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Enzymatic scavengers in the epididymal fluid: Comparison between pony and miniature breed stallions

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ABSTRACT

The use of stallion semen collected from cauda epididymis for AI has increased due to the new protocols available for cryopreservation. Preserving the genetic material from valuable males that suffer sudden death or other events that prematurely end the stallion's reproductive life is an important strategy for Stud breeding management. While protecting spermatozoa from oxidative stress and infectious agents, the epididymis promotes the enhancement of sperm cell morphology and changes in membrane protein profile, increasing its fertility potential. The epididymal fluid must be a balanced redox environment to allow sperm preservation and protein–protein and protein–lipids interactions to quantify. The aim of this study was quantify the enzymatic ROS scavengers in epididymal fluid of pony and miniature breed stallions. Epididymides from 8 pony stallions and 12 miniature breed stallions were dissected and fluid from caput, corpus and cauda epididymis collected. Spermatozoa were separated of epididymal fluid by 2-step centrifugation. The activities of catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) were measured and compared between stallion groups and epididymal regions. The three enzymes were present in all epididymal regions tested, with higher activities of catalase and SOD in cauda epididymis in miniature breed stallions ($P < 0.05$). GPx activity was higher in caput epididymis in pony stallions ($P < 0.05$), however with no difference to fluid from cauda epididymis of both breeds. These results show a difference in antioxidant enzymatic scavengers between pony and miniature breed stallions. Also, our data confirm the protective role of cauda epididymis, preserving spermatozoa integrity from oxidative damage. As glutathione peroxidase is involved in several signaling pathways, its constant activity during epididymal transit corroborates the importance of this enzyme for spermatozoa maturation.

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1. Introduction

The interest in stallion epididymis has increased in the last years not only because of its importance to sperm quality and fertility potential, but also due to the development of protocols for cryopreservation of epididymal semen. These protocols aim to preserve the genetic material from valuable males that suffer sudden death, castration or other events that prematurely end the stallion's reproductive life (Cary et al., 2004; Bruemmer, 2006; Papa et al., 2008). Investigations on the preservation on epididymal sperm for future use proposed methods for epididymal sperm collection (Bruemmer, 2006) and use of extenders for flushing and cryopreservation (Papa et al., 2008; Pasquini et al., 2008; Pucci et al., 2008; Weiss et al., 2008).

The epididymal environment regulates different biochemical processes that are fundamental for sperm maturation (Sostaric et al., 2008). Most of the biochemical characteristics of the spermatozoa are modulated during epididymal transit. Specifically in the plasma membrane, these modifications involve not only changes in the composition of glycoproteins and sterols (Retamal et al., 2000) but also results in a selective and progressive loss of phospholipids, elevating the proportion of cholesterol (Jones, 1998; Jones et al., 2007). Cholesterol, as the precursor of all sexual steroid hormones, has a pivotal role in sperm production. However, its importance goes further, since studies with epididymosomes (Rejraji et al., 2006) and the presence of different lipoprotein receptors and cholesterol transmembrane transporters in the epididymis show that cholesterol homeostasis is critical to ensure the normal function of the male gametes (Saez et al., 2011). In sperm physiology cholesterol is used in steroidogenesis and is an important part of the plasma membrane and is an endogenous ligand of LCN 1, a lipocalin that appears to be part of the complex defense system involved in protection against harmful molecules and microbial and fungal infections (Robaire et al., 2006). Therefore, intact lipid molecules are necessary for a functional sperm membrane.

The most frequent damage suffered by spermatozoa plasma membrane is the attack by reactive oxygen species (ROS) due to its high polyunsaturated fatty acids (PUFA) content (Alvarez and Storey, 1985; Ball and Vo, 2002; Brouwers et al., 2005). Consequently, loss of motility and fertilizing capacity are among the deleterious effects of lipid peroxidation on spermatozoa (de Laraminde et al., 1997; Engel et al., 1999; Lenzi et al., 2000; da Silva et al., 2011). As a natural spermatozoa reservoir, the epididymis possesses a complex antioxidant system aiming to prevent oxidative damages on sperm lipids, proteins and DNA (Vernet et al., 2004). The main enzymatic scavengers catalase, superoxide dismutase (SOD) and glutathione peroxidase (GPx) have been shown to be present along the epididymis in different mammals (Zini and Schlegel, 1997; Tramer et al., 1998; Drevet, 2006). In the stallion, catalase was described in cauda epididymal fluid in lower concentration when compared to seminal plasma (Ball et al., 2000). The same group described SOD and GPx in equine reproductive tissues, fluids and sperm, observing that equine seminal plasma contains high activity of SOD, although equine spermatozoa have limited GPx and SOD-like activity (Baumber and Ball, 2005). Aiming to better understand the antioxidant status during post-testicular maturation, the present work compared the activities of catalase, SOD and GPx in the epididymal fluid of standard size and miniature breed stallions.

2. Material and methods

2.1. Animals and samples

All chemicals were obtained from Sigma Chemical Co. (St. Louis, USA). For the experiment, epididymides from 8 pony size (PS group) stallions, aged between 3 and 5 years and 12 Brazilian Miniature Breed (MB Group) stallions, aged between 3 and 4 years were used. Tissues were collected in stud farms at Rio Grande do Sul State, Brazil, after routine castrations, and transported to the laboratory on ice. The epididymides were dissected, and epididymal fluid from cauda was flushed by use of a needle attached to a syringe filled with 0.1 M potassium phosphate buffer solution (pH, 7.8) that contained 1 mM EDTA. Caput and corpus epididymal fluid was obtained after chopping and centrifugation of dissected epididymal tubules in 100 μL of buffer. Sperm cells were separated from the epididymal fluid in 2 centrifugation steps ($300 \times g$ for 10 min, and $10{,}000 \times g$ for 1 h). After centrifugation, the supernatants were stored at −20 °C until analyzed. An aliquot was taken to determine protein concentration (Lowry et al., 1951).

2.2. Enzymatic assays

Catalase activity was measured at 240 nm using a double beam spectrophotometer with temperature control (Hitachi U-2001®). Thirty microliters of epididymal fluid was added to 720 μL of reaction medium consisted of 20 mM H_2O_2, 0.1% Triton X-100, and 10 mM potassium phosphate buffer pH 7.0. One unit is defined as 1 μmol of hydrogen peroxide consumed per minute, and specific enzyme activity is reported as units per milligram protein (Aebi, 1984; Bustamante-Filho et al., 2009).

SOD activity was carried out as described elsewhere (Marklund, 1985; Bhandari et al., 2007; Bustamante-Filho et al., 2009). The inhibition of autoxidation of pyrogallol occurs in the presence of SOD, whose activity was indirectly assayed spectrophotometrically at 420 nm (Hitachi U-2001). In a quartz cuvette were added 30 μL of epididymal fluid samples, 4 μL of 30 μM catalase, 958 μL 50 mM Tris 1 mM EDTA pH 8.2 buffer and 8 μL of 24 mM pyrogallol prepared in 10 mM HCl. Purified SOD was used for a standard calibration curve, in order to calculate the activity of SOD present in the samples. Results were reported as units of SOD/mg protein.

Glutathione peroxidase activity was measured using *tert*-butyl-hydroperoxide as substrate (Wendel, 1981; Munz et al., 1997; Bustamante-Filho et al., 2009). Ninety microliters of epididymal fluid sample were added to an incubation medium containing 790 μL of 100 mM potassium phosphate buffer containing 1 mM EDTA, pH 7.7, 20 μL of 2 mM glutathione, 30 μL of 0.15 U/mL glutathione reductase, 10 μL of 0.4 mM azide, 10 μL of 0.1 mM NADPH (nicotinamide adenine dinucleotide phosphate-oxidase)