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Original article

The effect of benign prostatic hyperplasia on total antioxidant capacity and protein peroxidation in canine prostatic fluid and spermatozoa

A. Domosławska[1], S. Zduńczyk[1], A. Bielecka[2] , M. Kankofer[2]

[1] Department of Animal Reproduction with Clinic,
University of Warmia and Mazury, 10-719 Olsztyn, Oczapowskiego 14, Poland
[2] Department of Biochemistry, Faculty of Veterinary Medicine,
University of Life Sciences, 20-033 Lublin, Akademicka 12, Lublin, Poland

Abstract

The aim of this study was to evaluate the antioxidative/oxidative status of spermatozoa and prostatic fluid in dogs with benign prostatic hyperplasia (BPH) by the determination of total antioxidant capacity and protein peroxidation markers. Study was conducted on 40 intact dogs of various breeds. The dogs were assigned to two groups: BPH group (n=20) and non-affected group (n=20). The second and third fractions of the ejaculate were collected separately by digital manipulation. Total antioxidant capacity (TAC) and the concentrations of SH-groups in sperm and prostatic fluid were determined spectrophotometrically, the concentrations of bityrosine and formylkynurenine were determined using spectrofluorimetric methods. The mean values of TAC in spermatozoa and prostatic fluid were significantly lower ($p<0.05$), whereas the mean contents of biotyrosine and formylkinurenine were significantly higher ($p<0.05$) in BPH dogs compared to control dogs. There was no statistically significant difference in the content of SH group between dogs with BPH and control dogs ($p>0.05$). In conclusion, the results indicate that BPH in dogs is associated with reduced total antioxidant capacity and increased protein oxidation in the prostatic fluid and spermatozoa, and suggest the importance of oxidative stress in the pathogenesis of this condition. The potential role of antioxidants in the prevention and therapy of canine BPH requires further studies.

Keywords: dog, BPH, prostatic fluid, total antioxidant capacity, protein peroxidation

Correspondence to: A. Domosławska-Wyderska, e-mail: anna.domoslawska@uwm.edu.pl

Introduction

Benign prostatic hyperplasia (BPH) is the most common and age-related prostatic disease in dogs (Smith 2008). BPH is histologically diagnosed in the dog as early as 2 years of age. More than 95% of intact male dogs over 9 years of age exhibit BPH (Berry et al. 1986). At the beginning, dogs with BPH are frequently asymptomatic. With the progressive enlargement of the prostate, sanguineous discharge from the urethra, dysuria, hematuria, hemospermia and tenesmus may occur. BPH is often associated with the decrease in libido and fertility (Smith 2008, Lévy et al. 2014, Cunto et al. 2022)

The pathogenesis of BPH is not completely understood. BPH involves both an increase in cell numbers (hyperplasia) and in cell size (hypertrophy) (Berry et al. 1986). It is considered that BPH develops under the influence of androgen metabolite dihydrotestosterone (DHT) (Barsanti and Finco 1986). With aging, estrogen/testosterone ratio and the activity of 5-α-reductase, the enzyme that converts testosterone to DHT, increase (Tunn et al. 1988). Estrogens induce nuclear DHT receptors and increase the sensitivity of the prostate to DHT (Gobello and Corrada 2022). In the prostatic tissue of BPH dogs DHT concentrations is elevated (Meikle et al. 1981). DTH stimulates enlargement of the canine prostate by enhancing growth of glandular epithelial cells, and, to a lesser extent, stromal cells (Schäfer-Somi 2023). BPH begins as glandular hyperplasia and transitions to cystic hyperplasia with the formation of multiple small cysts in the parenchyma (Berry et al. 1986, Gobello and Corrada 2002).

Oxidative stress due the imbalance between production and neutralisation of reactive oxygen species (ROS) is recently proposed to contribute to the pathogenesis of BPH in men (Srivastava and Mittal 2005, Aydin et al. 2006, Pace et al. 2010, Minciullo et al. 2015). However, studies on oxidative stress in dogs with BPH are limited and results are inconsistent. Dogs with BPH have been found to have reduced serum antioxidant enzyme activity (Dearakhshandeh et al. 2019). Total serum antioxidant activity (TAC) was reported to be lower in dogs with BPH than in non-affected dogs (Domosławska et al. 2022). However, other study found no significant differences in the oxidative profile between dogs with BPH and healthy dogs (Angrimani et al. 2020).

The prostatic fluid (PF) has a major role in the production of seminal plasma during ejaculation. It constitutes approximately 90% of seminal fluid volume and provides transport and sperm support (Ferré-Dolcet et al. 2022). The PF contains large amounts of proteins that may affect semen quality (Souza et al. 2007, Aquino-Cortez et al. 2017). Protein peroxidation, defined as a reaction causing the covalent modification of proteins, can cause damages to protein structure and changes in protein function (Stadtman and Levine 2000).

Oxidative stress can be assessed by the determination of total antioxidant capacity and the end products of peroxidation. The measure of antioxidant capacity (TAC) considers the cumulative action of all the antioxidants present in a biological sample (Ghiselli et al. 2000). A high level of ROS may results in the production of amino acids carbonyl derivatives such as bityrosine and formylkinourenine, and to decrease of sulfhydryl-groups (SH-groups) content. These measurements can be used as marker of protein peroxidation (Kankofer 2001, Halliwell and Whiteman 2004).

To now there are no data on TAC and protein peroxidation in the PF and ejaculated spermatozoa (ES) of dogs with BPH. Thus, the aim of this study was to evaluate the antioxidative/oxidative status of PE and ES in dogs with BPH by the determination of total antioxidant capacity and protein peroxidation markers.

Materials and Methods

Reagents

All of the chemicals used in the experiments were of analytical grade and, unless otherwise stated, purchased from Sigma-Aldrich (Poznań, Poland).

Animals

The study was conducted on 40 intact dogs of various breeds. The males were presented at the Department of Animal Reproduction with Clinic Faculty of Veterinary Medicine in Olsztyn because of sanguineous discharge from the urethra or for the evaluation of semen quality. The dogs were assigned to two groups: BPH group (n=20) and control group (n=20). The diagnosis of BPH was based on history, clinical symptoms like sanguineous discharge from the urethra, dysuria, tenesmus and enlargement of the prostate on rectal palpation and ultrasound examination (Mindray Bio-Medical 2 with a 7.5-MHz convex transducer). The control animals showed no clinical signs and the prostate was not enlarged. The age of the dogs ranged from 5 to 8 years and averaged 7.4±0.9 years in the BPH group and 6.7±0.7 years in the control group (Table 1). The dogs were fed with commercial premium dry diets.

Table 1. Breeds and age of healthy dogs and dogs with benign prostatic hyperplasia (BPH).

Healthy dogs		Dogs with BPH	
Breed	Age (years)	Breed	Age (years)
Akita	6.5	Akita	7
Cavalier king charles spaniel	6.5	Beagle	8.5
Cavalier king charles spaniel	6	Cavalier king charles spaniel	7
Cavalier king charles spaniel	7.5	Cavalier king charles spaniel	8
Continental bulldog	6	Cavalier king charles spaniel	8.5
Dachshund	7	Continental bulldog	6.5
Fox terrier smooth	5.5	Fox terrier smooth	6
Fox terrier smooth	5.5	Fox terrier smooth	6.5
German Shepherd	7.5	German Shepherd	8
German Shepherd	7	Golden retriever	8
Golden retriever	7.5	Golden retriever	8.5
Golden retriever	6.5	Golden retriever	7
Miniature schnauzer	7	Miniature schnauzer	8
Miniature schnauzer	6	Miniature schnauzer	7
Miniature schnauzer	7	Miniature schnauzer	7.5
Polish lowland sheepdog	7	Miniature schnauzer	7
Polish lowland sheepdog	6,.5	Polish lowland sheepdog	7.5
Scottish terier	7.5	Scottish terrier	8
Shih tzu	6	Shetland sheepdog	6.5
Springer spaniel	7.5	Springer spaniel	8
Mean ±SD	6.7 ± 0.7		7.4 ± 0.8

Ethical statements

The study was conducted according to good veterinary practice as part of clinical service. The animal owners consented to the use of their dogs in this study. The study does not require authorization from the Local Ethics Committee for its performance.

Semen collection and sample preparation

Semen was collected by manual manipulation as described by Linde-Forsberg (2001) in the presence of a teaser bitch in heat. The second and third fractions (prostatic fluid) of the ejaculate were collected separately into sterile glass tubes and stored at -20°C until further use. In order to obtain ES the second fraction of ejaculate was centrifuged at 700 g for 10 min at room temperature and the supernatant was removed (Strzeżek et al. 2009). ES were resuspended after centrifugation in a volume of 0.9% NaCl equal to the volume of the removed supernatant and 1% Triton X-100 was added.

Protein measurement

Protein concentration in the samples was determined according to the method based on the biuret reaction using a commercial colorimetric kit (Cormay, Lublin, Poland).

TAC measurement

Total antioxidant capacity was measured according to the method of Benzie and Strain (1996), which was based on ferric-reducing ability of samples. Working reagent consisting of 300 mM/L acetate buffer (pH 3.6), 10 mmol/dm^3 2,4,6-tri-pyridyl-s-triazine in 40 mM/L HCl and 20 mM/L $FeCl_3$ x $6H_2O$ mixed in the ratio of 10:1:1, was prepared immediately before use. Working reagent (2250 µL) was mixed with 25 µL of sample and absorbance was measured at 593 nm (Ultrospec 2000, Pharmacia, Sweden) against the working reagent alone. After exactly 10 min of incubation at room temperature, the absorbance was read again. The difference in absorbance at zero and 10 min time was compared with a standard curve prepared with different dilutions of Fe(II) between 0 and 1000 µM/L. The results were expressed in µmol per g protein in sample.

Bitirosine measurement

Bityrosine was determined by a spectrofluorimetric method (Rice-Evans et al. 1991, Worobiej and

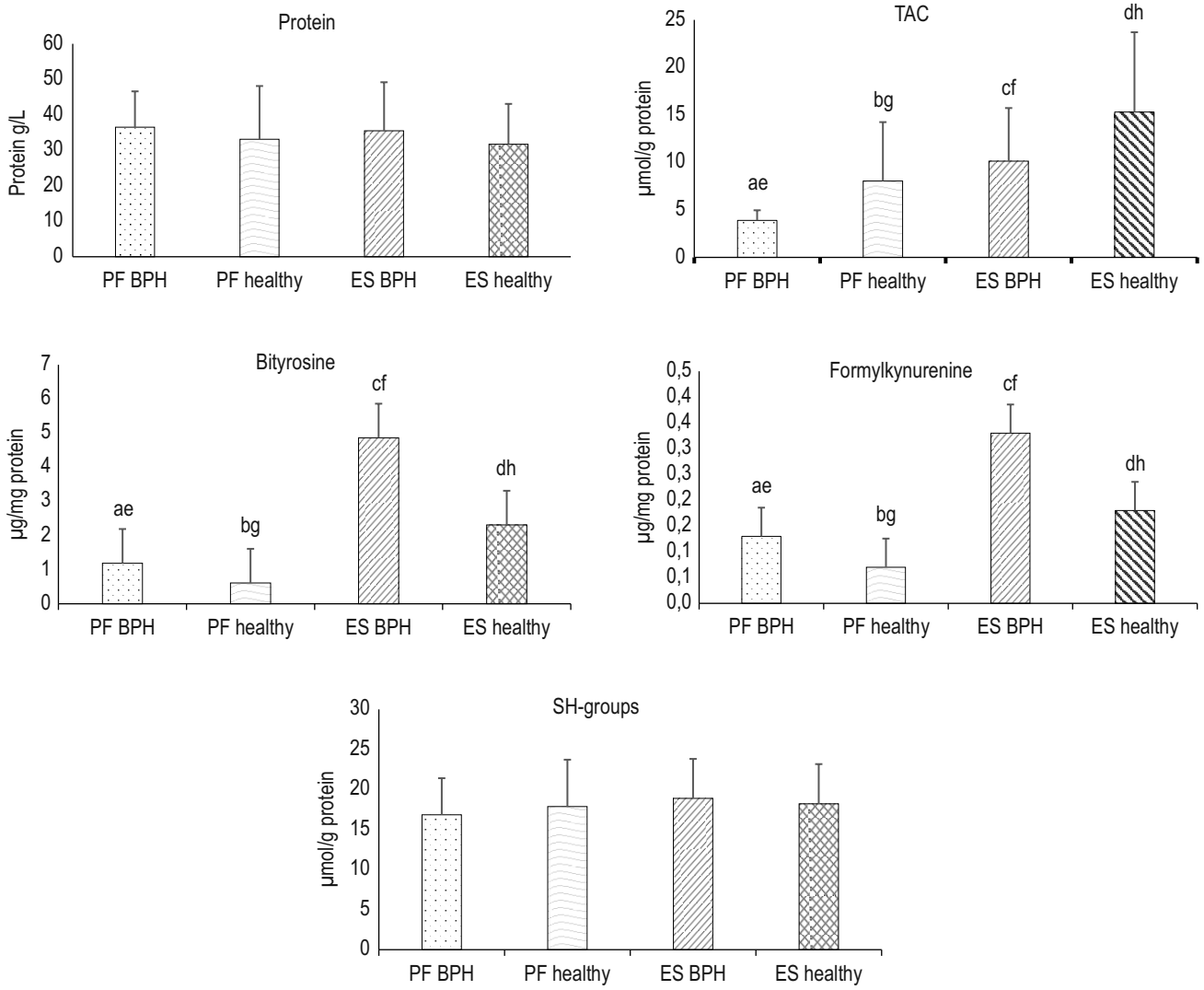

Fig. 1. Values (mean ± SD) of protein, total antioxidant capacitation (TAC), bityrosine, formylkynurenine and SH-groups in prostatic fluid (PF) and ejaculated spermatozoa (ES) of dogs with benign prostatic hyperplasia (BPH) and healthy dogs.
a, b – differences between PF of dogs with BPH and healthy dogs statistically significant at $p<0.05$.
c, d – differences between ES of dogs with BPH and healthy dogs statistically significant at $p<0.05$.
e-f – differences between PF and SE of dogs with BPH statistically significant at $p<0.05$.
g-h - differences between PF and ES of healthy dogs statistically significant at $p<0.05$.

Klepacka 2003). Samples were diluted with 0.9% NaCl. The fluorescence was measured at excitation 325 nm and emission 410 nm. The spectrofluorimeter (Jasco, Tokyo, Japan) was standardized to 100 deflections with chinine sulphate (0.1 µg/ml in 0.1 mol/ H_2SO_4) at excitation 350 nm and emission 445 nm. The results were expressed as µg/mg protein. The intra-assay and inter-assay coefficients of variation were 5.8% (n=10) and 5.9% (n=10), respectively.

Formylkinurenine measurement

Formylkinurenine was determined by a spectrofluorimetric method (Rice-Evans et al. 1991, Worobiej and Klepacka 2003). After previous 50-fold dilution of samples with 0.9% NaCl, the fluorescence was measured at excitation 360 nm and emission 454 nm. The spectrofluorimeter (Jasco, Tokyo, Japan) was standardized as described above. The results were expressed as µg/mg protein. The intra-assay and inter-assay coefficients of variation were 6.1% (n=10) and 6.4% (n=10), respectively.

SH-groups measurement

The content of SH-groups was determined by a spectrofluorimetric method (Rice-Evans et al. 1991). A volume of 300 µL of 10% sodium dodecyl sulphate in sodium phosphate buffer (10 mM/L, pH 8.0) was added to 300 µL of sample and mixed thoroughly. A 2.4 ml aliquot of the same buffer was added and absorbance was measured at 412 nm (Ultrospec 2000,

Pharmacia, Sweden). After measurement, 300 µL of DTNB (20 mg of 5,5'-dithiobis-2-nitro benzoate in 50 ml of buffer) was added and incubated for 1 hour at 37°C. The control contained 300 µL of buffer instead of DTNB. After incubation, absorbance was measured again at 412 nm. The difference in absorbance before and after incubation (after subtraction of adequate absorbance of control) referred to the content of the SH-groups. The content was calculated using a standard curve prepared with different dilutions of glutathione (GSH, 0-1 mM/L in buffer) and expressed in µmol per g of protein. The intra-assay and inter-assay coefficients of variation were 6.9% (n=10) and 7.1% (n=10), respectively.

Statistical analysis

The results were presented as mean and standard deviation and compared between both groups using t-Student's test or Mann-Whitney according to the distribution of variables (GraphPAD PRISM, Version 9.00, GraphPad Software, San Diego, CA, USA). The level of significance was set at $p<0.05$.

Results

The results of the study are detailed in Fig. 1. The mean protein concentration in PF and SE were similar in both groups. There were no statistical differences in protein concentrations between PF and SE of dogs with BPH and healthy dogs ($p>0.05$).

The mean values of TAC in PF were significantly lower ($p<0.05$) in dogs with BPH than in non-affected dogs (3.89 ± 1.05 µmol/g protein vs 8.04±8.26 µmol/g protein). The mean values of TAC in ES were significantly lower ($p<0.05$) in dogs with BPH than in healthy dogs (10.13±5.58 µmol/g protein vs 15.30±8.39 µmol/g protein). The mean values of TAC in ES were significantly higher than in PF in both groups ($p<0.05$).

The mean contents of bityrosine and formylkinurenine in PF in both groups were significantly higher ($p<0.05$) in dogs with BPH than in healthy dogs (1.18±0.96 µg/mg protein vs 0.61±0.38 µg/mg protein and 0.13±0.12 µg/mg protein vs 0.07±0.04 µg/mg protein, respectively). The mean contents of biotyrosine and formylkinurenine in ES were significantly higher ($p<0.05$) in dogs with BPH compared to healthy dogs (4.85±3.21 µg/mg protein vs. 2.31±1.28 µg/mg protein and 0.33±0.32 µg/mg protein vs 0.17±0.06 µg/mg protein, respectively). The mean contents of bityrosine and formylkinurenine in ES were significantly higher than in PF ($p<0.05$).

Compared to healthy dogs, the mean contents of SH-groups in PF and ES in dogs with BPH were numerically lower, but the differences were not statistically significant ($p>0.05$). There were no statistically significant differences in the content of SH between PF and ES in both groups ($p>0.05$).

Discussion

The pathogenesis of BPH is not well understood. DHT is accepted as a key hormone in stimulating enlargement of the canine prostate (Barsanti and Finco 1986). With advancing age, the estrogen/testosterone ratio increases. This leads to an increase in the concentration of androgen receptors in the prostatic tissue and an increase in the conversion of testosterone to DHT by 5α-reductase (Tunn et al. 1988). Overproduction of DHT and enhanced prostate sensitivity to androgens induce prostate cell hyperplasia and hypertrophy (Berry et al. 1986, Gobello and Corrada 2002).

Several studies indicate that oxidative stress may play also a role in the development of BPH in men (Srivastava and Mittal 2005, Aydin et al. 2006, Pace et al. 2010, Minciullo et al. 2015). Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and the ability of the antioxidant mechanism to neutralize these reactive products. Excessive ROS can cause damage of DNA and hyperplastic transformation of prostatic cells (Minciullo et al. 2015, Vital et al. 2016, Roumeguère et al. 2017). It is assumed that BPH is an immune-mediated inflammatory disease. Age-related hormonal imbalance or infection activate a chronic inflammatory response in the prostate. This causes the generation of free radicals and results in oxidative stress (Gandaglia et al. 2003, Tong and Zhou 2020).

The present study showed that BPH in dogs was associated with lowered TAC and increased protein peroxidation in PF and ES. This indicates the presence of oxidative stress in the prostate of dogs with BPH. Systemic oxidation stress (oxidative imbalance in the systemic circulation) and local oxidation stress (limited to the prostate) are not strongly correlated. In men with BPH, the increase in peroxides was more pronounced in samples taken locally during prostatectomy than taken systemically (Pace et al. 2010). No associations between oxidation stress biomarker concentrations in systemic plasma and seminal plasma (SP) in infertile men was found (Bergsma et al. 2020). In our previous study, we did not observe significant differences in the serum biomarkers of protein peroxidation, found in this study in PF (Domosławska et al. 2022).

In this study, the mean value of TAC in ES was significantly higher than in PF. Also, previous studies demonstrated that antioxidant defence varies within canine ejaculate fractions. High values of antioxidant

enzymes activity were observed in the ES and sperm reach fraction compared to the post-spermatic fraction (fraction III) (Strzeżek et al. 2009). The results of this study showed higher concentrations of protein oxidation biomarkers in ES than in PF. This may be associated with enhanced ROS production in sperm mitochondria and the nicotinamide dinucleotide phosphate oxidase (NOX) pathway in sperm plasma membranes (Castleton et al. 2022).

Oxidative imbalance in PF and ES indicates the importance of oxidative stress in the pathogenesis of BPH in dogs, as in men. Furthermore, oxidative stress associated with BPH may be responsible for the reduced fertility in dogs with this condition. Reduced TAC and increased protein peroxidation in SP were found in infertile stud dogs (Domosławska et al. 2019). BPH is a frequent cause of subfertility and infertility in male dogs (Memon 2007, Fontbonne 2011). It was found in 32.8% of infertile dogs (Domosławska et al. 2020).

This peroxidative damage of proteins may lead to the modification of amino acid residues, aggregation or fragmentation of protein molecules, altered conformation and loss of the biological activity of proteins (Stadtman and Levine 2000). Protein oxidation may result in dysfunction of mitochondria and low sperm motility (Nowicka-Bauer et al. 2018). Protein carbonyl expression in human semen was negatively correlated with sperm motility, fertility rate, and subsequent embryo quality in human intracytoplasmic sperm injection (ICSI) cycles (Al Smadi et al. 2021). Redox-dependent protein modification impaired also sperm capacitation (Morielli and O'Flaherty 2015).

Oxidative stress in dogs with BPH suggests the use of antioxidants in the prevention and therapy of this condition. In men, the clinical usefulness of antioxidants like polyphenols in BPH is widely discussed (Mitsunari et al. 2021, Stewart and Lephart 2023). The effect of antioxidants on BPH in dogs has not yet been studied. However, it has been shown that supplementation with essential fatty acids or selenium and vitamin E improves semen quality in dogs with reduced fertility (Da Rocha et al. 2009, Kawakami et al. 2015, Domosławska et al. 2018)

In conclusion, the present study showed that BPH in dogs was associated with lowered TAC and increased protein oxidation in PF and ES. This suggests the importance of oxidation stress in the pathogenesis of this condition and lowered fertility in BPH dogs. The potential role of antioxidants in the prevention and therapy of canine BPH requires further studies.

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