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Original article

Molecular forms of selected antioxidant enzymes in dog semen – electrophoretical identification

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Abstract

The aim of the study was the electrophoretical identification of molecular forms of selected antioxidant enzymes in dog semen. Ejaculates to be studied were chosen from five dogs, aged from two to eight years. Polyacrylamide gel electrophoresis was carried out under non-denaturing conditions and then gels were stained for the activity of the following enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Sperm homogenates and all fractions (pre-spermatic, spermatic and post-spermatic) of dog ejaculate demonstrated one protein band with SOD activity characterized by low electrophoretic mobility. Based on the confirmed sensitivity to H_2O_2, it can be assumed that the detected SOD is an enzyme containing ions of Zn^{2+} and Cu^{2+} (Cu,Zn SOD). In sperm homogenates one protein band with GPx activity was characterized by high electrophoretic mobility, whereas in the spermatic and post-spermatic fractions of dog ejaculate three protein bands with different (low, medium and high) electrophoretic mobility were identified.

CAT molecular forms were not found in either sperm homogenates or in the analyzed fractions of ejaculate.

Key words: dog, spermatozoa, semen, antioxidant, enzymes, electrophoresis

Introduction

Reactive oxygen species (ROS) are generated by sperm metabolism. On one hand, low concentrations of ROS are required for maturation, capacitation and acrosome reaction, and on the other, elevated ROS levels cause sperm pathology (ATP depletion leading to insufficient axonemal phosphorylation, lipid peroxidation and loss of motility and viability) (de Lamirande et al. 1997, Pasqualotto et al. 2000).

Mammalian spermatozoa and seminal plasma contain a number of ROS scavengers, including enzymes such as superoxide dismutase (SOD), catalase (CAT) and the glutathione peroxidase/glutathione reductase system (GPx/GR) (de Lamirande et al. 1997). These enzymes are present in various molecular forms, i.e. isoenzymes. The existence of these forms with different quaternary structures but the same catalytic activity is characteristic for animal semen (Strzeżek and Glogowski 1977). Isoenzymes have different physico-chemical properties; as a result their functions adapt to the metabolic needs of sperm or seminal plasma.

Our previous research showed that SOD is the main antioxidant enzyme of dog semen (Strzeżek et al. 2009). GPx activity was found in sperm

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homogenates as well as in spermatic and post-spermatic fraction of dog ejaculate. It was not confirmed whether CAT activity was present in the sperm homogenates or in any of the analyzed fraction of dog ejaculate.
The aim of the present study was the electrophoretical identification of molecular forms of SOD, GPx and CAT in sperm homogenates and individual fractions of dog ejaculate.

Materials and Methods

Animals

Five mixed-breed dogs (aged 2 to 8 years) were used in this study. They were clinically healthy and were under full veterinarian care. The dogs were kept in individual cages with access to water and food. This study was approved by the Local Ethics Committee.

Semen collection

A total of ten ejaculates were collected, using the masturbation method in the presence of female dogs in heat. Three fractions of the ejaculate (pre-spermatic, spermatic, post-spermatic) were separately collected into calibrated glass vessels at 37°C. The volume of individual fractions of ejaculate were evaluated in calibrated tubes. In the case of spermatic fractions, motility and concentration of spermatozoa were determined. Spermatozoal motility was evaluated with a phase-contrast light microscope (Olympus C011) equipped with a heated stage (37°C). The concentration of spermatozoa was counted in a hemocytometric chamber.

Ejaculate fraction treatment

Each fraction of the ejaculate was centrifuged at 12000 x g at room temperature for 15 minutes. The resulting supernatants were designed for studying isoenzyme profiles.

Sperm treatments

Spermatozoa from the spermatic fractions were washed by centrifugation (1000 x g, 5 minutes at room temperature). The sperm pellets were washed twice with 0.85% NaCl and later adjusted to a final concentration of 3 x 10^9 spermatozoa /ml. The sperm samples were homogenized at room temperature using an Ultra-turrax T8 homogenisator (IKA-Werke, Germany). The homogenized samples were centrifuged at 12000 x g at a room temperature. The resulting supernatants were designed for studying isoenzyme profiles.

Native-PAGE analysis

Electrophoresis was carried out in 7.5% polyacrylamide gel, using 0.05 M Tris-glycine buffer (pH 8.3) (Davis 1964). Separating gel and stacking gel used in the study were prepared according to the instructions. Glass tubes (0.5 x 8.7) were filled up to 7.5 cm with a degassed solution of the separating gel and 0.5 cm with stacking gel. The tubes were placed in an electrophoresis apparatus, electrode vessels were filled with 0.05 M Tris-glycine buffer (pH 8.3), and then each gel had a sample placed on it (loaded down with sucrose crystals). An electrophoretical separation was performed with a direct current of 5 mA. All the steps were carried out at 4°C.

After the completion of electrophoresis in the gels, protein fractions were identified and the gels were stained for the activity of SOD, GPx and CAT.

Staining of protein fractions with Amido black

For visualization of protein bands the gels were stained with 0.5% Amido black in 7% acetic acid for five minutes and next destained with a solution of 7% acetic acid (Schaffner and Weissmann 1973).

Staining for SOD activity

SOD activity was identified by using the staining method described by Beauchamp and Fridovich (1971). The gels were incubated in the dark for 20 min in 0.001 M nitroblue tetrazolium (NBT) and than transferred to a solution containing 0.028 M TEMED (tetramethyl ethylenediamine), 28 μM riboflavin and 0.036 M phosphate buffer (pH 7.8) for 15 min. The gels were developed over a fluorescent light for 10 min. Areas showing SOD activity remained unstained, whereas the remainder of the gel stained blue.

In order to identify SOD isoforms, the gels, before being stained as describe above, were incubated in a solution of 0.4% (v/v) H_2O_2, which is an inhibitor of SOD containing Zn^{2+} and Cu^{2+} ions (Fridovich, 1975).

Staining for GPx activity

The staining was done according to the method described by Pavlova et al (1994). The gels obtained after PAGE were soaked in a solution comprising

0.08% diaminobenzidine (DAB), 0.075% H_2O_2 and 0.06 M phosphate buffer (pH 7.2) for 60 min at room temperature. Areas showing GPx activity stained dark brown.

Staining for CAT activity

The gels were rinsed three times for 15 minutes in distilled H_2O and incubated for ten minutes in 0.003% (v/v) of H_2O_2 solution. After the incubation, the gels were rinsed with water and incubated for ten minutes in a solution with a composition of 2% (w/v) $FeCl_2$ and 2% (w/v) $K_3Fe(CN)_6$. The gel stained dark green and the areas containing an active enzyme remained coloruless (Woodbury et al. 1971).

Results

Only ejaculates with normospermia were used in these experiments (Table 1).

three bands were characterized by low electrophoretic mobility, whereas the other two bands exhibited medium and high mobility, respectively. In the pre-spermatic fraction, four proteins bands with different electrophoretic mobilities were identified: one band exhibited low mobility two bands showed medium mobility, whereas the other band was characterized by a high mobility. In the spermatic fraction one protein band migrated in a slow pattern of mobility, three protein bands exhibited medium mobility and only one protein band showed high mobility. The post-spermatic fraction was represented by six protein fractions: three fractions were characterized by low mobility, two bands were characterized by average mobility and the other band showed high mobility.

There were no differences in electrophoregrams of proteins which exhibited SOD activity in sperm homogenates as well as in all fractions of dog ejaculate. This was confirmed because only one protein band with low electrophoretic mobility showed SOD activity. After the incubation of gels in the solution of H_2O_2, the inhibition of SOD activity was confirmed.

Table 1. Macro and microscopic evaluation of dog semen (n = 5). Mean ± SD

Semen volume (cm^3)			Sperm concentration (x $10^6/cm^3$)	Sperm motility (%)
fraction				
Pre-spermatic	Spermatic	Post-spermatic	622 ± 40	83.15 ± 0.96
2.08 ± 0.19	1.00 ± 0,07	5.62 ± 0.38		

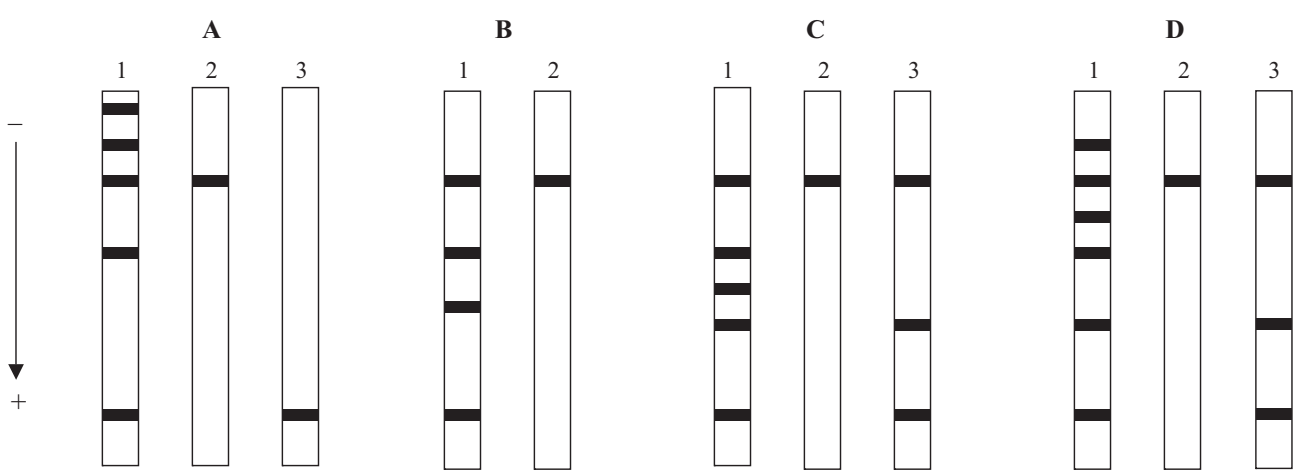

Fig. 1. Electrophoregrams stained for protein (1), SOD (2) and GPx (3) activity in sperm homogenates (A) and fluids of the fractions: pre-spermatic (B), spermatic (C) and post-spermatic (D) of dog ejaculate.

Figure 1 shows the different electrophoretic mobilities of protein bands obtained from sperm homogenates and various fractions of dog ejaculate. Electrophoretic analysis revealed five protein bands with different mobilities in the sperm homogenates:

Staining the gels with DAB and H_2O_2 showed one molecular form of GPx characterized by a high mobility to be present in sperm homogenates. However, in the fluids of the spermatic and post-spermatic fractions of the dog ejaculate, three protein bands were

identified as having activity of this enzyme: one showed low mobility and the other two exhibited medium and high electrophoretic mobility respectively. It was confirmed that there were no protein bands with peroxidase activity in the fluid of the pre-spermatic fraction.

CAT activity was not identified either in sperm homogenates or in the different ejaculate fractions.

Discussion

The fact of the presence of protein bands with SOD activity in sperm homogenates and fractions of ejaculate fluids indicates the mulitple sources of this enzyme in dog semen. This can mean that the source of SOD in dog seminal plasma is the epididymis as well as the prostate. However, confirmation of the inhibition of this enzyme by H_2O_2 proves that this is an isoform with Zn^{2+} and Cu^{2+} ions (CuZn SOD). Furthermore, another isoform of this enzyme – MnSOD, located in the mitochondria, does not demonstrate the sensitivity to this inhibitor (Fridovich 1975).

The occurrence of certain components of the antioxidant system in mammal semen depends on the species (Kowalowka et al. 2008). For example, one SOD molecular form was show to be present in spermatozoa and seminal plasma of boars (Strzeżek et al. 2000, Kowalowka et al. 2008). Based on biochemical studies, this enzyme was classified as an secretory extracellular form of SOD (EC-CuZn SOD) (Kowalowka et al. 2008). On the other hand, three protein bands showing SOD activity were identified in the semen of stallions (Strzeżek et al. 2000). This occurence is most likely caused by species-related differences in the anatomic structure of the reproductive system which directly influences the diversified profile of secreted seminal plasma.

Three protein bands with peroxidase activity in the spermatic fraction proves, that in dog semen GPx and not CAT plays the main role in the decomposition of hydrogen peroxide. Thus, the catalase is responsible for the decomposition of hydrogen peroxide only when it occurs in high, excessive physiological concentrations (Cohen and Hochstein 1963). This is also why GPx activity is the first line of protection responsible for the decomposition of H_2O_2 present in physiological concentrations (Drevet 2006).

As in our previous studies, GPx activity was not shown in the pre-spermatic fraction, which, like a post-spermatic fraction, originates from the prostate (Strzeżek et al. 2009). This occurrence is caused by the specific biochemical mechanism of the enzyme as well as the composition of the pre-spermatic fraction formed by the prostate secretion as well as the secretions from tubular glands (Zduńczyk and Janowski 2010). It could be suggested that the interaction of protein secretions originating from the prostate and tubular glands significantly inhibited GPx activity in the pre-spermatic fraction.

In electrophoregrams of proteins from sperm homogenates and analysed fluids of the different fractions from dog ejaculate no CAT molecular forms were identified. This confirms our early results (Strzeżek et al. 2009) which indicated the lack of CAT secretion in dog ejaculate. Importantly, the CAT activity was shown in the spermatozoa of several species of mammals, e.g. human, stallion and rat (Jeulin et al. 1989, Tramer et al. 1998, Strzeżek et al. 2000). In turn, high CAT activity was confirmed in stallion seminal plasma (Kuklińska et al. 2005).

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