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Activity of antioxidant enzymes and their mRNA expression in different reproductive tract tissues of the male roe deer (*Capreolus capreolus*) during the pre-rut and rut seasons

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ABSTRACT

Roe deer (*Capreolus capreolus*) are seasonal breeders with a short rut season during which there is a significant change in the secretory activity of the testis and epididymis. This study compared the activity of antioxidant enzymes and their mRNA expression levels in the reproductive tract tissues of the adult male roe deer during the pre-rut ($n = 6$ animals) and rut ($n = 6$ animals) seasons. Comparisons were made between tissues within seasons and within tissues between seasons. The activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) was evaluated in testicular and epididymal tissues; and real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was used to analyze mRNA expression levels of the antioxidant enzymes: copper/zinc SOD (SOD1), extracellular SOD (Ec-SOD), CAT, phospholipid hydroperoxide glutathione peroxidase (PHGPx) and GPx5. SOD activity was significantly higher ($P < 0.05$) in all the tissues in the rut season than in the pre-rut season. CAT activity was not significantly different between tissues in the pre-rut season, but was significantly ($P < 0.05$) higher in the caput and corpus epididymal tissues than the other tissues in the rut season and the same tissues in the pre-rut season. Testicular tissue GPx activity was significantly higher ($P < 0.05$) compared with the other tissues in both seasons. Significantly higher ($P < 0.05$) SOD1 mRNA levels were expressed in the three epididymal tissues than in the testicular tissue in both seasons. The caput epididymal tissue expressed higher ($P < 0.05$) levels of Ec-SOD mRNA compared with the other tissues during the pre-rut season, but the differences between the tissues in the rut season were not significant. The corpus and cauda epididymal tissue expression of Ec-SOD mRNA was significantly higher ($P < 0.05$) in the rut season compared with the pre-rut season. CAT mRNA expression level in the caput epididymis during the pre-rut season was significantly higher ($P < 0.05$) than in the other tissues and than in the rut season. Testicular tissue exhibited significantly higher ($P < 0.05$) expression level of PHGPx mRNA than the other tissues in both seasons. PHGPx mRNA expression in testicular tissue in the rut season was approximately 3× the level expressed in the pre-rut season ($P < 0.05$). The caput epididymal tissue expressed very high levels of GPx5 in both seasons, whereas the testicular tissue showed very low levels of GPx5 (both differences at $P < 0.05$). This study

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showed that the activity of antioxidant enzymes and their mRNA expression in the testis and epididymal tissues are dependent on the reproductive season in the male roe deer and suggests that the antioxidant enzyme system is more efficient during the rut season.

1. Introduction

The roe deer are typical seasonal breeders with a short rutting season (Goeritz et al., 2003). In the males the highest level of spermatogenic activity, which coincides with rut in the females, is observed in the summer (from mid-July to mid-August), whereas an arrest of spermatogenesis is observed in the winter (from October to February) (Blottner et al., 1996; Semperé et al., 1998; Goeritz et al., 2003; Martinez-Pastor et al., 2005; Schön and Blottner, 2009). Evidence has been shown that circannual changes in the male roe deer reproductive system are associated with fluctuations in sex hormone levels during the reproductive season (Roelants et al., 2002; Schön and Blottner, 2008; Kozioł and Koziorowski, 2013).

Normal spermatogenesis, sperm maturation and transit require favorable conditions to acquire functional competence (Blottner et al., 1996; Goeritz et al., 2003; Vernet et al., 2004). Protection of these processes in the testis and epididymis against the harmful effects of reactive oxygen species (ROS) is provided mainly by antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), which have complementary roles in cellular tissues and sperm cells (Drevet, 2006; Gancarczyk et al., 2006; Marti et al., 2007; Kowalowka et al., 2008; Koziorowska-Gilun et al., 2011). Several isoforms of GPx have been detected, however, phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) and an epididymal-specific GPx (GPx5) have been shown to play a significant role in the antioxidant enzyme defence system of the male reproductive tract of various animal species (Beiglböck et al., 1998; Vernet et al., 2004; Grignard et al., 2005; Drevet, 2006; Boitani and Puglisi, 2008; Chabory et al., 2010; Koziorowska-Gilun et al., 2013).

Previous studies showed that there were species-specific differences in seasonal variations in the antioxidant defence system of the male reproductive tract (Marti et al., 2007; Kowalowka et al., 2008; Asadpour and Tayefi-Nasrabadi, 2012; Koziorowska-Gilun et al., 2013). To our knowledge, no studies have yet been carried out to analyze the antioxidant enzyme system of the reproductive tract of the male roe deer. Therefore, the present study was designed to characterize the activity of antioxidant enzymes and their mRNA expression levels in the tissues of the testis and epididymidis of the free-ranging mature male roe deer during the pre-rut and rut seasons.

2. Materials and methods

2.1. Animals and collections of reproductive tract tissues

Tissues of the reproductive tract were collected immediately from hunted adult mature roe bucks (*Capreolus capreolus*), aged 3–7 years (defined based on dentition), at the Bieszczady, a region of north-eastern Poland (49°17′ N; 22°7′ E). Due to the limited availability of samples, we examined only two periods of the roe deer reproductive cycle: the pre-rut season (10th–31st May), without sexual display, and the rut season (from-mid July to mid-August), with very short sexual activity. Samples were collected from an equal number of animals in each of the pre-rut and rut seasons ($n = 6$ animals per season). The animals were treated in accordance with the Polish Animal Welfare Act. Tissues of the testis, and caput, corpus and cauda epididymides were excised immediately after hunting, wrapped in aluminum foil, shocked frozen in liquid nitrogen (−196 °C), transported to the laboratory and stored at −80 °C, until required for analysis.

2.2. Preparation of reproductive tract tissues for antioxidant assays and total RNA isolation

All chemicals were purchased from Sigma Chemicals Company (St. Louis, MO, USA), unless stated otherwise. The dissected tissues of the sperm-free testis and epididymides (caput, corpus and cauda) were subjected to homogenization, according to a previously described procedure (Koziorowska-Gilun et al., 2013). Following homogenization, the tissue samples were centrifuged at 15,000 × *g* for 15 min at room temperature, and the supernatants were collected and stored at −80 °C, for subsequent analysis.

2.3. Measurements of antioxidant enzyme activity

Prior to the measurements of the antioxidant enzyme activity, total protein content in the tissue homogenates was measured, according to the method of Lowry et al. (1951), using serum bovine albumin, BSA (Serum and Vaccine Production, Cracow, Poland), as a standard.

Superoxide dismutase activity was determined using the RANSOD assay (Randox Laboratories, Crumlin, UK). This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the condition of the assay (37 °C, pH 7.0). The activity of SOD was measured spectrophotometrically at 505 nm.

A commercial kit (Sigma Aldrich Corp., St. Louis, MO, USA) was used to measure CAT activity by monitoring the decrease in H_2O_2. The quinoneimine dye coupling product, which correlated to the amount of H_2O_2 remaining in the reaction mixture, was measured spectrophotometrically at 520 nm. One unit of CAT decomposed 1 μM H_2O_2 to oxygen and water per minute at a substrate concentration of 50 mM H_2O_2 at 25 °C (pH 7.0).

The Ransel Glutathione Peroxidase kit (Randox Laboratories, Ltd., London, UK) was used to measure GPx activity. In this assay GPx catalyzed the oxidation of GSH by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH, oxidized glutathione (GSSG) was converted to GSH with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance was measured spectrophotometrically at 340 nm. One unit of GPx catalyzed the oxidation of 1 μM NADPH per minute at 37 °C (pH 7.2).

All measurements were performed, using a Beckman Coulter spectrophotometer DU 800 (Beckman Coulter, Inc., Fullerton, CA, USA). The assays were run in duplicate and the results were expressed as U/mg protein.

2.4. Analysis for mRNA expression of antioxidant enzymes

2.4.1. Total RNA isolation

The tissues of the testis, caput, corpus and cauda epididymides were suspended in 1 ml TRI-Reagent (Sigma Aldrich Corp., St. Louis, MO, USA) and were homogenized in a FastPrep-24 apparatus (MP Biomedicals LLC, Ohio, USA) for 45 s. Following homogenization, total RNA was isolated from the tissue samples, according to a previously described method