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Research article

THIOBARBITURIC ACID REACTIVE SUBSTANCES (TBARS) IN DOG'S HAIR AS A SIGN OF OXIDATIVE STRESS – PRELIMINARY STUDY

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Various endogenous and exogenous factors influence the occurrence of oxidative stress in all organisms, as well as in dogs. An increase in reactive oxygen species (ROS) concentration and the occurrence of oxidative stress can lead to changes in the structure of proteins, lipids, and DNA. The level of oxidative stress can be determined by measuring the end products of lipid peroxidation known as reactive substances of thiobarbituric acid (TBARS) of which malondialdehyde (MDA) is the most important. The concentration of MDA can be easily measured in various tissues and body excretions, but also by a non-invasive method of hair sampling. In this research, we have collected dog hair in grooming saloons, fluorometrically measured TBARS levels and compared the obtained values with factors such as breed, sex, age, passive smoking, sterilization, and season. No significant difference between sterilized and non-sterilized dogs was observed. The intensity of lipid peroxidation differed between the sexes, dog breeds, status of smoking by owner and exposure to UV radiation.

Keywords: biomarkers, dogs, hair, lipid peroxidation, oxidative stress, ROS

INTRODUCTION

In domestic animals, oxidative stress is involved in a number of pathological conditions that affect production and general welfare and health of the animal [1]. Oxidative stress occurs when reactive oxygen species (ROS) are formed faster than they can be neutralized by physiological antioxidant mechanisms [2]. High ROS production and oxidative stress can alter DNA structure and modify lipid and protein structure [3], which can lead to cell degeneration and play a significant role in the pathogenesis of many diseases [4]. External sources of ROS (ionizing and non-ionizing radiation, air pollution, naturally toxic gases such as ozone, various chemicals, and toxins) greatly contribute to the oxidative stress of the organism [1]. In addition to the above, external

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sources of ROS include improper diet, UV light, medications (e.g., chemotherapeutics) and cigarette smoke [5]. Due to the presence of free radicals, cigarette smoke is one of the prominent factors responsible for the increase of lipid peroxidation [6].

As the half-life of ROS is short, the rate of oxidative stress can be assessed indirectly by measuring the products of oxidative stress and lipid peroxidation [7]. Lipid peroxidation is the best described consequence of excessive ROS generation [8]. Most methods for the assessment of lipid peroxidation are based on quantification of end products among which malondialdehyde (MDA) is of particular importance. MDA is most commonly measured by thiobarbituric acid (TBA) assay [9]. As TBA can react with a variety of substances (aldehydes, amino acids, and carbohydrates), the measured products of this assay are commonly referred to as thiobarbiturate acid reactive substances (TBARS) [9]. The increase of TBARS can be used to estimate the intensity of lipid peroxidation [8,10].

Concentration of TBARS as a biochemical biomarker has been used in numerous studies to determine the association of various diseases and the occurrence of oxidative stress in dogs. Thus, significantly higher serum MDA levels were found in dogs with different types of tumors than in clinically healthy dogs [4]. Furthermore, dogs with various parasitic and bacterial diseases, but also other conditions such as Cushing's syndrome, show higher values of TBARS in the blood serum compared to healthy dogs [11-14]. It can be concluded that in dogs, the TBARS method is predominantly used in research related to the pathogenesis and occurrence of oxidative stress, while there are only a few studies that have observed the influence of some other factors on the occurrence of oxidative stress. One such example is the study of the impact of short-term transport on the occurrence of oxidative stress in the serum of dogs [15].

A lipid peroxidation with TBARS assay is most often measured in blood (plasma), urine or tissue. However, due to their simplicity, non-invasive methods and sampling of saliva and hair are also used [16]. Determination of TBARS from hair and saliva has been predominantly used in human research [16-18]. A non-invasive method of saliva sampling has also been used in studies in rats and mice [19,20]. By using non-invasive methods, additional stress resulting from sampling can be avoided. Compared to other tissues and body fluids, hair is highly stable during storage and has high concentrations of most analytes making it suitable for research and diagnosis [21]. Also, the hair follicle is surrounded by numerous capillaries, so the same information found in the blood is also found in the hair [22]. As far as we know, this is the first study that used a non-invasive method of sampling and determination of TBARS values from dog hair.

The aim of this study was to explore the possibility of measuring the level of lipid peroxidation in dog hair in relation to different parameters such as breed, sex, castration/sterilization, age, and passive smoking. Additionally, samples were collected in different seasons (summer and winter) to assess the effect of solar UV radiation.

MATERIALS AND METHODS

Sample collection

Samples were collected during the summer (August-September) and winter (November-January) months. Hair was collected in collaboration with dog grooming salons. Hair samples were cut as close as possible to the dog's skin, without pulling it from the roots to avoid contamination of the hair with blood, taking care not to injure the dog. Samples were not taken from dogs infested with fleas or other external parasites. A total of 192 samples were collected (Table 1).

Table 1. The number of hair samples collected and analysed in the present research

	Breed			Sex		Sterilization		Owners		Living	
	Maltese	Shih-Tzu	Mongrel	M	F	Yes	No	Smokers	Non smokers	Indoors	Outdoors
Summer	40	26	36	66	36	56	46	49	53	91	11
Winter	39	32	21	48	44	43	49	47	45	89	3
Total	79	58	57	114	80	99	95	96	98	180	14

The hair of Maltese (Ma), Shih Tzu (Sh) and mixed breed dogs (mongrels (Mo)) was sampled. During sampling, data on the breed, sex, age, and castration/sterilization status of the individual were recorded, as well as whether the dog owners are smokers or non-smokers and whether the dog lives indoors or outdoors. The age of dogs ranged from 2 months to 14 years. The samples were categorized into age groups: <3 years (1); 3 and <6 years (2); 6 and <9 years (3); 9 and <12 years (4); 12+ (5) years.

Sample preparation

Samples were prepared according to Sheu [16]. To remove sebaceous gland products and other impurities the hair samples were washed in acetone, rinsed with distilled water, and left for 48 hours at room temperature to dry.

To prepare the digestion mixture, 2 M NaOH was added to the weighed hair (60-80 mg) and the mixture was incubated for 20 minutes at 80°C. After incubation, the digested samples were centrifuged for 10 minutes at 3500x*g* to remove hair residues. For further processing, the supernatant was used.

Determination of lipid peroxidation products (TBARS assay)

To estimate TBARS content in dog hair a method according to Sheu [16] was adapted. Briefly, a 9% aqueous solution of H_3PO_4 (85%) was added to a sample tube to prepare samples for staining. The reaction mixture for staining the blank and the standard is prepared in the same way, except 40% ethanol (for the blank) or the corresponding standard (for the preparation of the reaction mixture of the standard) is added instead

of samples. 1,1,3,3-tetraethoxypropane (TEP) was used as a standard and for the preparation of the calibration curve.

Each reaction mixture was stained by the addition of 30 mM thiobarbiturate acid (TBA), vortexed, capped and left in a water bath to incubate at 100°C for 60 minutes. After incubation the tubes were cooled to room temperature and 1-butanol (p.a.) was added. Samples were vortexed for 20 seconds before transferring for centrifugation for 20 minutes at 1000x*g*.

The alcoholic fraction (top layer of supernatant) of each sample, standard and blank was transferred to a microtiter plate. The blank, standards and samples were measured on a spectrofluorometer (Varian CaryEclipse) (ex = 537 nm; em = 553 nm). Using the obtained fluorescence values of the standard, a calibration curve is constructed for the calculation of TBARS in the samples. The results are expressed as μmol per milligram of hair (μmol/mg_{hair}).

Data analysis

Data were statistically processed in R version 3.5.0 software [23] and R Studio Team [24]. The Shapiro-Wilk test ($p<0.05$) was used to check the normality of the data distribution. Homogeneity of variance was determined by the Fligner-Killeen test. The Kruskal-Wallis test and the Wilcoxon test were used to determine the existence of a statistically significant difference ($p<0.05$) between certain parameters followed by a Gao post-hoc test [25].

RESULTS

A similar number of hair samples were collected in each season (summer n=102, winter n=92), of each investigated breed (79 Maltese, 58 Shih-Tzu and 57 mongrel) and sex (114 M, 80 F) (Table 1). Values were significantly higher in samples from the summer season ($p<0.05$) (Figure 1A). The highest value of TBARS (0.890 μmol/mg_{hair}) was measured in a Maltese male in summer, while the lowest value was measured in a Shih-Tzu male in winter (0.099 μmol/mg_{hair}). The difference between seasons was significant for all three tested breeds ($p <0.001$). Significant differences were found between breeds (Figure 1B), with the highest values in mongrels and the lowest in Maltese dogs ($p <0.05$).

Overall, male dogs had significantly higher TBARS values compared to females ($p <0.05$) (Figure 1C). When gender within season for a particular breed was analyzed the only significant difference was between Maltese males and females in the summer ($p=3.78 \times 10^{-6}$) (Figure 3). Samples analyzed within gender and breed but between seasons differed only for Shi-Tzu female dogs ($p=0.029$), Maltese ($p= 5.231 \times 10^{-7}$) and mongrel males ($p=0.015$) (Figure 3).

Most dogs belonged to the age group 2 (n = 92) and 1 (n = 42), while the least dogs belonged to the age group 5 (n = 9). No significant difference was observed between

age groups (Figure 1E). Within age groups, only in group 2 (3 and <6 years) Maltese dogs differed from the other two breeds and had the lowest TBARS values (Figure 4B).

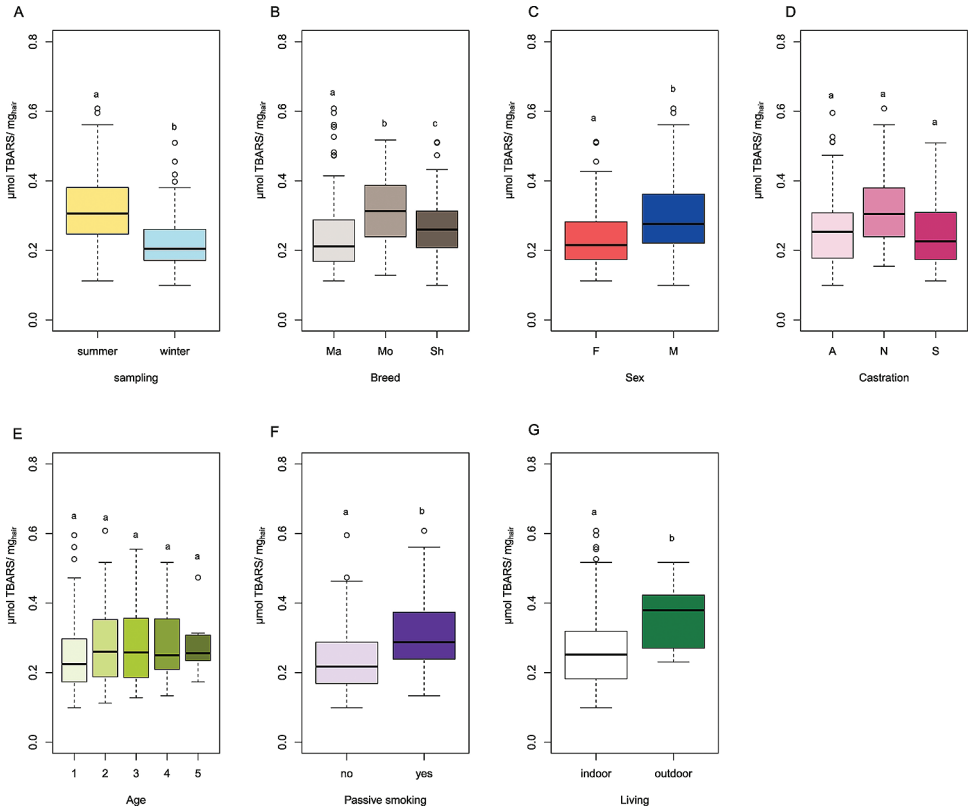

Figure 1. Differences in TBARS concentration measured in dog hair between seasons **(A)**, breeds **(B)**, sex **(C)**, castration status **(D)**, dog's age **(E)**, owner's smoking habit **(F)** and dog's housing **(G)**. Different letters show statistically significant differences ($p \leq 0.05$), circles represent outliers. Ma Maltese, Mo – Mongrel, Sh – Shih-Tzu, A – active, not castrated, N – neutered, S – spayed; Age: 1-< 3 years, 2-3 and <6 years, 3-6 and <9 years, 4-9 and <12 years, 5-12+ years.

One of the parameters observed was whether the dog owner was a smoker or a non-smoker. Overall, 96 samples came from dogs whose owners were smokers and 98 from non-smokers. Hair samples from dogs owned by smokers had a significantly increased TBARS value compared to dogs owned by non-smokers (Figure 1F). The mean value of TBARS in dogs owned by smokers was 0.314 ± 0.115 µmol/mg_{hair}, while in dogs owned by non-smokers was 0.247 ± 0.116 µmol/mg_{hair}. If values are analysed according to age only group 2 (3 and <6 years) differed significantly (Figure 4A).

Almost half of the hair samples came from dogs that had been sterilized (n=99). However, no significant difference between samples from sterilized and non-sterilized dogs was observed (p = 0.168) (Figure 1D). If samples are analyzed and compared separately between seasons then the only significant difference exists between sexually active (non-sterilized) Maltese (p=0.0024) and Shi-Tzu (p=0.0016) dogs (Figure 2).

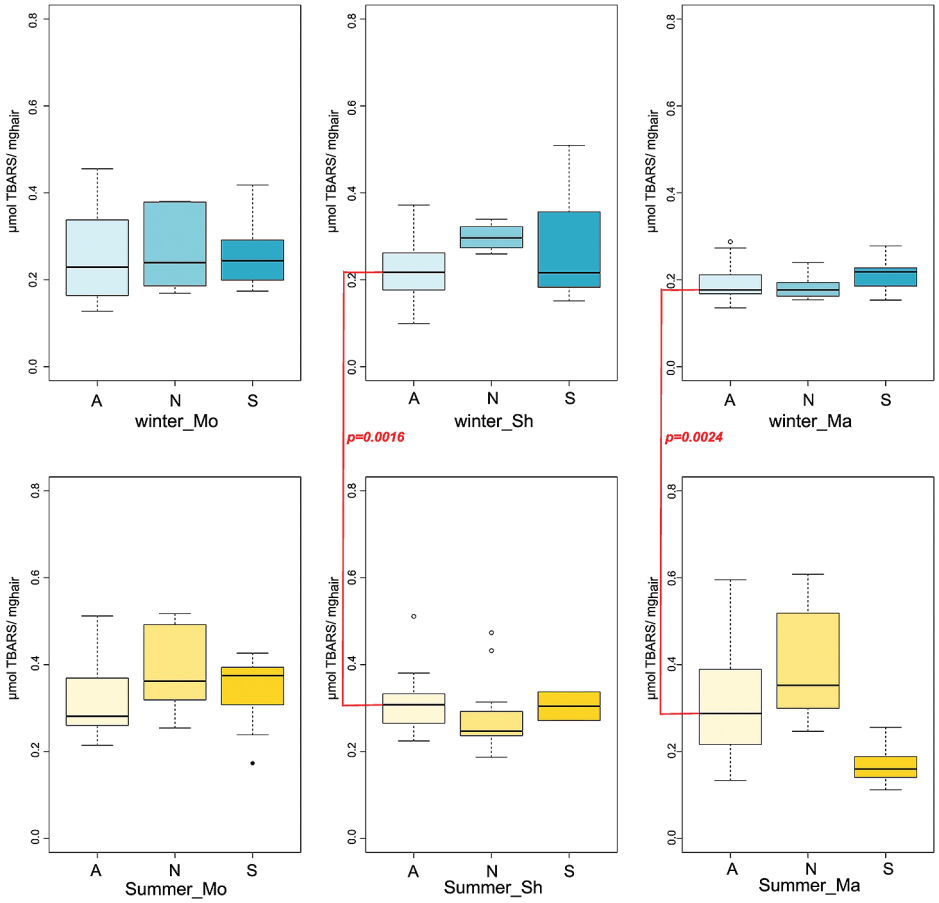

Figure 2. Differences in TBARS concentration measured in dog hair within a season and breed against castration status. Statistically significant differences between seasons are noted ($p \leq 0.05$), circles represent outliers. Ma – Maltese, Mo – Mongrel, Sh – Shih-Tzu, A – active, not castrated, N – neutered, S – spayed.

Only 14 dogs lived outdoors, while the rest spent most of their time indoors. The dogs that spent most of their time in backyards had higher TBARS values (0.370± 0.098 µmol/mg_{hair}) compared to those dogs living in houses and apartments (0.273 ±

0.119 µmol/mg_{hair}) ($p < 0.05$) (Figure 1G). Most dogs that lived outdoors were sampled in summer (only three samples in winter), but there was no difference between them.

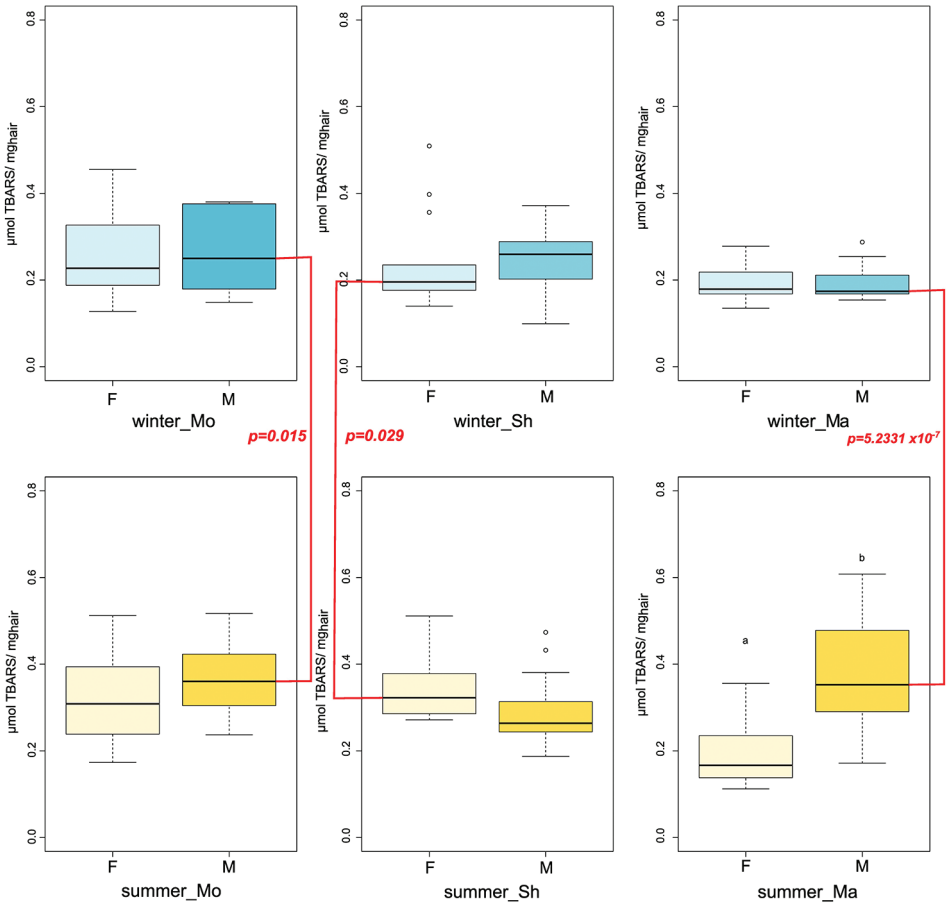

Figure 3. Differences in TBARS concentration measured in dog hair within a season and breed against sex. Statistically significant differences between seasons are noted ($p \leq 0.05$), circles represent outliers. Ma – Maltese, Mo – Mongrel, Sh – Shih-Tzu, F – female, M – male.

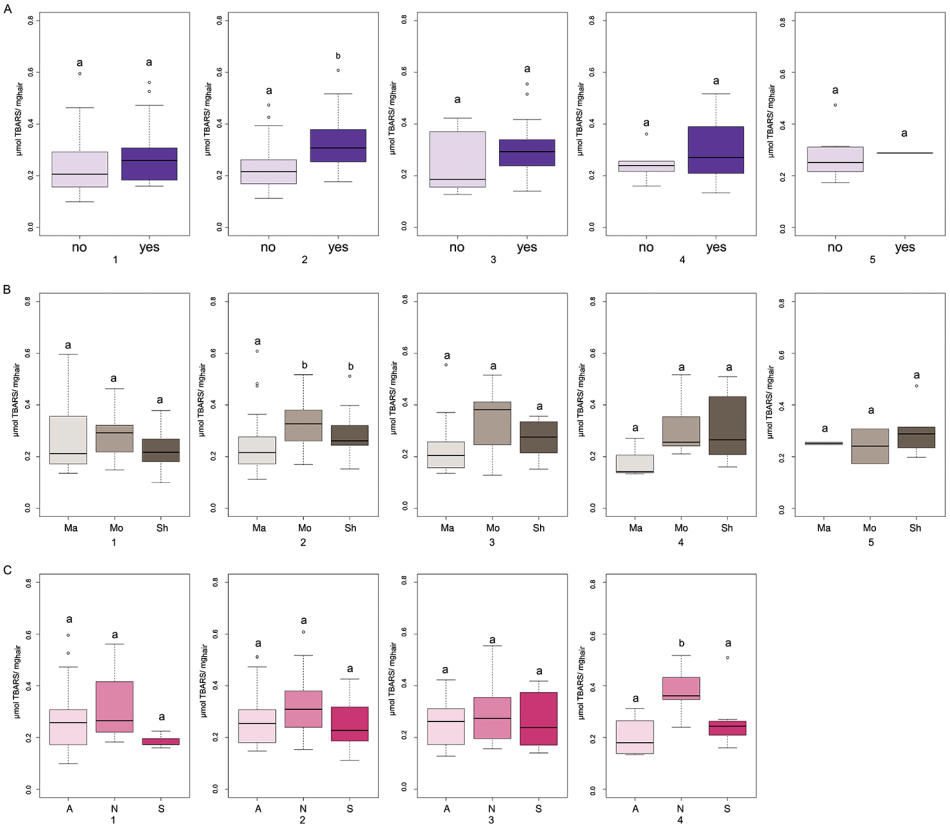

Figure 4. Differences in TBARS concentration measured in dog hair within certain age groups (1-< 3 years, 2-3 and <6 years, 3-6 and <9 years, 4-9 and <12 years, 5-12+ years) according to the owner's smoking status **(A)**, breed **(B)** or castration status **(C)**. Different letters show statistically significant differences ($p \leq 0.05$), circles represent outliers. Ma Maltese, Mo – Mongrel, Sh – Shih-Tzu, A – active, not castrated, N – neutered, S – spayed.

DISCUSSION

TBARS assay cannot be used with certainty as a diagnostic test for individuals as the type and strength of the acid used in the method, the duration of incubation of the samples and other factors may make it difficult to interpret the obtained results. However, the amount of MDA can serve as a biomarker for determining the degree of lipid peroxidation on a group basis [6]. Also, TBARS assay can be performed in combination with cortisol measurements in hair, given the established positive correlation between cortisol levels and TBARS, which can provide a better insight into the understanding of stress reactions caused by various external factors [8].

A significant difference was observed between samples from summer and winter. The difference can be attributed to higher air temperature and the amount of UV radiation

that could have a significant impact on TBARS values. Namely, the daily amounts of irradiated solar energy are the highest in summer and the lowest in winter months [26]. When the degree of heat gain exceeds the body's ability to lose heat, heat stress develops [27]. Bernabucci et al. [28] found that under the influence of heat stress, increased levels of superoxide dismutase, glutathione peroxidase, intracellular thiols and TBARS can be observed in dairy cattle exposed to heat stress in summer, as opposed to cattle exposed to lower temperatures in colder parts of the year. Furthermore, exposure to UV radiation causes changes in the structure of keratin, photo-oxidation of amino acids, sterols and fatty acids, and the breakdown of lipids. UV-B radiation affects the breakdown of disulfide bonds in the hair, while UV-A radiation affects the production of ROS. Exposure to this type of radiation leads to a significant increase in MDA. The duration of radiation exposure affects the production of lipid peroxides, where longer exposure results in production up to 233% higher than non-exposed hair [29].

Phenotype fixation and inbreeding have led to the emergence of breed-specific diseases and large variations in life expectancy [30,31], it is therefore not surprising that there is a difference in the level of oxidative stress between different dog breeds. At the cellular level, longer development of the individual is associated with an increase in the production of ROS, which can lead to mutations that can cause accelerated ageing and disease, which is especially noticeable in large dog breeds [32]. Large breeds are significantly more burdened by oxidative damage, such as damage caused by lipid peroxidation and DNA damage, leading to a higher rate of disease and thus earlier death [32,33]. Unfortunately, we did not have a sufficient number of samples from large dog breeds to compare hair TBARS levels with small breeds. The difference in TBARS values between mixed breeds and purebred dogs could be caused by a higher number of unpredictable genetically inherited mutations of mixed breeds, and thus greater susceptibility to diseases compared to purebred dogs whose breeding seeks to reduce the likelihood of disease.

The results obtained in this study are consistent with the results obtained by Todorova et al. [34], who observed higher concentrations of MDA in male dogs compared to females. Males are known to be more susceptible to cardiovascular disease, which is associated with higher levels of TBARS [35]. Females live longer than males in many species, which may be related to lower free radical levels in female mitochondria [36], but also due to the higher rate of expression of antioxidant genes and less oxidative damage to mitochondria in females [37]. Furthermore, estrogen shows strong antioxidant properties, which is not the case with progesterone and testosterone [38].

With age, the number of mitochondria decreases and mitochondria become larger, the respiratory activity of mitochondria itself decreases and the production of reactive particles increases, leading to increased oxidative damage [39]. Therefore, it is to be expected that TBARS values in older individuals will be significantly higher. However, this was not the case in this study. An increase in TBARS values can be observed in older age groups compared to younger age groups, but that difference is not statistically significant. The cause of such results could be a smaller number

of samples of individuals of older age groups. Studies in humans of different age groups have not found an association between age and lipid peroxidation, although, as in this study, higher values were observed in older age groups [40,41]. Similarly, given the positive correlation between cortisol levels and TBARS, it is assumed that cortisol levels in dog hair will increase with age, which has not been confirmed [42].

One of the factors influencing the increase in lipid peroxidation in this study is smoking, perhaps due to the presence of highly oxidizing free radicals in cigarette smoke [43]. Tobacco smoke contains a large number of oxidants and radicals in the tar and gas phases. It is estimated that one breath of a cigarette contains about 1015 radicals in the gaseous phase and 1014 radicals in the tar phase, which allows the modification of endogenous macromolecules such as lipids [44]. The results of this research are in line with other research conducted. Sheu et al. [16] found that TBARS values were significantly higher in people who were smokers as opposed to non-smokers. Nielsen et al. [6] found a significant correlation between the number of hours of cigarette smoke exposure and the plasma MDA concentration. Measurements of antioxidants and biomarkers of oxidative stress confirm that smokers suffer from increased levels of oxidative stress and are often used as a reference group that is continuously exposed to an increased exogenous source of oxidative stress, i.e., tobacco smoke [1]. Dogs exposed to the smoke of their owner's cigarettes passively inhale harmful substances from the smoke. Passive smokers also show higher concentrations of MDA than non-smokers, although slightly less than active smokers [40], so it is not surprising that smoker owners thereby affect their pets and increase the value of TBARS.

Castration can prevent some diseases of the genital system, but also unwanted behavior such as urinary marking and aggressive behavior. However, castration has some drawbacks and is thought to lead to changes in the body's normal physiological function, antioxidant status [45] and early side effects such as increased levels of oxidative stress caused by trauma during surgery [46,47]. In female dogs, plasma MDA levels were found to be significantly higher on the third day after surgery compared to a few hours after surgery or several days after surgery, indicating that dogs were most affected by oxidative stress on the third day after surgery [46]. Unlike females, no increase was observed in males and there was no difference in pre-operative and post-operative outcomes [45]. The increase in the level of oxidative stress occurs a few days after the operation itself, after which it decreases. In this study, dog hair samples were not taken immediately after castration, but in the period after the procedure when there was no noticeable increase in biomarkers of oxidative stress (several weeks, months, even years after the procedure), which explains the lack of difference between castrated and uncastrated individuals. The very occurrence of oxidative stress and lipid peroxidation due to castration could be caused by the action of anesthetics, inflammation, and post-operative pain [47], and not by the act of castration itself.

Dogs that have access to the yard have a positive effect on activity due to the large number of stimuli they encounter, and they move twice as much as dogs that only stay indoors [48]. Increased physical activity can lead to increased production of

ROS and thus cause oxidative stress. During strenuous physical exertion, the rate of skeletal muscle metabolism increases oxygen consumption which can lead to increased production of superoxide anion in the mitochondria [49]. Yet, it is unlikely that outdoor dogs are under such physical exertion that oxidative stress would manifest. Furthermore, an outdoor environment can never be fully controlled, especially when it comes to climatic conditions and encounters with other animal species (birds, rodents, etc.) [48]. Environmental factors can be defined as acute stressors, but it is questionable to what extent they really affect dogs, given their great ability to adapt to new conditions [50]. Due to the small number of individuals in this study living in outdoor conditions (n = 14), it is difficult to say with certainty the cause of the increased TBARS values. It is most likely that the cause of such results is high summer air temperature and the amount of UV radiation, given that most of the individuals living outside were sampled in summer.

CONCLUSION

Thiobarbituric acid reactive substances (TBARS) from the hair could serve as a complementary biomarker of oxidative stress in dogs, especially when testing is performed on larger groups. This is a simple and non-invasive method, and since the measurements are made from the hair, the consequences of acute stress that would arise from another type of sampling are eliminated. The TBARS method can serve as an indicator of oxidative stress to breeders, but also in veterinary medicine. TBARS values vary between breeds and genders, but a number of external influences, such as cigarette smoke, the amount of UV radiation, and climatic factors or high air temperatures have an influence. In future research, it would be good to establish a correlation between TBARS values obtained from hair samples and from blood serum, which would establish the influence of various factors on acute and chronic stress and their relationship. Also, it would be beneficial to include more breeds and other factors such as physical activity and dog nutrition.

Authors' contributions

NB carried out the sampling and measurement and drafted the manuscript. BKH participated in the design of the study and performed the statistical analysis. DKH conceived the study, and participated in its design and cowrite the paper. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Statement of Informed Consent

The owner understood procedure and agrees that results related to investigation or treatment of their companion animals, could be published in Scientific Journal Acta Veterinaria-Beograd.

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SUPSTANCE REAKTIVNE SA TIOBARBITURNOM KISELINOM (TBARS) U DLACI PASA KAO ZNAK OKSIDATIVNOG STRESA – PRELIMINARNA STUDIJA

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Različiti endogeni i egzogeni faktori utiču na pojavu oksidativnog stresa kod svih organizama, pa tako i kod pasa. Povećanje koncentracije reaktivnih vrsta kiseonika (ROS) i pojava oksidativnog stresa mogu dovesti do promena u strukturi proteina, lipida i DNK. Nivo oksidativnog stresa se može odrediti merenjem krajnjih proizvoda peroksidacije lipida poznatih kao reaktivne supstance tiobarbiturne kiseline (TBARS) od kojih je najvažniji malondialdehid (MDA). Koncentracija MDA se lako može meriti u različitim tkivima i telesnim izlučevinama, ali i neinvazivnom metodom uzorkovanja dlaka. U ovom istraživanju prikupljali smo pseću dlaku u salonima za negu, fluorometrijski izmerili nivoe TBARS i uporedili dobijene vrednosti sa faktorima kao što su rasa, pol, starost, pasivno pušenje, sterilizacija i godišnje doba. Nije primećena značajna razlika između sterilisanih i nesterilisanih pasa. Intenzitet peroksidacije lipida razlikovao se između pola, rase pasa, statusa pušenja kod vlasnika i izloženosti UV zračenju.