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Dehydroepiandrosterone improves hepatic antioxidant reserve and stimulates Akt signaling in young and old rats

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ABSTRACT

This study examined, in the liver of young and old (3- and 24-month-old, respectively) healthy Wistar rats, the *in vivo* effect of dehydroepiandrosterone (DHEA) (10 mg/kg body weight) administered subcutaneously for 5 weeks. Reduced (GSH) and oxidized (GSSG) glutathione levels, glucose-6-phosphate dehydrogenase (G6PDH), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and catalase (CAT) activities, hydrogen peroxide concentration, GST and p-Akt/Akt immunocontent ratio were assessed in hepatic tissue. DHEA treatment significantly increased total glutathione content (17%) and GSH (22%) in 3- and 24-month-old treated groups when compared to control groups. The aging factor increased G6PDH (51%) and GPx (22%) activities as well as the hydrogen peroxide concentration (33%), independently of treatment. DHEA treatment increased p-Akt (54%) and p-Akt/Akt ratio (36%) immunocontents in both treated groups. Increased serum levels of alanine aminotransferase (ALT) in aged rats were reduced by DHEA treatment (34%).

1. Introduction

Dehydroepiandrosterone (DHEA) is an endogenous steroid synthesized in the adrenal cortex, gonads, brain, and gastrointestinal tract [1,2]. Levels of DHEA are maximal between the second and third life decades, and then start to decline 2% per year [3]. DHEA declinium associated with age may lead to autoimmune diseases, sexual dysfunction, osteoporosis, deterioration of lipid metabolism, type 2 diabetes mellitus and cardiovascular diseases [4]. Also the incidence of liver disease increases with age and older individuals are more susceptible to most acquired liver disorders and are more vulnerable to its consequences [5].

The differential diagnosis of specific liver diseases is aided by the measurement of aspartate (AST) and alanine (ALT) aminotransferases levels. These aminotransferases are enzymes involved in the reactions of nitrogen removal from amino acids (transamination reaction). In addition, such enzymes are also known as important biomarkers of hepatic function, specially, ALT [6]. Evidences indicate important age-related changes in liver function through the demonstration of significant diminished liver regenerative capacity in animal models [7–9].

Aging is a progressive deterioration in physiological functions and metabolic processes. In aging process, the reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide, and the highly reactive hydroxyl radical can be formed and be toxic at high concentrations [10]. To protect cells, defense mechanisms utilize non-enzymatic and enzymatic antioxidants to avoid or to retard cellular damage arising from oxidative stress—an imbalance between free radicals generation and antioxidant systems.

An important antioxidant cellular defense is the reduced glutathione (GSH), which participate as a substrate in the detoxification of xenobiotics and in the control of hydrogen peroxide and other peroxides concentrations. These processes are catalyzed by enzymes such as glutathione-S-transferase (GST) and glutathione peroxidase (GPx) [11]. Glutathione is a ubiquitous molecule that is produced in all organs, especially in liver [12]. Glucose-6-phosphate dehydrogenase (G6PDH) is an important

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enzyme involved in the production of NADPH. NADPH is a cofactor required for the conversion of GSSG to GSH by glutathione reductase. Therefore, G6PDH plays key role to restore the intracellular thiol redox status [13].

The balance ROS/antioxidants exerts a key role in the induction and maintenance of signal transduction pathways [14]. Akt, a serine/threonine kinase, is a redox-sensitive protein that can be stimulated by ROS [15] and activated as an answer to DHEA chronic treatment [16].

Several evidences support the potential DHEA utility as a therapeutic intervention based on human trials [17–19], *in vitro* experiments [20] and animal models [21,22]. An important clinical interest in DHEA is based on many observations, including a significant decline production since early adulthood, literature evidences showing changes in this steroid levels related to multiple pathologies and a pronounced replacement therapy that may alleviate age-associated declines in many functions [20].

Besides that, DHEA was considered to be a possible "youth fountain" hormone. However, many questions concerning its efficacy still remains and no sufficient data are available in literature to support its recommendation. Taken all these points together, we strongly believe that there is need to better understand DHEA's role in oxidative stress and aging in liver. Therefore, this study was undertaken to investigate chronic DHEA effects on redox status and on the Akt survival protein activation in liver during the aging process and its repercussion in hepatic function.

2. Materials and methods

2.1. Animals and treatments

Twenty-four Wistar male rats (3 and 24 months) obtained from the Instituto de Ciências Básicas da Saúde (ICBS) of the Universidade Federal do Rio Grande do Sul (UFRGS) were randomly assigned into control or DHEA (dehydroepiandrosterone; Calbiochem) (10 mg/kg) groups treated once a week, subcutaneously, for 5 weeks. Control groups received vehicle (vegetal oil). DHEA protocol administration was based on other studies [16,21,23,24]. Animals were housed in plastic cages (four animals in each) and received water and pelleted food *ad libitum*. They were maintained under standard laboratory conditions (controlled temperature of 21 °C, 12 h light/dark cycle). After 5 weeks, animals were killed by decapitation and their livers were quickly removed. Liver samples (approximately 100 mg) were frozen on dry ice and stored at −80 °C for western blotting. Remaining liver tissue was used to measure hydrogen peroxide, glutathione concentration, and antioxidant enzymes. Special care was taken to minimize the number of animals used and their suffering. All animal procedures used in this study were in accordance with the Principles of Laboratory Animal Care (COBEA – Brazilian College of Animal Experimentation) and the experimental protocol was approved by UFRGS Animal Care Committee (http://www.cobea.org.br project number 2006535).

2.2. Determination of oxidized and reduced glutathione concentration

To determine oxidized and reduced glutathione concentration, liver tissue was deproteinized with 2 mol/L perchloric acid, centrifuged for 10 min at 1000 × *g* and the supernatant was neutralized with 2 mol/L potassium hydroxide. The reaction medium contained 100 mmol/L phosphate buffer (pH 7.2), 2 mmol/L nicotinamide dinucleotide phosphate acid, 0.2 U/mL glutathione reductase, 70 μmol/L 5,5′-dithiobis (2-nitrobenzoic acid). To determine reduced glutathione, the supernatant was neutralized with 2 mol/L potassium hydroxide, to react with 70 μmol/L 5,5′-dithiobis (2-nitro benzoic acid), and the absorbance values measured at 420 nm [25].

2.3. Hydrogen peroxide concentration

Hydrogen peroxide was measured via its horseradish peroxidase (HRPO)-mediated oxidation of phenol red. Slices of fresh liver tissue were incubated for 30 min at 37 °C in phosphate buffer 10 mmol/L (NaCl 140 mmol/L and dextrose 5 mmol/L). The supernatants were transferred to tubes with phenol red 0.28 mmol/L and 8.5 U/mL HRPO. After 5 min incubation, 1 mol/L NaOH was added and the solution's absorbance values measured at 610 nm. The results were expressed in nmoles H_2O_2/g tissue [26].

2.4. Antioxidant enzyme activities

Glucose-6-phosphate dehydrogenase (*G6PDH*) assay was measured by the method of Leong and Clark [27], in which the reaction mixture (1 mL) contained: 100 mmol/L Tris–HCl pH 7.5, 10 m mol/L $MgCl_2$, 0.5 m mol/L NADP+, and sample. The reaction was started by the addition of 1 mmol/L glucose-6-phosphate and was followed in a spectrophotometer at 340 nm. One G6PDH unit corresponds to 1 mmol of substrate transformed per minute and the specific activity is represented as units per mg protein.

Glutathione-S-transferase (*GST*) activity, expressed as μmoles per milligram of protein, was measured by the rate formation of dinitrophenol-S-glutathione at 340 nm. The reaction medium consisted of 19 mmol/L sodium phosphate buffer (pH 6.5), 1 mmol/L GSH, and 1 mmol/L chloride dinitrobenzene [28].

Glutathione peroxidase (*GPx*) catalyzes reduced glutathione oxidation in the presence of peroxides. Its activity was expressed as μmoles of NADPH reduced per minute per milligram of protein. Selenium-dependent and -independent GPx activities were measured by following NADPH oxidation at 340 nm in a reaction medium containing 0.17 mmol/L reduced glutathione, 0.2 U/mL glutathione reductase, and 0.5 mmol/L tert-butyl hydroperoxide (which reacts with both the Se-dependent and non-Se-dependent GPx), as described by Flohé and Gunzler [29].

Catalase (*CAT*) activity was determined by following the decrease in 240 nm absorption in a reaction medium containing 50 mmol/L phosphate buffer (pH 7.2), and 10 mmol/L hydrogen peroxide (H_2O_2) [30]. It was expressed as μmoles of H_2O_2 reduced per minute per milligram of protein.

2.5. Western blotting

Liver samples were homogenized (Ultra-Turrax) with lysis buffer, pH 7.4, proportionally to weight (20 mL/g of tissue). Electrophoresis and protein transference were performed as described elsewhere [31,32]. The membranes were processed for immunodetection using rabbit polyclonal antibodies for p-Akt (ser 473) (60 kDa), Akt (60 kDa) (isoform Akt 1) (Santa Cruz Biotechnology) and GST (26 kDa) (Chemicon). The bound primary antibodies were detected using goat anti-rabbit horseradish peroxidase-conjugate secondary antibody and membranes were revealed for chemiluminescence. The autoradiographies generated were quantitatively analyzed for the protein levels with an image densitometer (Image-master VDS CI, Amersham Biosciences Europe, IT). The molecular weights of the bands were determined by reference to a standard molecular weight marker (Rainbow full range Bio-Rad, CA, USA). The results from each membrane were normalized to the Ponceau values (5% in acetic acid) instead of using actin or tubulin as control, once DHEA could alter cytoskeleton proteins [33]. To minimize interassay variations, samples from all experimental groups were

Table 1
Effects over GSH total levels, GSH (reduced glutathione), GSSG (oxidized glutathione) and GSH/GSSG ratio evaluated in liver homogenates of the different groups after DHEA treatment (10 mg/kg) or control.

Measurement/group	3-month-old Control	3-month-old DHEA	24-month-old Control	24-month-old DHEA
Total glutathione (nmol/mg prot)	0.6292 ± 0.046	0.7509 ± 0.143[a]	0.7001 ± 0.149	0.8120 ± 0.056[a]
GSH (nmol/mg prot)	0.5812 ± 0.056	0.7390 ± 0.055[a]	0.6383 ± 0.139	0.765 ± 0.121[a]
GSSG (nmol/mg prot)	0.0479 ± 0.012	0.0515 ± 0.005	0.062 ± 0.005	0.056 ± 0.011
GSH/GSSG ratio	12.93 ± 1.59	13.72 ± 1.77	10.35 ± 1.25	10.41 ± 1.44

Values are expressed as mean ± SD ($n = 6$ per group). Two-way ANOVA followed by SNK.
[a] DHEA groups significantly different from control groups ($P < 0.05$).

Table 2
Antioxidant enzyme activities of glucose-6-phosphate dehydrogenase (G6PDH), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and catalase (CAT) in liver homogenates from the different groups after DHEA treatment (10 mg/kg) or control.

Enzyme activity/group	3-month-old Control	3-month-old DHEA	24-month-old Control	24-month-old DHEA
G6PDH (U/mg prot)	3.41 ± 0.35	3.35 ± 0.75	5.11 ± 0.83[a,b]	5.08 ± 0.85[a,b]
GST (μmol/min mg prot)	0.2182 ± 0.019	0.2198 ± 0.015	0.2247 ± 0.016	0.2342 ± 0.016
GPx (μmol/min mg prot)	189.5 ± 5.87	176.2 ± 9.84	229.2 ± 13.27[a,b]	216.0 ± 28.35[b]
CAT (μmol/mg prot)	1.55 ± 0.23	1.71 ± 0.14	1.59 ± 0.25	1.96 ± 0.39

Values are expressed as mean ± SD ($n = 6$ per group). Two-way ANOVA followed by SNK.
[a] Significantly different from 3 m, same treatment ($P < 0.001$).
[b] Significantly different from 3 m, independently of treatment ($P < 0.05$).

Table 3
Values of serum aminotransferases evaluated the different groups after DHEA treatment (10 mg/kg) or control.

Measurement/group	3-month-old Control	3-month-old DHEA	24-month-old Control	24-month-old DHEA
AST	69.80 ± 7.28	67.50 ± 6.58	51.73 ± 8.54[b]	61.11 ± 8.28
ALT	28.8 ± 8.72	37.42 ± 9.38	40.12 ± 1.87[b]	26.65 ± 6.36[a]
AST/ALT ratio	2.01 ± 0.19	1.95 ± 0.52	1.09 ± 0.35	2.36 ± 0.48

Values are expressed as mean ± SD ($n = 6$ per group). Two-way ANOVA followed by SNK.
[a] Significantly different from its control, same age ($P < 0.05$).
[b] Significantly different from 3 m, same treatment ($P < 0.05$).

processed in parallel ($n = 4$ in each group). Protein expression values were expressed as arbitrary densitometric units.

2.6. Alanine and aspartate aminotransferases activity

Trunk blood samples were collected into tubes containing heparin as anticlotting agent. After centrifugation at 1000 × *g* for 10 min, between 0 and 4 °C (Sorval RC 5B-rotor SM24, Du Pont Instruments, EUA), plasma was separated for aminotransferases activity evaluation. The method is based on the reaction of aspartate or alanine with α-ketoglutarate, leading to oxalacetate formation. This product reacts with NADH and the absorbance reduction, at 340 nm, is direct proportional to the aminotransferases activity [34]

2.7. Protein determination

Protein was measured by the method of Lowry et al. [35], using BSA as standard. The results were expressed in mg of protein/mL. In Western blot analysis, protein was measured by the method of Bradford [36].

2.8. Data analysis

Two-way analysis of variance (ANOVA) followed by the post hoc Student-Newmann–Keuls (SNK) test was used for the statistical analysis of data, which are presented as the mean ± SD. Values of $P < 0.05$ were considered significant.

3. Results

Changes in GSH total levels, GSH, GSSG and GSH/GSSG ratio evaluated in liver samples, in the different groups, are reported in Table 1. There was an increase in total GSH (17%) and in reduced GSH (22%) levels in liver homogenates of 3- and 24-month-old groups treated with DHEA. There were no changes in GSSG levels nor in GSH/GSSG ratio in liver samples of 3- or 24-month-old groups, considering age or treatment factors.

Table 2 shows the antioxidant enzymes activities of G6PDH, GST, GPx and CAT in liver homogenates in the different groups. An increase of G6PDH (51%) and GPx (22%) activities were observed in liver homogenates of 24-month-old groups when compared to 3-month-old groups, independently of treatment. No changes were found between the two 24-month-old groups regarding to G6PDH or GPx activities. Considering GST activity, there were no changes in liver homogenates of 3- or 24-month-old groups. Also, any changes were observed in CAT activity in liver homogenates of 3- or 24-month-old groups considering age or treatment factors.

As is evident from Table 3, AST presented diminished levels (26%) in 24-month-old control group when compared to 3-month-old control group. In contrast, ALT levels were augmented (39%) in the 24-month-old control when compared to 3-month-old control group. DHEA treatment reduced ALT levels (34%) in the 24-month-old group when compared to 24-month-old control. The AST/ALT ratios were not different among the groups, considering age or treatment.

Fig. 1 presents liver hydrogen peroxide concentration. An increase of hydrogen peroxide concentration (33%) was observed in liver samples of 24-month-old groups when compared to 3-month-old groups, independently of treatment.

Immunocontent of p-Akt (Fig. 2A) was enhanced in DHEA treated groups (54%) when compared to control groups, independently of age. An increase of p-Akt concentration (38%) was found in 24-month-old treated group when compared to 24-month-old control group as well as in the 3-month-old treated group (46%) when compared to 3-month-old control group. Fig. 2B presents Akt concentration ratio (p-Akt/Akt), which was also different between

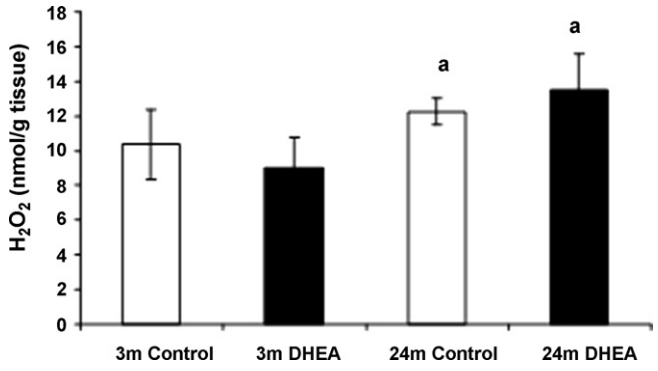

Fig. 1. Hydrogen peroxide concentration (nmol/g tissue). a: Significantly different considering age, independently of treatment ($P < 0.05$).

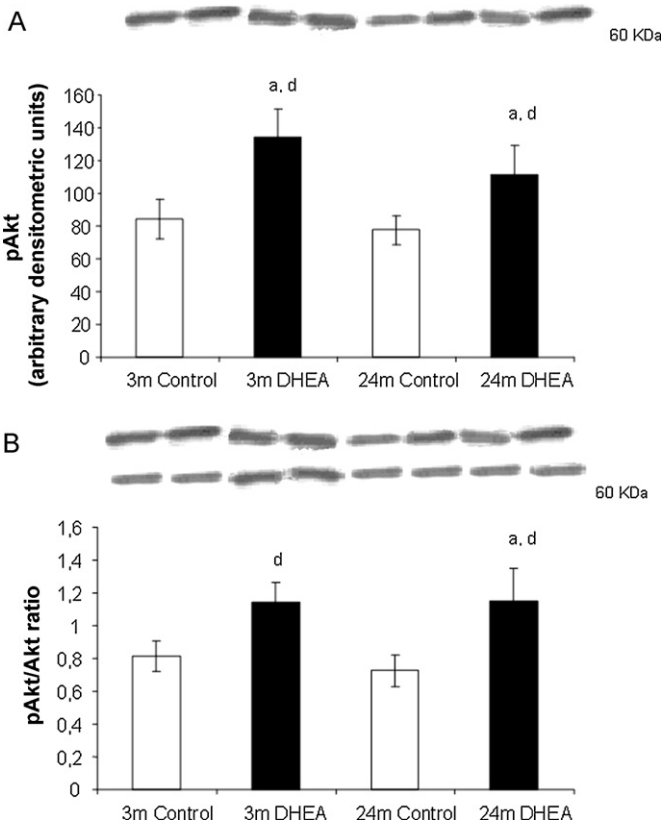

Fig. 2. Western blot analysis using p-Akt antibody and the ratio pAkt/Akt. a: Significantly different from its control, same age ($P < 0.05$); d: DHEA groups significantly different from control groups ($P < 0.05$).

groups: DHEA groups presented increased relation (36%) when compared to control groups–independently of age- and the treated 24-month-old group had increased Akt ratio (58%) when compared to its control.

Immunocontent of GST (Fig. 3) did not present any significant change in liver homogenates of 3- or 24-month-old groups when considering age or treatment factors.

4. Discussion

The major outcome of this paper was to demonstrate that the improved redox status was associated with an increased Akt activation and with a better hepatic function in aged rats. These changes would be related to DHEA treatment once its effects over oxidative stress have been largely described in literature [16,24,32,37,39,40].

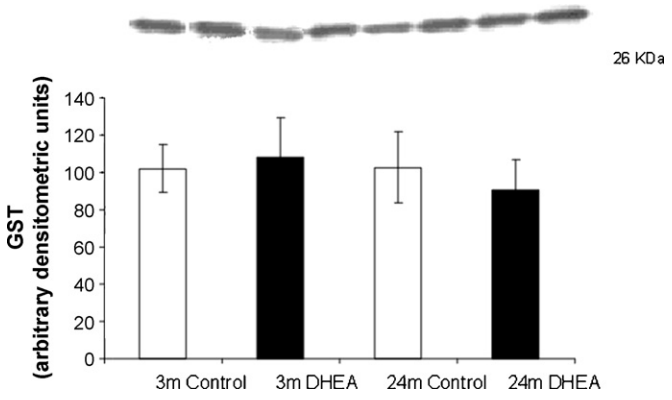

Fig. 3. Western blot analysis using GST antibody.

Mastrocola et al. [37] demonstrated the dual effect of DHEA in the liver, depending on the dosage. The pro-oxidant effect was mediated by the induction of peroxisome proliferator-activated receptors (PPARs), which appeared when DHEA tissue concentration reached pharmacological levels (50 or 100 mg/rat for 7 days). At lower concentration (4 or 10 mg/rat for 7 days), the steroid was unable to affect the levels of pro-oxidant species, in order that DHEA chronic administration exerted a protective effect regarding to lipid peroxidation.

Cellular GSH levels could be modified by oxidative stress through alterations in its biosynthesis or in the ratio of reduced to oxidized forms of glutathione, which might affect multiple physiological responses [38]. In this liver study, DHEA treatment in both groups (3 and 24 month) increased the total content of glutathione and GSH levels when compared to control groups. These DHEA effects over GSH levels seem to be positive, and characterized as an antioxidant action. Pelissier and others have also found increased GSH levels in the liver after 7 days of DHEA (50 mg/kg) injected in healthy and young male Wistar rats [39], corroborating our report. In our study, the amount of GSSG and the GSH/GSSG ratio were not modified in any group, neither by the aging process nor by the xenobiotic.

These results are completely different from myocardial analysis [24], in which DHEA augmented the GSSG levels in the aged group when compared to its control, and also when compared to 3-month-old treated group. The overall data reinforce that DHEA treatment may provide distinct answers depending on tissue and its susceptibility to the redox imbalance.

GSSG levels depend on its recycling capacity, on GPx activity and on the quantity of peroxides to be metabolized. According to our results, we noted that GPx is activated by the aging factor. In the 24-month-old groups, GPx activity was increased when compared to the young ones. These aged groups have also augmented hydrogen peroxide concentration when compared to the young ones, independently of treatment. GPx is a glutathione-dependent enzyme which provides a second line of defense against ROS and catalyzes the conversion of hydrogen peroxide to water. Probably, GPx activity was increased due to these variations in hydrogen peroxide concentration in the aged groups. However, after 5-week-treatment, CAT enzyme activity was not changed in any group, in accordance with other study that has also shown no change in CAT activity after DHEA treatment over healthy liver rats (4 mg/day for 3 weeks) [40]. This result suggests that the increment of hydrogen peroxide levels has not sufficient magnitude to activate CAT. In fact, there is no worsening in redox status, reinforcing the previous statement. In addition, redox status maintenance could be also a result of the demanded G6PDH activity. Studies with DHEA suggest the importance of G6PDH inhibition in reducing inflammation, hyperplasia and carcinogenesis, showing evidence to indicate

that G6PDH inhibition may retard the development of age-related diseases. In our study, the old groups did present a significantly increased G6PDH activity when compared to young groups. This increased G6PDH activity probably generates higher NADPH levels, and then contributing to the GSH recycling from GSSG.

Evidences have shown that serine/threonine kinases (including Akt) can be regulated by oxidants [41], suggesting new horizons to investigate the relation between DHEA, aging and Akt in different tissues. Indeed, one of the mechanisms that would promote changes in some proteins phosphorylation is ROS concentration, by altering the intracellular redox status and by oxidative modification of proteins [42]. Fig. 2 presents the increase of Akt immunocontent and Akt ratio in DHEA treated groups when compared to control groups, independently of age. Akt protein, when activated, phosphorylates a range of intracellular substrates that regulate growth, metabolism and survival [43]. Jacob et al. [32] have already shown that DHEA treatment (50 mg/kg) resulted in elevation in protein levels of pAkt/Akt ratio in heart tissue of young and healthy male Wistar rats. Yet, pAkt expression was enhanced in heart homogenates of 3- and 18-month-old groups treated with DHEA (10 mg/kg for 5 weeks) [16], supporting our findings. These results reinforce the idea that DHEA can act as a survival factor by triggering the galpha-PI3K/Akt-Bcl2 pathway against apoptosis [44]. Thus, considering our results, DHEA treatment influenced Akt phosphorylation in liver homogenates—and this action could also means that the Akt activation in this organ is a protective answer which would contribute to a preserved function. Although DHEA increased total glutathione content and GSH levels, and those effects occurred in parallel to augmented Akt phosphorylation in both ages (3- and 24-month-old rats), we have not found a strong statistic correlation between such measurements. It does not mean necessarily a cause-consequence interaction, but since Akt is a redox sensitive protein [15,24,42] such treatment effects in these important antioxidant sources could be related to its activation. Corroborating this idea, one previous study from our group have already demonstrated correlation between p-Akt expression and GSSG levels in rat hearts [24].

Once using a xenobiotic in this protocol, we have chosen to study GST activity and concentration once it belongs to a multifunctional proteins family which detoxifies a variety of electrophilic xenobiotics by catalyzing their conjugation to glutathione and reduces many organic hydroperoxides into alcohols [45]. Once using a xenobiotic, it was expected that GST would be changed. However, in our study GST activity and concentration did not present any significant change in liver homogenates of 3- or 24-month-old groups considering both variables studied. This finding suggests that this regimen (dose and via) of DHEA administration may not represent a toxic potential to liver. In addition, hepatic function, injured during aging, was preserved by DHEA administration as observed by normalized ALT levels in the 24-month-old DHEA group.

DHEA production varies among people within a normal population, and this should be taken into consideration once there are differences in susceptibility to free radical-mediated tissue damage [39]. This point supports that a deeper overview of DHEA mechanisms of action and its use need additional studies through different models. In spite of the very wide and uncontrolled use of DHEA as a dietary supplement, it has to be emphasized that there is no established protocol to guarantee the safety of DHEA therapy regarding redox imbalance and its implications in many tissues.

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