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Oxidative stress markers and antioxidant defense in hibernating common Asian toads, *Duttaphrynus melanostictus*

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Abstract: To assess the oxidative assaults and antioxidant defense, oxidative stress markers, including lipid peroxidation level, protein carbonylation level, GSSG/GSH ratio and nonenzymatic antioxidants such as total glutathione, ascorbic acid and uric acid, in liver and brain tissues of hibernating common Asian toads, *Duttaphrynus melanostictus*, were compared with toads during active periods. Oxidative stress was found in both liver and brain tissues of hibernating common Asian toads in spite of depressed metabolism and low oxygen consumption. Significantly higher lipid peroxidation, protein carbonylation and an increased GSSG/GSH ratio were found in liver and brain tissues of hibernating toads, indicating oxidative stress. To counteract the stress, ascorbic acid was increased significantly in the liver and brain tissues of hibernating individuals in comparison to individuals during active periods. The uric acid level decreased in both the liver and brain tissues of hibernating toads, which may be due to its decreased rate of synthesis because of low xanthine oxidase activity at low body temperature and hypometabolism. The common Asian toad faced oxidative stress during hibernation, which was counteracted by augmented nonenzymatic antioxidant defense.

Keywords: hibernation; oxidative stress markers; nonenzymatic antioxidant defense; glutathione redox ratio

INTRODUCTION

In all aerobic organisms, reactive oxygen species(ROS) such as superoxide radicals ($O_{2}^{\cdot -}$), hydroxyl radicals (OH^{-}) and hydrogen peroxide (H_2O_2) that are produced during cellular metabolism promote the oxidation of biomolecules, and their damaging effects are minimized by antioxidant defenses comprising antioxidant enzymes and nonenzymatic antioxidants. When the rate of ROS production overcomes the natural capacity of cellular antioxidant defenses a situation called oxidative stress results [1]. ROS-mediated oxidation of amino acid residues, inparticular proline, arginine and lysine of protein in animal cells to their carbonyl derivatives, increases exponentially with exposure to stressed conditions [2,3]. Similarly, oxidative damage of lipids, resulting in a wide variety of lipid peroxidation products such as malondialdehyde, hexanol, and 4-hydroxyalkenals, has been reported to increase during stressed conditions in insects, rotifers, fishes, amphibians, reptiles and mammals [4-6]. Thus, lipid peroxidation and protein carbonylation are considered to be oxidative stress markers in a variety of living organisms [7-8].

Aerobic organisms adopt different strategies to deal with oxidative stress. One of them is to minimize the level of oxygen uptake or to deter its conversion to ROS [9]. Another way is by evolving an antioxidant defense system to counteract the oxidative stress. This is again either by catalytic removal of ROS by antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase), or by scavenging ROS by nonenzymatic antioxidants (α-tocopherol, ascorbic acid, reduced glutathione, uric acid) [10]. Reduced glutathione (GSH), a nonenzymatic hydrophilic endogenous antioxidant, acts as a scavenger of ROS in combination with antioxidant enzymes glutathione peroxidase(GPx) and glutathione reductase (GR)[11,12]. GSH is converted into its oxidized form, glutathione disulfide (GSSG), by donation of a reducing equivalent ($H^{+}+e^{-}$) to the ROS

for its neutralization in the presence of GPx, followed by regeneration from GSSG by GR. Accordingly, the GSSG/GSH ratio has been considered as a marker of oxidative stress [13]. Nonenzymatic antioxidants such as ascorbic acid and uric acid, are water-soluble ROS scavengers that have been reported to increase in physiologically stressed conditions [14]; however, reports regarding their status during hibernation are limited to some endothermic mammals and are almost nonexistent in ectothermic animals.

The common Asian toad, *Duttaphrynus melanostictus*, hibernates as an adaptation to cold during the winter season, characterized by metabolic depression, low body temperature, slow breathing and decreased heartbeat rate. They usually hibernate inside their burrows in moist and loose soil and under leaves and debris. Although cycles of torpor and arousal, depending on changes in body temperature, have been reported during hibernation in endothermic mammals, studies related to hibernation in ectothermic animals are limited. A reduction in oxygen consumption to nearly 20%of normal resting rate and depressed metabolism during hibernation were reported [15], most likely resulting in a decrease in ROS production and lower oxidative stress. However, increased oxidative stress during hibernation has been reported mainly in endothermic mammals [16-18]. Reports on oxidative stress and antioxidant defenses during the hibernation of ectothermic vertebrates are limited [2,19-21], and investigations into oxidative stress markers and antioxidant defense in the hibernating common Asian toad have not been undertaken in detail. In the present work we examined whether oxidative stress markers increase during hibernation in spite of low oxygen consumption and depressed metabolism, and what happens to the antioxidant defense status, specifically to selected nonenzymatic antioxidants, during hibernation.

MATERIALS AND METHODS

Ethics statement

Animal treatment followed the directives of the Institutional Animal Ethics Committee, Berhampur University, India, Registration No. 2020/GO/Re/S/18/CPCSEA, and Resolution No. 01.

Chemicals

GR, 5,5'dithio-bis (2-nitro benzoic acid)(DTNB) and thiobarbituric acid(TBA) were purchased from Sigma Chemical Co (USA); NADPH, GSH, GSSG,2-vinyl pyridine, guanidine hydrochloride ,EDTA and ascorbic acid were obtained from HiMedia Laboratories Pvt. Ltd., India. All other chemicals and reagents were of analytical grade.

Sample collection

Middle-aged (2-to 4-year-old) male toads (*Duttaphrynus melanostictus*) found along with toads of different age in a well-protected area with broken houses, bushes and swampy areas located in Paralakhemundi (10˚ 45' N, 84˚ 6'E), India, were selected after determining their age by skeletochronology[22]. Males were identified by observing a brick red or orange colored hue on the throat region, the subgular vocal sac and black nuptial pads on the inner sides of the first two fingers of the forelimb. In this study, 14 male toads with a snout-vent length of 7.5-8 cm and body weight of 35-47 g,collected fromtheir natural habitat, were used. Oxidative stress markers and the nonenzymatic antioxidant defense status were assessed in brain and liver tissues of 7 hibernating toads collected during the winter season (December and January) and compared to 7 active toads collected during the early rainy season(July to August).

Tissue preparation

Toads were collected from their natural habitat and immediately decapitated to dissect out the whole liver and brain. Adherent tissues were removed in ice-cold (2°C) amphibian Ringer's solutions, and they were weighed and processed immediately for different estimations of oxidative stress markers and nonenzymatic antioxidants.

Lipid peroxidation assay

The level of lipid peroxidation (LPO) in terms of thiobarbituricacid reactive substances (TBARS) formed was estimated by the thiobarbituric acid (TBA) test as described[23]; 0.5 mL 2.5% (w/v) ice-cold aqueous tissue homogenate, 1.5 mL of 1% orthophosphoric acid and 0.5 mL of 0.6% TBA were heated in a hard glass test tube for 45 min at 95°C. After cooling to room temperature, 3mL of chloroform and 1mL of glacial acetic acid were

added to the mixture. Extinction of the upper phase of the supernatant containing TBARS after centrifugation at 1000×g for 10 min was measured at 535nm against the control containing 0.5 mLof distilled water instead of the tissue homogenate. The formed TBARS was expressed as μmol/g tissue wet weight using a molar extinction coefficient of $1.56 \times 10^5 M^{-1}cm^{-1}$.

Protein carbonylation assay

The carbonyl content of proteins as a marker of oxidative stress [24] in a sucrose soluble tissue homogenate was estimated following the methods of Uchida and Stadtman [25]. In the experimental tube, 0.8 mL of 0.25 M sucrose soluble tissue supernatant (2.5% w/v) and 0.8 mL of 0.1% (w/v) 2,4 dinitrophenyl hydrazine (DNPH) in 2 N HCl was incubated at room temperature (25±2°C) for 1 h in the dark. A control tube was also run simultaneously along with the experimental tube with 0.8 mL of 2 N HCl instead of DNPH. After the incubation period, protein fractions were obtained by centrifugation (1000 × g for 10 min) with 0.8mL of 20% trichloroacetic acid (TCA). Protein fractions were washed with an ethanol/ethyl acetate mixture (1:1V/V) and then dissolved in 2 mL of 8Mguanidine hydrochloride prepared in 133 mMTrisbuffer (pH 7.2) containing 13 mM EDTA. Extinction of the experimental sample was measured at 365 nm against the control, and the carbonyl content was expressed as nanomoles of DNPH incorporated per mg protein, based on the molar extinction coefficient of 22×10^3 $M^{-1}cm^{-1}$. The protein content of the tissue homogenate was estimated following the method of Lowry et al. [26] using bovine serum albumin as standard.

Total and oxidized glutathione

Total glutathione equivalents (GSH_{eq}) consisting of both GSH and GSSG were measured following the method of Griffith [27]. A protein-free supernatant obtained by centrifugation (10000×g for 15 min) of the tissue homogenate (1:5w/v) in ice-cold (2°C) sulfosalicylic acid, was divided into two parts. One part was used to measure GSH_{eq} by observing the rate of reduction of DTNB at 412 nm containing 0.2 mM NADPH, 0.6 mM DTNB, 5 mM EDTA, 125 mM sodium phosphate buffer (pH 7.5) and tissue extract in a final volume of 1mL.To ensure the rate of reaction as zero, the reaction was started by adding GR (0.5U). The rate of reaction is proportional to the concentration of GSH_{eq} and was compared with the standard curve of GSH (0-6 μM). Another part of the protein-free supernatant was treated with 170mM2-vinyl pyridine for 1h to derivatize GSH. The rest of the GSSG was measured, and the total GSH was calculated from the equation GSH_{eq}=GSH+2GSSG, and the result was expressed as μmol/g tissue wet weight. The levels of GSH (GSH=GSH_{eq}-2GSSG) and percent oxidized GSH (GSSG/GSH) were also calculated.

Ascorbic acid

A deproteinized supernatant obtained from centrifugation (1000×g for 10 min) of 2.5%(w/v) of tissue homogenate prepared from 6% ice-cold TCA was used to estimatethe ascorbic acid content following the method by Roe [28]. Ascorbic acid present in deproteinized tissue extract was oxidized to dehydroascorbic acid (DHAA) using bromine water, which transformed irreversibly to 2,3-diketogulonic acid (DKA). The DKA coupled with 2, 4-dinitrophenyl hydrazine (DNPH) to form a colored product with H_2SO_4. Extinction of the colored product was measured at 530nm and the result was obtained from the standard curve of ascorbic acid and expressed as μg ascorbic acid/g wet tissue.

Uric acid

The uric acid content in the deproteinized supernatant obtained by centrifugation (1000×g for 10 min) of 0.5 mLof 2.5%(w/v) tissue homogenate prepared in ice-cold 50 mM phosphate buffer (pH 7.0), 4 mL of N/23 H_2SO_4 and 0.5 mL of 5.6% sodium tungstate was estimated as described [29]. Extinction of the light blue-colored product formed by adding 0.2 mL of phosphotungstic acid reagent and 1mL of 0.6 N NaOH to 3 mL of deproteinized supernatant was measured at 720 nm, and the result was obtained from the standard curve of uric acid. The uric acid content was expressed as μg uric acid/g wet tissue.

Statistical analysis

Data were expressed as the means±SEM. Student's t-test was performed to evaluate the statistical significance of the data from active vs hibernating individuals. Differences were considered significant at $P<0.05$.

Table 1. Glutathione status in liver and brain tissues of the common Asian toad, *Duttaphrynus melanostictus.*

Tissue	Condition	GSH (µmol/g Tissue)	GSSG (µmol/g Tissue)	GSH_{eq}(2GSSG+GSH) (µmol/g Tissue)	GSSG/GSH Ratio
Liver	Active period	1.93±0.03	0.28±0.006	2.50±0.04	0.147±0.003
	Hibernation	1.50±0.04*** (22% decrease)	0.33±0.004*** (17% increase)	2.18±0.04*** (12% decrease)	0.225±0.006*** (53% increase)
Brain	Active period	0.28±0.006	0.026±0.001	0.332±0.006	0.093±0.003
	Hibernation	0.22±0.005*** (21% decrease)	0.041±0.004** (57% increase)	0.304±0.008* (8% decrease)	0.186±0.004*** (100% increase)

GSH – reduced glutathione, GSSG – oxidized glutathione, GSH_{eq} – glutathione equivalent.
Data are expressed as the means±SEM, (n=7). Significant differences calculated using Student's t-test from animals during active period are designated as *(P<0.05), ** (P<0.01), *** (P<0.001).

RESULTS

Oxidative stress

Table1 shows the glutathione status of different organs during hibernation vs the active period. GSH was significantly low in both liver (P<0.001) and brain (P<0.001) tissues in comparison to toads during the active period. A significantly higher GSSG level was observed in both liver (P<0.001) and brain (P<0.01) tissues of hibernating toads in comparison to toads during the active periods. However, there was a significantly (P<0.001 and P<0.05) lower level of total glutathione equivalent (GSH_{eq}=2GSSG+GSH) in both liver and brain tissues of hibernating toads (Fig. 2C) in spite of significant (P<0.001 and P<0.01) increases in GSSG. The decrease in the GSH_{eq} was due to a decrease in GSH, which led to a significant increase (P<0.001) in the GSSG/GSH ratio in both liver and brain tissues, indicating oxidative stress.

Oxidative damage

Compared with the active period, the level of lipid peroxidation measured in terms of TBARS was significantly higher in both liver (P<0.001) and brain (P<0.001) tissues during hibernation (Fig. 1A).Similarly, a significantly higher level of protein carbonylation was also found in both liver (P<0.01) and brain (P<0.01) tissues of hibernating toads in comparison to active toads (Fig. 1B).

Antioxidant defense

Ascorbic acid, a potent prooxidant scavenger, was significantly higher in both liver (P<0.05) and brain (P<0.001) tissues in comparison to toads during the active period (Fig. 2A). Uric acid, a nonenzymatic antioxidant, significantly deceased in both liver (P<0.05) and brain (P<0.05) tissues of hibernating toads in comparison to toads during the active period (Fig. 2B).

DISCUSSION

The status of oxidative stress markers and the nonenzymatic antioxidant defense was investigated in the liver and brain tissues of hibernating common Asian toads. Hibernation, which is accompanied by hypometabolism and low body temperature, has a potent effect on ROS

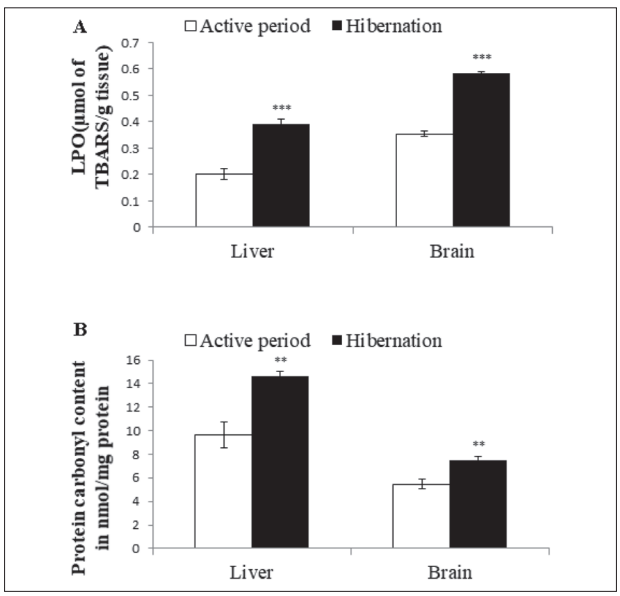

Fig. 1. Effect of hibernation on the level of lipid peroxidation (LPO) (**A**) and the protein carbonyl content (**B**) of liver and brain tissues of male common Asian toad, *Duttaphrynus melanostictus*. The data are expressed as the means±SEM, (n=7). Significance was calculated relative to animals in the active period; **(P<0.01), ***(P<0.001).

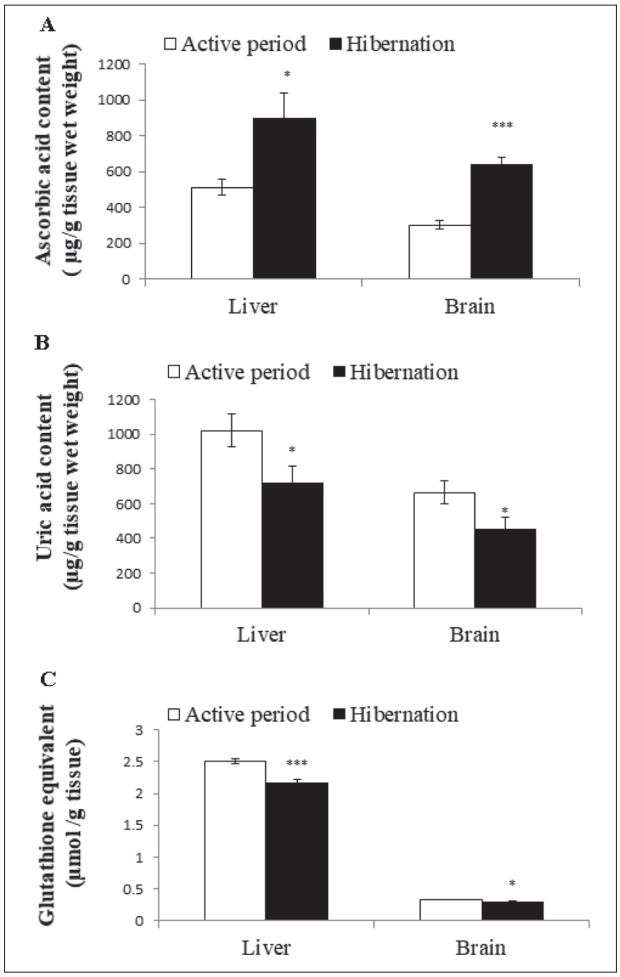

Fig. 2. Effect of hibernation on the ascorbic acid (**A**), uric acid (**B**) concentrations and the glutathione equivalent (**C**) of liver and brain tissues of male common Asian toad, *Duttaphrynus melanostictus*. The data are expressed as the means±SEM, (n=7). Significance was calculated relative to animals in the active period; *($p<0.05$), ***($P<0.001$).

generation and oxidative stress [16,30-33].Data from our investigation supports the assumption that the level of oxidative stress increases during hibernation. Significantly lower levels of GSH and GSH_{eq} were found in both liver and brain tissues of hibernating toads. In addition, a significantly higher GSSG level was found in both tissues during hibernation. This increase in GSSG and the reduction in GSH lead to a significant increase in the GSSG/GSH ratio, an indicator of oxidative stress [34, 35] and overall susceptibility towards oxidative damage [2]. The reduction in GSH could be due to its decreased biosynthesis [30] and decreased regeneration from GSSG [2]. As biosynthesis of GSH is an energy-consuming process [24], its synthesis probably diminished in the hypometabolic state during hibernation. Similarly, regeneration of GSH from GSSG by GR activity was probably decreased due to low GR activity and decreased NADPH supply in the hypometabolic condition and low body temperature during hibernation [2,36]. GSH-linked enzymes (GPx and GR) have also been reported to possess decreased activities in tissues of aestivating toad *Scaphiopus couchii*[2]. Our findings regarding oxidative stress in liver and brain tissue, as indicated by an increased GSSG/GSH ratio, also support the observed significantly higher levels of lipid peroxidation and protein carbonylation during hibernation. The difference in GSH content found between liver and brain tissues of the hibernating toad was probably because of the role played by the liver in its biosynthesis and inter-organ homeostasis [37]. A higher GSSG/GSH ratio indicating oxidative stress has also been reported in the intestinal mucosa of endothermic animals such as ground squirrels (*Spermophilus tridecem lineatus*) during hibernation [30]. Similarly, oxidative stresses during hibernation and activation of the redox-sensitive transcription factor NF-κB have also been reported in intestinal tissue of ground squirrels [16]. The GSH status of the common Asian toad during hibernation is in good agreement with findings obtained in different ectothermic animals, such as *Scaphiopus couchii* [2] and *Nanorana parkeri* [33].

Our investigation showed a significantly higher level of lipid peroxidation in terms of TBARS and protein carbonyl content in both tissues studied in toads during hibernation in comparison to toads during the active period. Hypometabolism and reduced oxygen consumption during hibernation are supposed to produce low levels of ROS that causes low level of oxidative damage [38]. The augmented lipid peroxidation level in the studied tissues points to oxidative damage due to reduced lipid peroxide scavenging mechanisms and continuous accumulation of damaged products in lipid reserves that are not replenished by dietary intake and biosynthesis during hibernation. The increased carbonyl protein content in the liver and brain during hibernation, indicating oxidative damage to proteins, may be due to elevated lipid peroxide products [25] and low protease activities removing oxidized proteins [39]. Reduced oxygen consumption maintains the redox state of the mitochondrial electron transport system towards a reduced state, favoring the production of superoxide radicals [40] and thus increased cellular oxidant produc-

tion [41]. Hibernating toads with hypometabolism and low oxygen consumption probably produced considerable oxidants that caused increased lipid peroxidation and protein carbonylation in the examined tissues. Also, the increase in polyunsaturated fats (PUFA) in membranes during cold exposure maintains membrane fluidity [42]. Accordingly, the increase in PUFA content in the body fat of heterothermic mammals has also been reported before their entry into torpor [43]. Low body temperature during hibernation could have increased the PUFA content in the cell membrane, making them more susceptible to lipid peroxidation. Our findings regarding oxidative stress and oxidative damage conform with results obtained in different ectothermic [2,33] and endothermic animals [16,30,31].

During hibernation, the toads were dormant inside their burrows, without food intake until arousal, with increased oxygen intake and rewarming. With increased oxygen consumption for rewarming during arousal, it is tempting to speculate that the animals would be exposed to oxidative stress in the absence of an augmented antioxidant defense system [44]. Our investigation showed a significantly higher ascorbic acid content in both liver and brain tissues of hibernating toads in comparison to toads during the active period. In amphibians, including the common Asian toad, ascorbic acid is usually synthesized in the kidney [45,46] and distributed to other tissues where it is transported into cells through sodium-dependent uptake [47]. Ascorbic acid is a well-established antioxidant [48,49] and has been reported to act as a free radical trap [50]. It has also been reported that ascorbic acid acts as a protective antioxidant during hibernation and rewarming from hibernation by scavenging free radicals produced during hibernation and the oxidative burst associated with rewarming [51,52]. Our results showing an increased ascorbic acid content in liver and brain tissues corroborate this. This augmented ascorbic acid content in liver and brain tissues could be a preparatory mechanism to minimize the potential injury of ROS during hibernation and rewarming after hibernation [17,24,53,54]. The rise in ascorbic acid indicates the ability of the common Asian toad to adapt to oxidative stress. A comparatively higher increase in ascorbic acid content in brain tissue as compared to liver tissue observed in this study may be an adaptive response to counteract lipid peroxides that are produced due to its high PUFA content and to act as a neuroprotectant [55].

Like ascorbic acid, uric acid is a water-soluble antioxidant due to its ability to scavenge $^{\cdot}O_2^-$, H_2O_2 and peroxy radicals [56]. It also maintains ascorbic acid in its reduced state [57]. Unlike ascorbic acid, uric acid is produced locally in tissues as a product of purine metabolism catalyzed by xanthine oxidase in response to oxidative stress [58]. We found significantly lower uric acid contents in both liver and brain tissues of hibernating toad in comparison to active toads. The low uric acid content in the examined tissues during hibernation may be due to its decreased synthesis because of low xanthine oxidase activity at low body temperature and hypometabolism. A low uric acid content has also been reported during hibernation in the liver of the ground squirrel because of low AMP deaminase 2 activity [59].

GSH is a water-soluble, endogenous antioxidant tripeptide that is also capable of neutralizing free radicals and maintaining exogenous antioxidants such as ascorbic acid and tocopherol in their reduced state [60]. We observed its decrease in both liver and brain tissues of hibernating toads. This may have been due to its decreased biosynthesis and regeneration from GSSG during the hypometabolic state. Moreover, the biosynthesis of GSH is an energy-consuming process [24] and probably decreased in several organs during hibernation [18]. The increased GSSG observed in the present study might be due to its increased production because of the higher neutralization of ROS by reduced GSH and its decreased turnover into GSH due to low GR activity during the hypometabolic state of hibernation.

CONCLUSION

Hibernation in the common Asian toad, *Duttaphrynus melanostictus* is an adaptive response to low temperature and scarcity of food during the winter season. We found increased oxidative stress markers, such as lipid peroxidation, protein carbonylation and GSSG/GSH ratio, in both liver and brain tissues of hibernating toads. Increased oxidative damage by raised lipid peroxidation and protein carbonylation are in good agreement with increased oxidative stress. Adaptive responses to counteract the oxidative stress were observed as augmented nonenzymatic antioxidant, i.e. increased ascorbic acid level, during hibernation. A decrease in uric acid in both tissues during hibernation points to its low rate

of synthesis in the hypometabolic condition. The comparatively higher level of ascorbic acid in brain tissue points to its neuroprotective role during oxidative stress.

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Conflict of interest disclosure: The authors declare that there is no conflict of interest.

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