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Comparison of the antioxidant system response to melatonin implant in raccoon dog (*Nyctereutes procyonoides*) and silver fox (*Vulpes vulpes*)

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Abstract: The aim of this work was to investigate whether melatonin implant may modify the response of the antioxidant systems of raccoon dog and silver fox. Animals of each species were divided into 2 equal groups: implanted with 12 mg of melatonin in late June and not implanted (control). During the standard fur production process in late November, samples of tissues (liver, kidney, spleen, and heart) were collected and specific activities of superoxide dismutase (SOD) and catalase (CAT), and the contents of reduced glutathione (GSH), retinol, α-tocopherol (TCP), and total tissue protein, were determined in tissue samples. Activity of antioxidant enzymes SOD and CAT as well as concentrations of GSH and TCP were considerably higher in organs of raccoon dogs in comparison with silver foxes at the end of autumn fattening. Melatonin implants had no significant effect on the fox antioxidant system in contrast to the raccoon dog. The SOD activity in the liver, kidney, and heart of melatonin-treated raccoon dog considerably decreased, by 25% to 70%. The CAT activity was reduced in the kidney and heart, but it increased in the liver and spleen. Simultaneously, concentrations of GSH in the examined organs of the raccoon dog showed an inverse relationship with CAT activity. In summary, raccoon dogs and silver foxes differ not only in the function of the antioxidant system but also in the response of this system to exogenous melatonin. The rapid fattening evokes oxidative stress, which stimulates the activity of the antioxidant system in this species.

Key words: Catalase, glutathione, superoxide dismutase, metabolic syndrome, Canidae

1. Introduction

The raccoon dog (*Nyctereutes procyonoides*) and the silver fox, a color variety of the red fox (*Vulpes vulpes*), both belong to the family Canidae. However, the silver fox belongs to the tribe Vulpini (true foxes), while the raccoon dog belongs to the basal Caninae, which are considered more primitive than the tribes Canini and Vulpini (1). The genetic distance (p-distance) between these species is estimated at 0.0282 (2). Moreover, the biology and ecology of these 2 species are different. Both species are omnivorous, but plant food forms a greater part of raccoon dog diets than fox diets. In addition, the raccoon dog is the only Canidae to spend several winter months in a burrow in hibernation (winter dormancy); therefore, its autumnal fattening and natural weight loss in winter and spring are greater in comparison to other Canidae (3–5). Between the late autumn and spring the body weight of the wild raccoon dog changes from about 6.5 to 4.5 kg (3), but in farm animals it changes from about 12 to 7 kg (6,7), while in this same period the body weight of wild and farmed silver fox varies from about 6 to 4 kg (8) and from about 7 to 5 kg (9), respectively.

The raccoon dog and the silver fox are seasonal animals, producing a winter coat in the autumn. Molting processes are regulated by the secretion of melatonin, the pineal hormone encoding photoperiod information and orchestrating the seasonal changes in body functions (10). Exogenous melatonin induces changes in the same physiological parameters as the shortening photoperiod. It is therefore possible to raise the circulating melatonin levels with subcutaneous implants and thus accelerate the maturation of a winter fur coat (11,12).

It is known that melatonin can act as an antioxidant (12–15), the functions of which are to reduce the level of reactive oxygen species (ROS) and to stimulate the activity of antioxidant enzymes. Knowledge regarding the influence of melatonin implants on the antioxidants of farmed canines is very limited. Moreover, taking into account that the raccoon dog and the silver fox are different types of canine species, it seems interesting to investigate whether melatonin implants may modify the response of the antioxidant systems of these animals.

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2. Materials and methods

2.1. Experimental design

The study was conducted on raccoon dogs and silver foxes at a fur farm in Poland (Permission No. 32/2010, First Local Ethical Committee on Animal Testing at the Jagiellonian University in Krakow). The animals of each species, 1.5 years old, were divided into 2 equal groups (n = 20, sex ratio 1:1). Experimental animals received a subcutaneous continuous-release melatonin implant with 12 mg of melatonin in late June. Control animals were not implanted. The samples of tissues (liver, kidney, spleen, and heart) were collected from carcasses after pelting during the standard fur production process in late November. They were snap-frozen at –25 °C and stored for further analysis.

2.2. Processing

The specific activities of superoxide dismutase (SOD) and catalase (CAT), and the contents of reduced glutathione (GSH), retinol, α-tocopherol (TCP), and total tissue protein, were determined in tissue samples. Tissue samples for measurement of enzyme activities and protein content were homogenized in 0.05 M phosphate buffer, pH 7.0, and centrifuged at 6000 × *g* for 15 min.

The total SOD activity was measured by the adrenochrome method based on the spontaneous autoxidation of epinephrine with the formation of product with an absorbance peak at 480 nm (16). This reaction depends on the presence of superoxide anions and is specifically inhibited by SOD. The amount of enzyme that caused 50% inhibition of epinephrine autoxidation is defined as 1 unit (U). The catalase activity was evaluated by measuring the decrease in H_2O_2 concentration at 240 nm (14). One enzyme unit (IU) is defined as the amount of catalase capable of transforming 1.0 μmol/min of H_2O_2. Specific activities of these enzymes were calculated by dividing total activity of each enzyme by protein content. The total tissue protein level was determined by the Lowry method.

Tissue samples for measurement of GSH were homogenized in 0.02 M EDTA and centrifuged at 5000 × *g* for 15 min. Next, the following mixture (supernatant, distilled water, and 50% trichloroacetic acid) was centrifuged at 3000 × *g* for 15 min. The GSH content was determined using the Ellman method in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid).

Tissue samples for measurement of retinol and α-tocopherol were homogenized in 0.25 sucrose solution (pH 7.4), and then centrifuged at 3000 × *g* for 10 min. The levels of retinol and α-tocopherol were measured in tissues by high performance liquid chromatography (17). Internal error of method was less than 1.5% for GSH and less than 1% for the other parameters.

2.3. Statistical analysis

All the collected and calculated numerical data were transformed into SI units and processed statistically as mean ± standard error of the mean. Multidimensional analysis of variance was employed with subsequent use of cluster analysis. Statistical analysis was performed using the Tukey test. Differences between samples were considered to be significant when the P-value was less than 0.05. The statistical analyses were performed using SigmaStat 2.03 (SPSS Science Software Ltd., USA), while figures were prepared using Grapher 7.0 (Golden Software Inc., USA).

3. Results

Results of the experiment showed that the silver fox and raccoon dog differed in the response of their antioxidant systems to the exogenous melatonin implant.

The activity of SOD in the liver, kidney, heart, and spleen of the control silver fox was 2.0, 4.3, 2.5, and 1.6 IU/mg protein, respectively, whereas it was 2.4 and 1.8 times higher in the raccoon dog's liver and heart ($P \leq 0.05$) and 1.6 times lower ($P \leq 0.05$) in the kidney (Figure 1a). Exogenous melatonin released from implant decreased SOD activity in the liver and heart of the raccoon dog by 23% and 44% ($P \leq 0.05$), respectively, but increased its activity in the spleen by 79% ($P \leq 0.05$). However, it did not evoke any changes in SOD activity in the fox's organs ($P > 0.05$; Figure 1a).

The CAT activity in the liver, kidney, heart, and spleen of the control silver fox was 4.2, 2.3, 0.5, and 0.3 IU/mg protein, respectively. The control raccoon dog in comparison to the silver fox demonstrated 2.5, 3.0, and 4.2 times higher CAT activity in the liver, heart, and spleen ($P \leq 0.05$) and 1.4 times lower activity ($P \leq 0.05$) in the kidney (Figure 1b). Exogenous melatonin released from implants increased activity of this enzyme in the liver and spleen of the raccoon dog by 23% and 150% ($P \leq 0.05$) but decreased its activity in the kidney and heart by 29% and 68%, respectively ($P \leq 0.05$). In melatonin-treated fox organs no changes in CAT activity were observed ($P > 0.05$; Figure 1b).

The GSH concentration in the liver, kidney, heart, and spleen of the control silver fox was 9.4, 8.1, 12.1, and 10.8 μmol/g tissue, respectively. The level of GSH in the raccoon dog's liver was 3.5 times lower in comparison to the silver fox but 2.0 times higher in the kidney ($P \leq 0.05$; Figure 2). Exogenous melatonin increased concentrations of this enzyme in the spleen of the raccoon dog by 26% ($P \leq 0.05$), but it did not evoke significant changes in GSH concentration in other analyzed organs in both species ($P > 0.05$; Figure 2).

The concentration of retinol in the kidney, liver, and heart of the control silver fox was 101.1, 10.6, and 1.0 μg/g

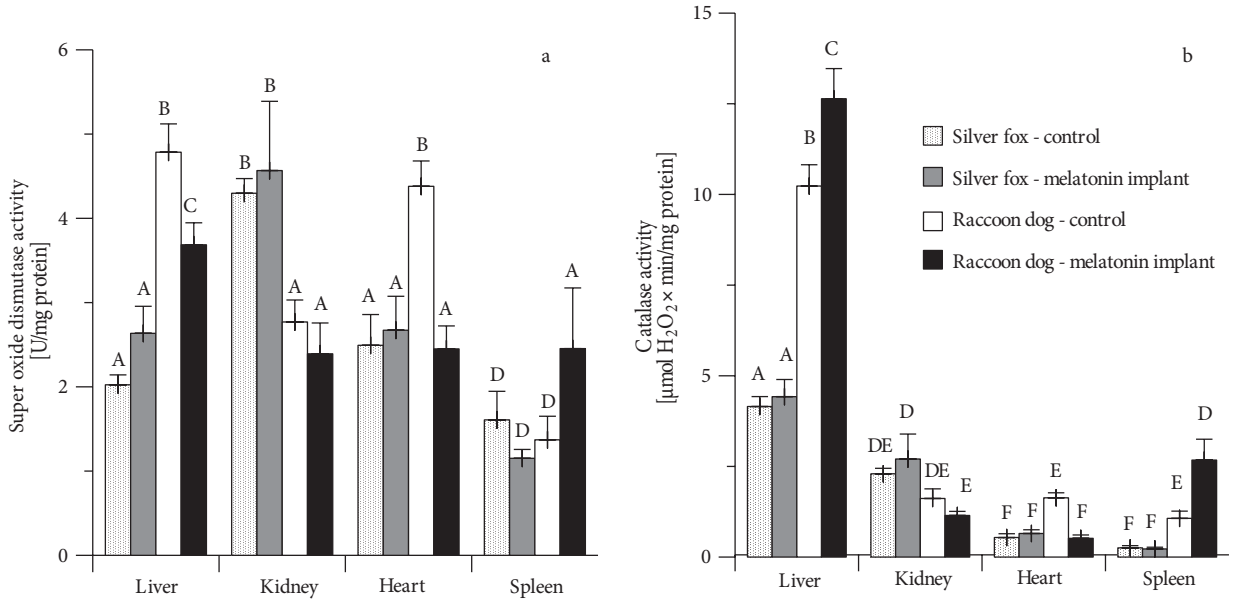

Figure 1. Activity of a) superoxide dismutase (SOD) and b) catalase (CAT) in organs of silver fox (n = 20) and raccoon dog (n = 20) that received a subcutaneous continuous-release melatonin implant (12 mg). A, B, C, D, E, F: bars with different letters differ significantly (P ≤ 0.05).

tissue, respectively, while the level of this vitamin in the raccoon dog's organs was 13.1, 8.1, and 3.3 times lower (P ≤ 0.05), respectively (Figure 3a). The exogenous melatonin increased concentrations of retinol in the silver fox's heart by 136% (P ≤ 0.05); meanwhile, retinol increased by 331% in the kidney and by 35% in the heart of the raccoon dog (P ≤ 0.05; Figure 3a).

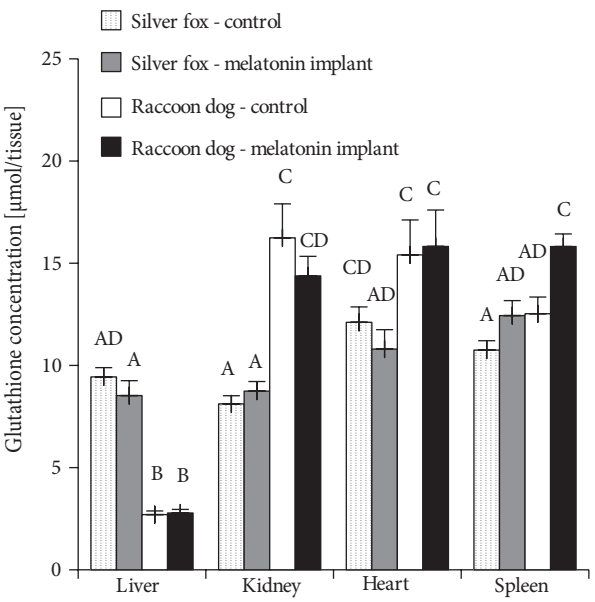

Figure 2. Concentration of glutathione in organs of silver fox (n = 20) and raccoon dog (n = 20) that received a subcutaneous continuous-release melatonin implant (12 mg). A, B, C, D: bars with different letters differ significantly (P ≤ 0.05).

The α-tocopherol level in the liver, kidney, heart, and spleen of control silver foxes was 22.2, 16.5, 10.7, and 16.0 µg/g, respectively. It was 3.5 (P ≤ 0.05) and 1.1 (P > 0.05) times lower in comparison to the kidney and heart of the raccoon dog and 1.4 higher compared to the liver and spleen (P > 0.05; Figure 3b). Administering exogenous melatonin from an implant decreased concentrations of this substance in the silver fox's liver by 35% (P ≤ 0.05) and increased it by 70% in the kidney of the raccoon dog (P ≤ 0.05; Figures 3a and 3b).

4. Discussion

The present study showed that activities of antioxidant enzymes SOD and CAT, as well as concentrations of GSH and α-tocopherol, are considerably higher in organs of raccoon dogs in comparison with silver foxes at the end of autumn fattening. This phenomenon mostly concerns the liver and the heart, the organs particularly exposed to ROS. Differences in the basal level of the antioxidant system between the 2 investigated species can be explained by differences in their ecology and physiology. The raccoon dog is a middle-sized canine with profound autumnal fattening followed by winter sleep (7), while the silver fox remains active during the winter (8). Seasonal accumulation of fat in the raccoon dog is characterized by low plasma free fatty acids, diacylglycerol and triacylglycerol levels, and rapid increase in insulin (3-fold in comparison to the summer concentration), leptin, and glycogen (but not glucose) (3,7). It seems that the rapid fattening accompanied by insulin resistance can

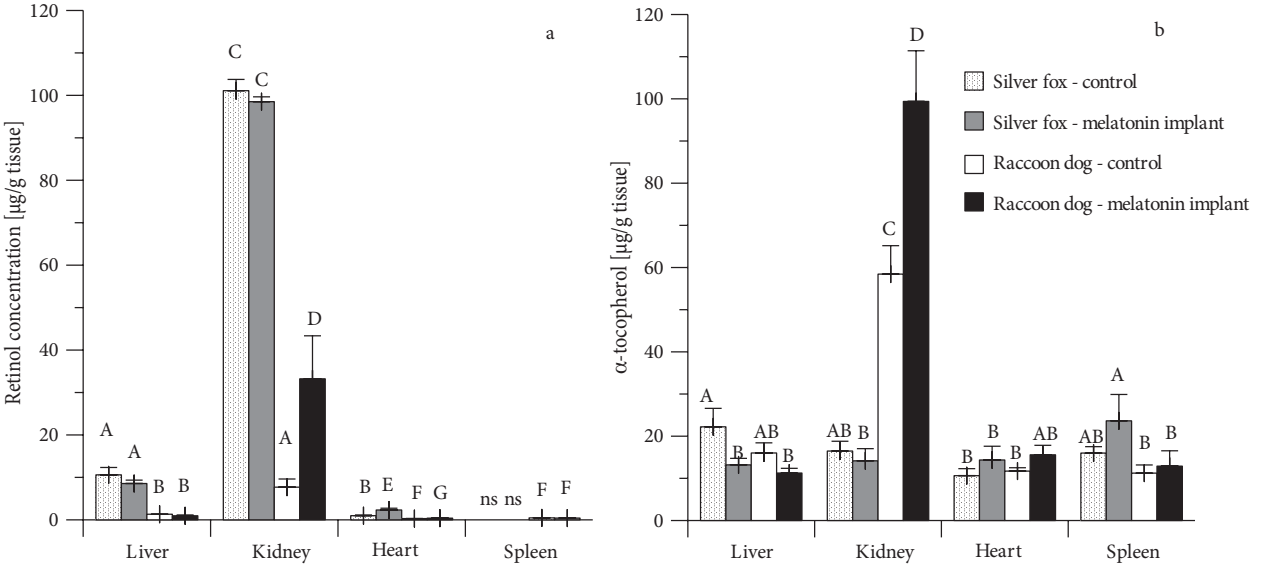

Figure 3. Concentration of a) retinol and b) α-tocopherol in organs of silver fox (n = 20) and raccoon dog (n = 20) that received a subcutaneous continuous-release melatonin implant (12 mg). A, B, C, D, E, F: bars with different letters differ significantly ($P \leq 0.05$).

evoke oxidative stress, which stimulates the activity of the antioxidant system in this species. It is worth noting that the reaction of the raccoon dog's organism resembles symptoms of metabolic syndrome (MS), which appears in humans.

MS is defined by the presence of insulin resistance, hyperinsulinemia, and some combination of cardiometabolic risk factors such as obesity, hypertension, and dyslipidemia (18,19). MS mostly affects societies leading a sedentary lifestyle and having continuous access to food (20), while exercise, diet, and fasting, which result in weight reduction, are well-documented ways to prevent insulin resistance and associated diseases (21). A similar effect can be observed in farmed raccoon dogs kept under conditions of reduced physical activity, which consume food and do not hibernate during the winter season. In these animals, plasma levels of insulin, glucose, and cortisol are significantly higher in comparison to the animals that undergo winter fasting and hibernating (7).

Some experimental studies indicated that prolonged administration of melatonin may be beneficial for patients with MS and/or diabetes and may improve the antioxidant defense of the organism (18,22). This is consistent with the opinion that melatonin influences the activity and expression of the antioxidative enzymes, i.e. glutathione peroxidase, SOD, and CAT (13,23–25), as well as antioxidant vitamins (26).

Melatonin implants are commonly used in fur animal practice. The release of melatonin from the implant is continuous and does not follow the diurnal rhythm of endogenous melatonin. Moreover, measurements of melatonin in blood plasma of implanted raccoon dogs (7) and silver foxes (27) show that the implants are capable of maintaining supraphysiological plasma levels of this hormone, several times exceeding endogenous concentration. They may reflect an increase in the endogenous secretion and/or a change in the metabolic clearance of the hormone. Such an increase or change would indicate that the constant supply of melatonin from the implant does not block the endogenous secretion of melatonin (27).

Taking into consideration the physiological role of melatonin, enhanced antioxidant activity in the organs of both investigated species could be expected. However, the results obtained did not confirm this assumption. Melatonin implants had no significant effect on the fox antioxidant system in contrast to the raccoon dog.

Response of the antioxidant system of the raccoon dog to melatonin implant reflects the results of studies performed in humans with MS (22). Patients with MS treated with melatonin for 2 months showed a significant increase in CAT activity, but SOD activity and GSH concentration in the blood plasma were not changed in comparison to the nontreated group. Similarly, mice fed with a hypercholesterolemic diet that received water with or without melatonin did not show changes in hepatic SOD activity and GSH levels. On the other hand, hepatic α-tocopherol and ascorbic acid levels in melatonin-treated animals significantly increased (28). However, these results are inconsistent with other experimental data obtained in rodents since Ohta et al. (24) observed in acutely liver-injured rats that a high oral dose of melatonin

attenuated the decrease in hepatic activity, while all applied melatonin doses did not affect hepatic CAT activity and GSH concentration in blood circulation. Nevertheless, Szaroma and Dziubek (29) found that a single injection of melatonin could prevent the decrease in GSH content and hepatic SOD and CAT activity in mice.

In order to explain the results obtained in our study it is necessary to take into consideration that the most definitive physiological role of melatonin is to convey information about day length (photoperiod) to body physiology for the organization of functions that vary with season, such as reproduction, pelage (coat growth and color), and winter sleep, but it also affects appetite, energy intake and expenditure, and adiposity. These effects may vary according to the species, breed, and/or even the genotype (30). In rats and mice melatonin reduces body weight while in raccoon dogs and silver foxes the effect of this hormone is opposite (7,12,14,15). In our study, animals of both species with melatonin implants had a better appetite and higher body weight, and their winter coat matured 3 weeks earlier in comparison with control animals (results not presented). It is possible that increase of melatonin concentration in blood plasma following implant application is interpreted by the organism of the raccoon dog that "a winter sleep is very near but fat has not been stored yet". It can excessively accelerate fattening and paradoxically trigger stress. The silver fox remains active during winter and its reaction to exogenous melatonin is not so strong.

In summary, raccoon dogs and silver foxes differ not only in the function of the antioxidant system but also in the response of this system to exogenous melatonin. It seems that melatonin administration reduces the level of activity of the antioxidant system in the raccoon dog. It can be assumed that the winter farm feeding raccoon dogs exhibit symptoms similar to those observed in MS. Therefore, it seems interesting to use these animals as an experimental model for medical studies of this disease.

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