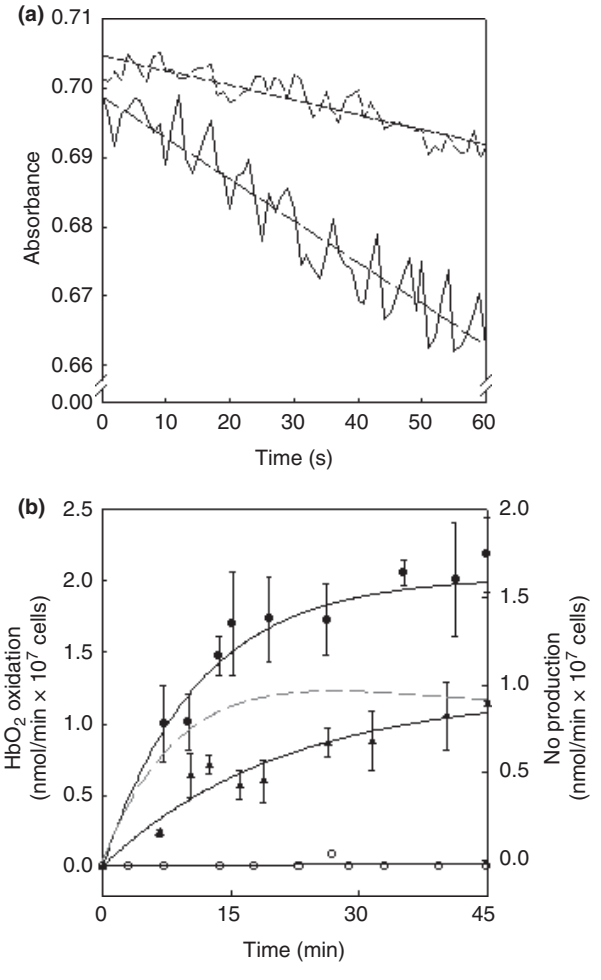

Fig. 3. HbO_2 oxidation during 45 min of sperm incubation ($1–2 \times 10^7$ sp/ml). (a) Representative traces of HbO_2 oxidation kinetics obtained at 15 min of sperm incubation with heparin (full lines) or with heparin + L-NAME (dashed lines). (b) Control (white circles), heparin (black circles), heparin + L-NAME (black triangles). NO production of heparin capacitated spermatozoa (grey dashed curve), obtained by the difference between HbO_2 oxidation by heparin-incubated spermatozoa and HbO_2 oxidation by heparin + L-NAME incubated spermatozoa

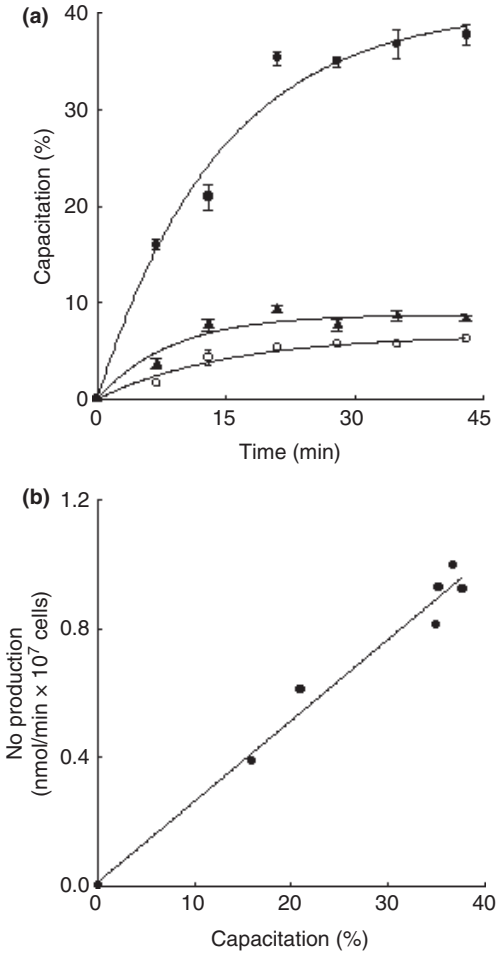

Fig. 4. (a) Sperm capacitation during 45 min of sperm incubation ($1–2 \times 10^7$ sp/ml). Control (white circles), heparin (black circles), heparin + L-NAME (black triangles). (b) Correlation between NO production and sperm capacitation ($r^2 = 0.98$; $p < 0.05$) of spermatozoa suspension incubated in the presence of heparin

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Superoxide anion production

Figure 1c illustrates a trace of partially acetylated cytochrome *c* reduction by O_2^- during heparin-induced capacitation. Superoxide anion production rate was increased during the first 15 min of incubation, showing a decrease from this time until 45 min (Fig. 5). Superoxide anion production at 15 min of incubation in heparin induced spermatozoa, was five times higher (1.3 nmol/min × 10^7 cells) than in control samples (Table 1). The addition of 2 μM DPI to the incubation medium significantly diminished O_2^- production rates at 15 min by about 80%, whereas the supplementation with L-NAME did not produce significant differences as compared to the spermatozoa incubated only with heparin (Table 1).

Progressive motility and sperm viability

Progressive motility (evaluated by light microscopy) and sperm viability (assessed by the supravital eosin/nigrosin technique) were not affected ($p \leq 0.05$) by any of the treatments (data not shown).

Discussion

Sperm capacitation is an energy-dependent process in which the ATP generated by the respiratory chain is employed during the hyperactivation phase, which is needed to fertilize the oocyte (Dalvit et al. 1995). Although cryopreservation modifies plasma membrane (Watson 1996) and mitochondrial integrity needed for sperm function (Windsor 1997), cryopreserved spermatozoa retain an adequate respiratory chain function (Beconi et al. 1990). In this study, capacitation with heparin generated an increase in O_2 uptake that may be partially due to the production of O_2^- by a membrane NAD(P)H oxidase previously described in mammalian spermatozoa (Aitken 1997; Aitken et al. 2003), but also to an enhancement in the activity of the mitochondrial respiratory chain, mainly evidenced by the slightly effect of L-NAME on O_2 uptake during the first 15 min of incubation of spermatozoa with heparin (Fig. 2). Respiratory chain activity and ATP production are related to changes in the motility patterns and to hyperactivation, this latter observed in this study as a change in the variability of the absorbance signal during the measurement of NO production (Fig. 3a). When the fraction of total O_2 uptake involved in O_2^- production and the one implicated in NO generation were calculated, it was observed that 21% of the consumed O_2 is used to produce O_2^- (Equation 1) and 19% is utilized to produce NO (Equation 2).

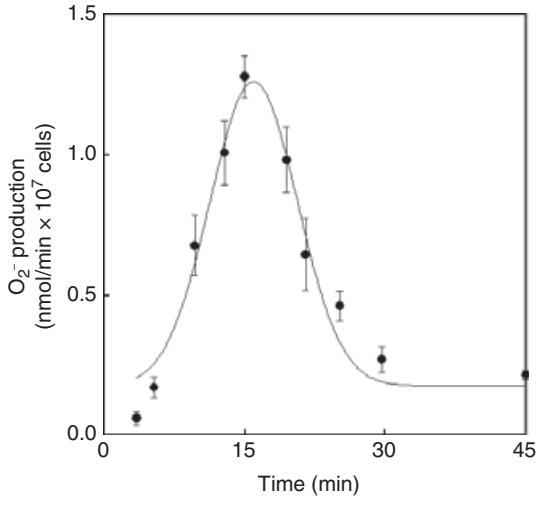

Fig. 5. Superoxide production during 45 min of incubation of heparin capacitated spermatozoa (1–2 × 10^7 sp/ml).

$$NADPH + 2\,O_2 \rightarrow NADP^+ + H^+ + 2O_2^- \quad (1)$$

$$\text{L-arginine} + 2\,O_2 + 1.5\,NADPH \rightarrow \text{L-citruline} + NO + 1.5\,NADP^+ + 2H_2O \quad (2)$$

The rest of the O_2 (60%) could be consumed in the mitochondrial respiratory chain to produce the ATP necessary to support the sperm energy requirements. In our experimental model, the energy obtained from oxidative substrates (pyruvate and lactate) would be enough to support *in vitro* sperm capacitation in cryopreserved bovine spermatozoa.

Exogenous NO induces capacitation in bovine (Rodriguez et al. 2005) and human (Herrero and Gagnon 2001) spermatozoa. Both the inhibition of NOS by L-NAME and the scavenging of NO by hemoglobin, diminish the capacitation induced by heparin (Herrero et al. 1994; Donnelly et al. 1997; Rodriguez et al. 2005). In this work, we showed that cryopreserved bovine spermatozoa produce endogenous NO during heparin-induced capacitation. As HbO_2 oxidation was inhibited by the addition of L-NAME to spermatozoa suspension incubated with heparin, we conclude that NO is actually produced by sperm NOS in a process stimulated by intracellular mechanisms triggered by heparin. This finding is coincident with previous results described in boar (Hou et al. 2008; Moran et al. 2008) and human (Zhang et al. 2007) spermatozoa. The presence of NOS in bull spermatozoa was recognized by immunohistochemistry, indicating the existence of both neuronal NOS (nNOS) and endothelial NOS (eNOS) protein (Meiser and Schulz 2003). The nNOS and eNOS isoforms are calcium dependent and constitutively expressed (Burnett et al. 1995). As calcium is one of the major necessary factors for capacitation (Herrero and Gagnon 2001), this cation may modulate the activity of sperm NOS (Hou et al. 2008). Endogenous NO produced during capacitation participates in intracellular mechanisms that lead to the activation of PKA, PKC and PTK (Rodriguez et al. 1995; Herrero et al. 2003; De Jonge 2005; O'Flaherty et al. 2006a) and tyrosine phosphorylation of proteins involved in capacitation (Herrero et al. 2003; Thundathil et al. 2003; O'Flaherty et al. 2006b).

Considering that the increase in O_2 consumption observed in heparin capacitated spermatozoa could be due to the activation of a membrane NAD(P)H oxidase (Vernet et al. 2001), O_2^- generation was measured. Cryopreserved bovine spermatozoa produced O_2^- during the first 15 min of capacitation. These findings are in agreement with the spermatozoa O_2^- production

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reported for several mammalian species (O'Flaherty et al. 1999; Vernet et al. 2001; Sabeur and Ball 2006; Burnaugh et al. 2007; Roy and Atreja 2007; De Iuliis et al. 2006). As it is shown in Fig. 5, the rate of O_2^- production increased during the first 15 min and diminished from this time until 45 min of capacitation. This pattern is not coincident with the one observed for O_2 uptake (Fig. 2), NO production (Fig. 3b), capacitation (Fig. 4a) or calcium uptake (Córdoba et al. 2006). All these parameters showed a hyperbolic increase up to 45 min of capacitation. It has been previously reported that at low concentrations, DPI is a recognized inhibitor of NAD(P)H oxidase (Cross and Jones 1986) and prevents the respiratory burst in cryopreserved bovine spermatozoa (Córdoba et al. 2006). Taking these data into account, the inhibition of O_2^- generation observed with 2 μmol/l DPI (Table 1) indicates that the source of O_2^- detected is the NAD(P)H oxidase of sperm plasma membrane.

The simultaneous NO and O_2^- generation during bovine crypreserved spermatozoa capacitation presented in this study suggests that $ONOO^-$ is also formed. This may be part of the molecular mechanism involved in capacitation. The rate of $ONOO^-$ production at 15 min of sperm capacitation can be calculated using the differential equation (3), and taking into account that the reaction between NO and O_2^- to yield $ONOO^-$ has a second-order rate constant, $k_1 = 1.9 \times 10^{10}$/M/s (Kissner et al. 1997):

$$d[ONOO^-]/dt = k_1[NO][O_2^-] \quad (3)$$

The steady-state concentrations of NO and O_2^- in the capacitating incubation medium are estimated from the experimentally assessed production rates (Table 1) at 15 min of heparin-induced capacitation. The double steady-state approach considers that both NO and O_2^- production rates equal the consumption rates of these species (Equations (4) and (5)):

$$d[NO]/dt = -d[NO]/dt = 1.1\,\text{nmol/min} \times 10^7\text{cells} = 3.8 \times 10^{-8}\ \text{M/s} \quad (4)$$

$$d[O_2^-]/dt = -d[O_2^-]/dt = 1.3\,\text{nmol/min} \times 10^7\text{cells} = 4.3 \times 10^{-8}\ \text{M/s} \quad (5)$$

These production rates, the differential Equations (6) and (7), and the corresponding second-order rate constants for the formation of $ONOO^-$ (k_1) and for the spontaneous dismutation of O_2^- ($k_2 = 5.0 \times 10^5$ M/s; Bielsky 1985), were used to estimate the steady-state concentrations of NO and O_2^-:

$$d[NO]/dt = k_1\,[NO][O_2^-] \qquad [NO] = 1.8 \times 10^{-11}\ \text{mol/l} \quad (6)$$

$$d[O_2^-]/dt = k_1\,[NO]\,[O_2^-] + k_2\,[O_2^-]^2 \qquad [O_2^-] = 1.1 \times 10^{-7}\ \text{mol/l} \quad (7)$$

The rate of $ONOO^-$ production results about 4×10^{-8} M/s. This value indicates that NO concentration is limiting or determining for $ONOO^-$ formation in the incubation medium. We have recently reported that $ONOO^-$ is involved in heparin-induced capacitation and induces capacitation in cryopreserved bovine spermatozoa (Rodriguez et al. 2009). The fact that uric acid impairs heparin-induced capacitation (Rodriguez and Beconi 2009) and the above estimated $ONOO^-$ production rate, suggest the involvement of $ONOO^-$ in physiological sperm capacitation.

According to our results we conclude that heparin-induced capacitation-like changes of cryopreserved bovine spermatozoa activates (i) mitochondrial O_2 uptake by high ADP levels due to increased energy requirements, (ii) NO production by a constitutive NOS and (iii) O_2^- production by a membrane-bound NAD(P)H oxidase. The products of both enzymes are released to the extracellular space and could be involved in sperm capacitation.

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Author contributions

M beconi had the original idea, was involved in experimental design and wrote initial draft. P Rodriguez, L Valdez and T Zaobornyj performed much of the laboratory work including suggestions to procedures and edited the draft. A Boveris was involved in discussions of experimental design and results and edited the draft.

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