Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 70 (2008) 91–96
Theriogenology
www.theriojournal.com

Nitric oxide induces caspase activity in boar spermatozoa

J.M. Moran, L. Madejón, C. Ortega Ferrusola, F.J. Peña *

Service of Reproduction and Obstetrics, Veterinary Teaching Hospital, Avd. de la Universidad s/n, 10071 Cáceres, Spain

Received 11 April 2007; received in revised form 12 February 2008; accepted 13 February 2008

Abstract

Nitric oxide (NO) is a highly reactive free radical that plays a key role in intra- and intercellular signaling. Production of radical oxygen species and an apoptotic-like phenomenon have recently been implicated in cryodamage during sperm cryopreservation. The objective of the present study was to evaluate the effect of sodium nitroprusside (SNP), an NO donor, on boar sperm viability. Semen samples were pooled from four boars that were routinely used for artificial insemination. Flow cytometry was used to compare semen incubated with SNP to control semen. Specifically, NO production was measured using the NO indicator dye diaminofluorescein diacetate, and caspase activity was determined using the permeable pan-caspase inhibitor Z-VAD linked to FITC. SNP induced a significant increase in the percentage of sperm cells showing caspase activity, from 9.3% in control samples to 76.2% in SNP-incubated samples ($P < 0.01$). This study suggests that NO is a major free radical involved in boar sperm damage.

Keywords: Boar sperm; NO; Betulinic acid; Caspases

1. Introduction

Technologies such as cryopreservation cause major damage to sperm. During this process, freezing leads to mechanical damage due to the formation of ice crystals and to chemical damage due to osmotic stress [1]. An advancing ice front (i.e. the ice crystal surface) binds water molecules while physically excluding other molecules: ice therefore damages sperm both because it acts like an agricultural plow and because its formation leads to the accumulation of salts, solutes, and gases that can have osmotic and toxic effects. The increased volume of ice versus water may exacerbate damage. Biochemical damage such as lipid peroxidation, premature ageing, and phase membrane transitions have been identified as important factors in sub-lethal damage of the spermatozoa that survive freezing and thawing [2]. Recently, the role of an ''apoptosis like'' phenomena in cellular injury induced by cryopreservation was reported [3,4]. Cryopreservation induces the formation of an excess of reactive oxygen species (ROS) that reduce subsequent sperm performance [2]. Peroxidation induces structural alterations, a fast and irreversible loss of motility, profound changes in metabolism, and a rapid release of intracellular components [5]. Spermatozoa are particularly susceptible to ROS-induced damage because their plasma membranes contain relatively large percentages of polyunsaturated fatty acids and their cytoplasm contains relatively low concentrations of scavenging enzymes [6–8]. Moreover, the intracellular antioxidant enzymes cannot protect the plasma membrane that surrounds the acrosome and the tail, forcing spermatozoa to depend, due to their limited intrinsic antioxidant capabilities, on the protection provided by the seminal plasma [9,10]. While the roles of free radicals such as the superoxide anion (O_2^-), peroxide (H_2O_2), and the

* Corresponding author at: Section of Reproduction and Obstetrics, Department of Medicine, Faculty of Veterinary Medicine, Avd. de la Universidad s/n, 10071 Cáceres, Spain.
E-mail address: fjuanpvega@unex.es (F.J. Peña).

0093-691X/$ – see front matter
doi:10.1016/j.theriogenology.2008.02.010

hydroxyl free radical ($^{\bullet}OH$) have been extensively studied, the role of NO has received much less attention in veterinary and human andrology. In a biological context, an important NO reaction is the direct bimolecular reaction with O_2 that yields peroxynitrite (ONOO–), a very potent oxidant that has been show to react in vitro with virtually all classes of biomolecules. During procedures where there is high ROS production, such as during cryopreservation, NO may be one of the major free radicals causing cryodamage. In fact, it has been demonstrated that human semen produces peroxynitrite and that its production is increased in infertile men [11]. The aim of the present study was to evaluate the effect of NO on boar spermatozoa and to determine its impact on the plasmalemma.

2. Materials and methods

2.1. Media

Tyrode's complete medium (TCM) was used. This consisted of 96 mM NaCl, 4.7 mM KCl, 0.4 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 5.5 mM glucose, 1 mM sodium pyruvate, 21.6 mM sodium lactate, 0.5 mM $CaCl_2$, 10 mM $NaHCO_3$, 20 mM HEPES (pH 7.45), and 3 mg/ml BSA. The medium was equilibrated with 5% CO_2. All media were made on the day of use and maintained at an osmolarity of 310 mOsm kg^{-1} and at pH 7.45 at 38 °C.

2.2. Collection and washing of semen

Commercial semen doses that had been processed for artificial insemination (AI) were obtained from CENTROTEC (Campo de Villavidel, León, Spain) and were received at the Laboratory of Andrology in the Veterinary Teaching Hospital of the University of Extremadura within 12 h of collection. Samples were from four boars and contained 3×10^9 spermatozoa in 100 ml of a commercial extender. The diluted semen of all boars was pooled to avoid individual effects during experiments. Pooled semen was centrifuged (10 min, $800 \times g$) and washed twice with TCM. Samples (1.5 ml) containing approximately 1×10^8 spermato-spermatozoa/ml were incubated in TCM at 38 °C. All experiments were repeated five times.

2.3. Staining for detection of activated caspases

The caspase FITC-VAD-FMK in situ marker (Molecular Probes Europe, Leiden, The Netherlands) was used to detect active caspases. This cell permeable caspase inhibitor peptide is conjugated to FITC and binds covalently to activated caspases, thus serving as an in situ marker for apoptosis [3]. Spermatozoa were diluted to 1×10^6 in 1 ml of PBS; 1 μl of FITC-VAD-FMK was added and the sample was incubated at room temperature in the dark for 20 min. Sperm were then washed in PBS and resuspended to the initial cell concentration. Ethidium homodimer was added (1 μl of a 1.167 mM solution; Molecular Probes Europe, Leiden, The Netherlands) to detect dead spermatozoa, and flow cytometry and fluorescence microscopy were conducted within 10 min.

2.4. Staining for assessment of subtle membrane changes and sperm viability

Membrane changes and sperm viability were determined as described by Peña et al. [12]. In brief, the following stock solutions were prepared in DMSO: YO-PRO-1 (25 μM) and ethidium homodimer-1 (1.167 mM; Molecular Probes Europe, Leiden, The Netherlands). Next, 500 μl of a sperm suspension containing 5×10^6 spermatozoa/ml were stained with 3 μl of YO-PRO-1 and 1 μl of ethidium homodimer. After thorough mixing, the sperm suspension was incubated at 37 °C in the dark for 16 min. The staining patterns were examined using combined phase contrast and fluorescence microscopy (Nikon-E80, Nikon, Tokyo, Japan).

2.5. Staining for detection of NO

NO was detected and quantified using an NO detection reagent, DAF-2 DA (Molecular Probes Europe, Leiden, The Netherlands). After washing, spermatozoa were resuspended to a concentration of 3×10^6 cells ml^{-1} in Tyrodes. DAF-2 DA was added to the suspension at a final concentration of 10 mM and incubated at 38 °C for 1 h DAF-2. It is a cell-permeable derivative of DAF-2. Upon entry into the cell, DAF-2 diacetate is transformed into the less cell-permeable DAF-2 by cellular esterases thus preventing loss of signal due to diffusion of the molecule from the cell. In the presence of oxygen, DAF-2 reacts with NO to yield the highly fluorescent triazolofluorescein (DAF-2T) After centrifugation at $750 \times g$ for 3 min, spermatozoa were resuspended to a concentration of $1–3 \times 10^6$ cells and then cultured in an atmosphere of 5% CO_2 at 39 °C. NO was detected by flow cytometry analysis of spermatozoa by monitoring emission fluorescence at 522 nm.

2.6. Flow cytometry

Flow cytometric analyses were carried out with a Coulter EPICS XL (Coulter Corporation Inc., Miami,