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Effect of Hibernation on Oxidative and Antioxidant Events under Laboratory Conditions in Anatolian Ground Squirrel, *Spermophilus xanthoprymnus* (Bennett, 1835) (Mammalia: Sciuridae) from Central Anatolia

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Abstract.- The oxidative stress is an integral part of the metabolic depression machinery associated with hibernation, estimations and tolerance to freezing, dehydration, hypoxia and anoxia In the present study, we aim to investigate the role of hibernation on lipid peroxidation, nitric oxide production and glutathione levels in various tissues of Anatolian Ground Squirrel *Spermophilus xanthoprymnus*. A total of 9 female ground squirrels were collected from different localities in Nigde, Bolkar Mountains, Turkey. All squirrels were in three different conditions: at hibernation, aroused and non-hibernation stage. They were sacrificed under anesthesia. Glutathione (GSH), reactive nitrogen oxide species (NOx) and malondialdehyde (MDA) levels were measured spectrophotometrically. GSH levels of almost all tissues were lower in the hibernating group compared with the aroused group. NOx levels were found to decrease in all tissues except the brain tissue examined on hibernating group compared to non-hibernating group. MDA levels were found to increase in brain, lungs and heart tissues examined on hibernating group compared to non-hibernating group. In conclusion, our data show that an impaired balance exists between oxidative stress and antioxidant systems in most organs and tissues during hibernation.

Key words: Hibernation, *Spermophilus xanthoprymnus*, oxidative stress, antioxidant.

INTRODUCTION

The ground squirrel, *Spermophilus xanthoprymnus*, is a terrestrial diurnal rodent; an excellent hibernator of the family Cricetidae. Bennet first described *S. xanthoprymnus* from Erzurum Turkey in 1835 (Yigit *et al*., 2000), followed by many studies done on different aspects including hibernation (Dogramacı *et al*., 1995; Yigit *et al*., 2000). Hibernation is a physiological adaptation characterized by entry into torpor, which involves profound decrease in metabolism, heart rate, respiration, and body temperature (a few degrees above the ambient temperature) (Geiser, 2004; Okamoto *et al*., 2006). During hibernation, mammals undergo repeated cycles of torpor and arousal and several physiological and biochemical parameters are reversed to normal euthermic levels without obvious damages in a short period when they return to aroused state (Okamoto *et al*., 2006).

Mammalian hibernators are exposed to hypoxia due to rapid increase in oxygen consumption while increasing to 37°C in a few minutes the body temperature during waking from hibernation outside receive apneic oxygenation in a deep hibernation (Storey and Storey, 2004). Besides that, dramatic changes due to wake and deep hibernation cycles result in increasing risk of oxidative stress in sensitive tissues of mammalian hibernators (Carey *et al*., 2000). Oxidative stress occurs when intracellular concentrations of free radicals increase over the physiologic values. Mammalian cells have developed elaborate antioxidant defense systems to prevent oxidative damage caused by free radicals (O'Duffy and Goldstein, 1976; Raborn and Grace, 2003). However, hibernators clearly tolerates the cycles of torpor and arousal (Carey *et al*., 2003a; Okamoto *et al*., 2006). The mechanisms of adaptation to the oxidative stress during re-warming of hibernators is not yet clearly understood. Markers of oxidative

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stress such as glutathione (GSH), nitrous oxide (NO) and malondialdehyde (MDA) are likely to provide such explanation. GSH is an antioxidant whose role is to prevent damage caused by reactive oxygen species (ROS) (Pompella *et al.*, 2003). In this process, glutathione is converted to its oxidized form glutathione disulfide. The ratio of reduced glutathione to oxidized glutathione within cells is often used as a measure of cellular toxicity. An increase in glutathione disulfide is considered as an indicator of oxidative stress (Pastore *et al.*, 2003). NO is both a gas and a free radical that reacts with many biological molecules (Bosca *et al.*, 2005). MDA is widely used as an indicator of increased lipid peroxidation. It is a stable end-product of peroxidation of membrane lipids by ROS (Saral *et al.*, 2005).

In the present study, we aim to investigate the effects of hibernation on GSH levels, NO production and lipid peroxidation in various tissues of aroused and non-hibernating of Anatolian ground squirrel.

MATERIALS AND METHODS

Chemicals

All chemicals were of highest analytical grade, and purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO, USA).

Animals

Nine female ground squirrels were collected from different localities in Nigde, Bolkar Mountains, Turkey. The squirrels were divided into three groups: hibernating (n=3), aroused (n=3) and non-hibernating (n=3) housed individually in a room with an ambient temperature of 21°C with a 12 h light/12 h dark cycle, and were fed standard rodent diet and tap water *ad libitum*. They were sacrificed under anesthesia, their brain, heart, kidney, liver and lung tissues were excised, immediately frozen in liquid nitrogen and stored at -80°C until used.

Determination of GSH level

The GSH levels were determined by the modified Elman method (Aykac *et al.*, 1985). Tissues were homogenized in 10% ice-cold trichloroacetic acid in tissue homogenizer Heidolph Diax 900 (Heidolph-Instruments GMBH & COKG, Scwabach, Germany). After centrifugation at 3000xg for 10 min by Hettich D-78532 (Andreas Hettich GmbH & Co. KG, Tutlingen, Gemany), 0.5 ml of supernatant was added to 2 ml of 0.3 M Na_2HPO_4 $2H_2O$ solution. Then 0.2 ml of dithiobis nitrobenzoic acid solution (0.4 mg/ml 1% sodium citrate) was added and the absorbance at 412 nm was measured by Spectro UV-VIS RS (Labomed, Inc., CA, USA) immediately after mixing. The GSH levels were calculated unit using an extinction coefficient of 13.600 mol/cm.

Determination of NO levels

NOx (nitrite+nitrate) levels in tissues, stable end products of nitric oxide, were measured by the Griess reaction (Green *et al.*, 1982). The tissue samples were homogenized in five volumes of phosphate buffer (pH 7.5) and centrifuged at 2,000×g for 5 min and 0.25 ml of 0.3 M NaOH were added to the 0.5 ml supernatant. After incubation for 5 min at room temperature, 0.25 ml of 10% $ZnSO_4$ was added for deproteinization. This mixture was then centrifuged at 14 000 × *g* for 5 min, and then the supernatants were used for the Griess assay. The nitrate levels in tissue homogenates were determined spectrophoto-metrically, based on the reduction of nitrate to nitrite by VCI_3 (Miranda *et al.*, 2001). The nitrite levels were measured by a standard curve prepared by different concentrations of (1, 10, 50, 100 µM) sodium nitrite and nitrate solutions.

Measurement of lipid peroxidation

Lipid peroxidation was estimated as MDA levels reacting with thiobarbituric acid, according to methods described by Casini *et al.* (1986) and expressed as nanomole of MDA per gram wet weight. In brief, tissue samples were homogenized in 10% ice cold trichloroacetic acid in a tissue homogenizer. After centrifugation of the homogenate at 3 000 rpm for 10 min, 750 µl of supernatant was added to an equal volume of 0.67% thiobarbituric acid and heated at 100°C for 15 min. The absorbance of the samples was measured at 535 nm with a spectrophotometer.

Statistical analysis

The statistical differences between the mean values were evaluated by one-way analysis of variance by SPSS 15.0 (SPSS Inc., Chicago). Values of $p<0.05$ were considered to be significant. All data were expressed as the mean ± standard deviation.

RESULTS

Table I shows GSH, NOx and MDA levels in hibernating, aroused and non-hibernating Anatolian ground squirrel.

GSH levels

GSH levels of almost all tissues were lower on hibernating group compared with aroused group. Moreover, GSH levels decreased in all tissues except the brain in hibernating group compared to non-hibernating group. The GSH level of brain tissue was the lowest in non-hibernating group (1.705±0.78 μmol/g tissue) whereas those of kidney, lung, liver and heart were the lowest in hibernating group (2.260±1.01, 1.988±0.78, 11.442±2.75 and 1.988±0.94 μmol/g, respectively). GSH levels increased however, in all tissues in aroused state compared with hibernating. When all tissues were evaluated separately, the GSH level of kidney was the lowest in hibernating group (2.26±1.01 μmol/g tissue) compared to both aroused (3.638±1.13 μmol/g tissue) and non-hibernating group (6.345±1.98 μmol/g tissue). GSH level of lung was the lowest in hibernating group (1.988±0.78 μmol/g tissue) compared to both aroused (5.584±1.96 μmol/g tissue) and non-hibernating group (4.081±1.79 μmol/g tissue). Likewise, GSH level of liver was the lowest in hibernating group (11.442±2.75 μmol/g tissue) compared to both aroused (12.076±2.78 μmol/g tissue) and non-hibernating group (13.726±2.98 μmol/g tissue). GSH level of heart was the lowest in hibernating group (1.988±0.94 μmol/g tissue) compared to both aroused group (2.136±0.98 μmol/g tissue) and non-hibernating group (3.955±1.12 μmol/g tissue).

NOx levels

Table I shows that NOx levels increased in brain and kidney tissues (0.183±0.02 and 0.582±0.05 μmol/g tissue, respectively), but decreased in lung, liver and heart (0.189±0.01, 1.238±0.12 and 0.115±0.01 μmol/g tissue, respectively) in hibernating group compared with aroused group. NOx levels were found to decrease in all tissues except the brain in hibernating group compared to non-hibernating group. NOx levels on the other hand, increased in the brain tissue of hibernating group (0.183±0.02 μmol/g tissue) compared with both aroused (0.148±0.05 μmol/g tissue) and non-hibernating group (0.156±0.04 μmol/g tissue).

MDA levels

MDA levels were found to increase in brain and liver tissues, and decrease in kidney, lung and heart of aroused group compared with hibernating group. Table I shows that MDA levels increase in brain, lung and heart tissues in hibernating group compared to non-hibernating group. In contrast, MDA levels decreased in kidney and liver in hibernating group (19.531±3.21 and 21.745±3.71 μmol/g tissue, respectively) compared to non-hibernating group (28.402±3.01 and 30.745±7.81 μmol/g tissue, respectively). The MDA level of brain tissue was the lowest in non-hibernating group (10.875±1.19 μmol/g tissue). MDA level of heart tissue was observed the highest in hibernating group (45.942±9.16 μmol/g tissue).

DISCUSSION

Hibernation is widely regarded as an adaptation to seasonal energy shortage, but the actual influence of energy availability on hibernation patterns is rarely considered. The presumed benefits of metabolic depression for hibernators have led some authors to interpret factors that lead to increased torpor expression as eliminating constraints that prevent torpor from being expressed at its ideal, maximal level (Frank *et al*., 2000; Harlow and Frank, 2001). Hibernating mammals exhibit oxidative stress resistance in brain, liver and other tissues. In many animals, cellular oxidative stress resistance is associated with enhanced expression of intracellular antioxidant enzymes (Page *et al*., 2009).

Table I.- MDA, GSH and NOx levels of the brain, kidney, lungs, liver and heart tissues

Tissues	GSH (μmol/g tissue)	NOx (μmol/g tissue)	MDA (nmol/g tissue)
Brain			
Hibernation[a]	1.776 ± 0.29	0.183 ± 0.02	11.663 ± 1.89
Aroused[b]	3.169 ± 1.11	0.148 ± 0.05	29.997 ± 3.27
Non-hibernation[c]	1.705 ± 0.78	0.156 ± 0.04	10.875 ± 1.19
Kidney			
Hibernation[a]	2.260 ± 1.01	0.582 ± 0.05	19.531 ± 3.21
Aroused[b]	3.638 ± 1.13	0.339 ± 0.07	19.134 ± 2.27
Non-hibernation[c]	6.345 ± 1.98	0.644 ± 0.12	28.402 ± 3.01
Lung			
Hibernation[a]	1.988 ± 0.78	0.189 ± 0.01	17.846 ± 2.89
Aroused[b]	5.584 ± 1.96	0.289 ± 0.06	12.058 ± 1.97
Non-hibernation[c]	4.081 ± 1.79	0.617 ± 0. 21	13.753 ± 1.73
Liver			
Hibernation[a]	11.442 ± 2.75	1.238 ± 0.12	21.745 ± 3.71
Aroused[b]	12.076 ± 2.78	1.385 ± 0.24	25.042 ± 4.61
Non-hibernation[c]	13.726 ± 2.98	1.515 ± 0.65	30.846 ± 7.81
Heart			
Hibernation[a]	1.988 ± 0.94	0.115 ± 0.01	45.942 ± 9.16
Aroused[b]	2.136 ± 0.98	0.154 ± 0.03	29.979 ± 3.14
Non-hibernation[c]	3.955 ± 1.12	0.162 ± 0.07	16.344 ± 2.21

GSH, glutathione; NOx, Nitric oxide; MDA,Malondialdehyde
All data are expressed as the mean ± SD
Statistical significance in all groups of squirrels sacrificed on the same day;
*The values marked not statistically different; P>0.05
** The values marked statistically different; p<0.05

The statistical difference found between groups

in brain
GSH values; a-c*; a-b**; b-c**;
NOx values; a-b*; a-c*; b-c*;
MDA values; a-b**; a-c;* b-c**;
in kidney
GSH values; a-b*; a-c**; b-c**;
NOx values; a-b*; a-c*; b-c*;
MDA values; a-b*; a-c*; and b-c*;

in lung
GSH values; a-b**; a-c**; b-c*;
NOx values; a-b*; a-c**; b-c**;
MDA values; a-b*; a-c*; *b-c*
The statistical different not found between groups
GSH values a-b*; a-c*; b-c*;
NOx values a-b*; a-c*; and b-c*;
MDA values; a-b*; a-c*; b-c*;
in heart
GSH values; a-b*; a-c*; b-c*;
NOx values a-b*; a-c*; b-c*;
MDA values; a-b**, a-c**; b-c**

A hibernator needs to protect its cells against oxidative damage occurring both over long periods while in torpor and during arousal as a result of the huge increase in oxygen consumption. Several studies have indicated seasonal and state-dependent changes in antioxidant defenses in ground squirrels (Buzadzic *et al*., 1997; Blagojevic *et al*., 1998). However, only a few studies have monitored oxidative stress during hibernation or following arousal in different tissues (Carey *et al*., 2003b; Ma

et al., 2005) or different animals (Osborne and Hashimoto, 2007). The effect of oxidative stress on hibernating animals of this species is limited.

ROS are highly damaging to biomacromolecules, hence, effective antioxidant defense mechanisms are needed. Indeed, early reports showed an increase in the activities of superoxide dismutase and glutathione peroxidase, two major antioxidant enzymes, in arousing hibernators (Chance *et al*., 1979). GSH is the major thiol-disulfide redox buffer within cells that plays a key role in the detoxification of endogenous and exogenous ROS (Aw *et al*., 1997). Cellular oxidative stress is often manifested as a shift in the ratio of glutathione from its reduced (GSH) to oxidized (GSSG) forms. Several events that occur during the hibernation season place the intestine at particular risk for oxidative stress including drastic alterations in blood flow, thermal stress and nutritional deprivation (Granger and Korthuis, 1995; Jonas *et al*., 1999). GSH levels of almost all tissues were lower on hibernating group compared with aroused group in our study. The increase in GSH levels may be protective against any oxidative stress resulting from arousal.

Organisms are exposed to oxidative stress with continuous generated ROS. Organisms use enzymatic and non-enzymatic defense mechanisms to protect themselves from these products. These ROS listed as superoxide radicals, hydrogen peroxide, hydroxyl radicals, molecular oxygen, ozone, nitric oxide and peroxynitrite (Hermes-Lima and Zenteno, 2002). NO is a bioactive molecule known to be involved in inflammation as well as having a demonstrated stimulatory effect on vascular cells (Coskun *et al*., 2007). It is also a potential candidate for such regulation because it is capable of modulating contractile function under a variety of circumstances (Canty, 2000). It is released by the endothelium in response to shear stress and plays an important role in flow-mediated vasodilatation (Avcı *et al*., 2008). In this study, NOx levels determined to increase in the brain tissue at hibernating group when compared with both aroused and non-hibernating groups. The increase of NOx levels is an indicator of increased oxidative stress on brain tissues during hibernating.

If the balance between the productions of free oxygen radicals and antioxidant defense mechanisms becomes corrupted, free oxygen radicals increase and cause tissue damage (Yarıktas *et al*., 2004). Free radicals harm such basic molecular as nucleic acids, proteins, free amino acids, lipids and carbohydrates. Furthermore, the free radicals affect the permeability of the cell membrane via lipid peroxidation and oxidation of protein sulfhydryl groups by starting (Kurata *et al*., 1993; Portoles *et al*., 1996). Lipid peroxidation by free radicals results in the formation of several end products, including MDA. This presence is commonly used as an indicator of oxidative damage (Baker *et al*., 2007). In our study, MDA levels increased in brain, lung and heart tissues in hibernating group. We show that there is more oxidative stress in these tissues during hibernation. Orr *et al*. (2009) evaluated lipid peroxidation in brown adipose tissue during hibernation. Lipid peroxidation was higher in eurotherm period but declined during hibernation. However, lipid peroxidation increased during the period of waking up late. At the same time, lipid peroxidation damage was found higher in liver tissue during hibernation than eurotherm period. L'vova and Gasangadzhieva (2003) reported high MDA levels during hibernating period compared to before hibernation. Carey *et al*. (2003b) showed that hibernation is associated with increased lipid peroxidation as well as the expression of stress-activated signaling pathways in ground squirrel intestine.

In conclusion, hibernation is being increasingly recognized as a valuable model for natural resistance to stress induced by extreme changes. Studies show that the effect of hibernation on oxidative stress in this species is limited, and there is an impaired balance between oxidative stress and antioxidant systems in most organs and tissues during hibernation.

Conflict of interest declaration

The authors have no conflict of interests to declare.

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