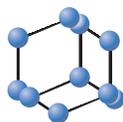


RESEARCH ARTICLE

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SCIENCE

DHEA Treatment Effects on Redox Environment in Skeletal Muscle of Young and Aged Healthy Rats



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Abstract: Background: Dehydroepiandrosterone (DHEA) is an important precursor of active steroid hormone, produced abundantly by the adrenal cortex with an age-dependent pattern.

Objective: We investigated whether chronic DHEA administration impacts on redox status and on Akt protein activation in skeletal muscle during the aging process (3 and 24 months-old rats).

Methods: Rats received one weekly dose/5 weeks of DHEA (10 mg/kg) or vehicle. Gastrocnemius muscle was removed to evaluate glutathione system, hydrogen peroxide, antioxidant enzymes, and expression of Akt kinase protein.

Results: In the 3-months-old rats DHEA induced an increase in hydrogen peroxide when compared both to its control (276%) and the 24-months-old DHEA group (485%). Moreover, in the 24-months-old rats DHEA caused an increase in GSSG (41 and 28%), a decrease in reduced-GSH (55 and 51%), and a more oxidized redox status (reduction in GSH/GSSG ratio, 47 and 65 %) when compared to 3-month-old DHEA and to 24-months-old control groups, respectively. Both older groups had increased G6PDH (2.7 fold) and GST (1.7 fold) activities when compared to younger groups, independently of any DHEA treatment. However, there was no modulation of Akt protein (phosphorylated/total isoform).

Conclusion: The results show that chronic DHEA administration to 3 and 24-months-old rats may not present positive effects regarding the redox environment in skeletal muscle without modulation of pro-survival Akt kinase. Due to the large-scale self-administration of DHEA as an “anti-aging” dietary supplement, it is crucial to investigate its molecular mechanisms over oxidative stress-induced related diseases.

Keywords: DHEA, aging, skeletal muscle, glutathione, hydrogen peroxide, Akt.

1. INTRODUCTION

Dehydroepiandrosterone (DHEA) is a steroid produced by the adrenal zona reticularis and is also synthesized in the gonads, brain, and gastrointestinal tract [1]. Levels of DHEA start to reduce 2% per year after the third life decade in humans [2]. Decay of age-related DHEA levels may lead to autoimmune diseases, sexual dysfunction, osteoporosis, lipid metabolism impairment, obesity, insulin resistance, atherosclerosis and cardiovascular diseases [3, 4]. DHEA treatment to rodents has demonstrated beneficial effects including the diminished incidence of obesity, heart disease, cancer and diabetes [3], notwithstanding the fact that circulating levels of DHEA in rats do not follow the human pattern [5].

Studies have shown an inverse association for DHEA/DHEA-sulfate levels and muscle loss, of which the primary cause is accelerated protein breakdown, contributing for morbidity outcomes in older population [6-8]. DHEA has been considered a strategy to preserve muscular mass in older people [6, 9]. Besides the positive and null effects observed when DHEA is associated with exercise [10-12], DHEA's use as a single strategy has shown no effects in young and elderly healthy subjects [13]. A recent investigation in myotubes performed *in vitro* showed that DHEA presented positive effects over AMPK and PGC-1 α , important signaling proteins related to metabolism, mitochondrial biogenesis and protein synthesis [14]. However, little is known regarding the cellular effects and mechanism of action of this steroid hormone *in situ* in skeletal muscle.

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Although aging is likely to be a multifactorial process, there is large evidence implicating the generation of Reactive Oxygen Species (ROS) and oxidative stress as key factors in determining longevity [15]. In the aging process, species like the superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radical can be generated from a variety of sources and exert toxic effects at high concentrations [15]. A pivotal antioxidant support is reduced glutathione (GSH), which participates as a substrate in the detoxification of xenobiotics and H_2O_2 reduction [16, 17]. The ratio between glutathione reduced and oxidized forms (GSH/GSSG) is largely used as a determinant of redox status [18, 19], which together with ROS levels exert a fundamental responsibility in the modulation of signal transduction, such as pro-survival PI3K/Akt [18, 19]. Redox-sensitive proteins such as Akt, a serine/threonine kinase, involved in surveillance and proliferation, can be modulated by ROS [20, 21]. This kinase was already shown to be activated by chronic DHEA-treatment as observed in heart tissue [22] and in Leydig cells by Ding and colleagues [23]. DHEA intervention has also demonstrated to upregulate Akt and other related pro-survival proteins in skeletal muscle in chronic disorders [24, 25]. Moreover, chronic DHEA administration affected steroid hormone levels and antioxidant parameters in different tissues of aged rats as well as induced a postponement of the aging process [26]. To the best of our knowledge, this is the first study to investigate whether chronic DHEA administration impacts on redox status and on Akt protein activation in skeletal muscle during the aging process (3 and 24 month-old rats).

2. MATERIAL AND METHODS

2.1. Animals and Treatment

Twenty-four male Wistar rats (3 and 24 months) acquired from the Instituto de Ciências Básicas da Saúde (ICBS) of the Universidade Federal do Rio Grande do Sul (UFRGS) were randomly allocated to control or DHEA (dehydroepiandrosterone; Calbiochem) (10 mg/kg) groups treated once a week, subcutaneously, for 5 weeks. Control groups received vehicle (vegetal oil). DHEA experimental management was based on previous studies [22, 27-29]. DHEA administration protocol was based on previous experiments of our group [22, 27, 30, 31] and on other studies [29, 32, 33]. Animals were accommodated in plastic cages (four animals per cage), receiving water and pelleted food *ad libitum*. They were maintained under standard laboratory conditions (controlled temperature of 21 °C, 12h light/dark cycle). After 5 weeks, animals were killed by decapitation and their gastrocnemius muscles were quickly separated. The skeletal muscle samples were frozen on dry ice and stored at -80°C for western blotting. Remaining gastrocnemius tissue was used to measure H_2O_2 , glutathione concentration and antioxidant enzymes activities. Particular care was given to reduce 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 (CONCEA - National Council of Animal Experimentation Control) and the experimental protocol was approved by the UFRGS Animal Care Committee (project number 2006535).

2.2. Oxidized and Reduced Glutathione Concentration

Skeletal muscle tissue was deproteinized with 2 mol/L perchloric acid, centrifuged for 10 min at 1000 x g and the supernatant was neutralized with 2 mol/L potassium hydroxide to measure oxidized and reduced glutathione concentration. 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 and 70 μ mol/L 5,5' dithiobis (2-nitrobenzoic acid). The supernatant was neutralized with 2 mol/L potassium hydroxide to react with 70 μ mol/L 5,5' dithiobis (2-nitro benzoic acid), and the absorbance values determined at 420 nm to measure reduced glutathione [34].

2.3. Hydrogen Peroxide Concentration

H_2O_2 was quantified via its horseradish peroxidase (HRPO)-mediated oxidation of phenol red. Fresh gastrocnemius muscle portions were hatched for 30 min. at 37°C in phosphate buffer 10 mmol/L (NaCl 140 mmol/L and dextrose 5 mmol/L). The supernatants were moved 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 determined at 610 nm. The measures were expressed in nmoles H_2O_2 /mg tissue [35].

2.4. Antioxidant Enzyme Activities

Using the method of Leong and Clark [36], glucose-6-phosphate dehydrogenase (G6PDH) assay was determined. The reaction mixture (1 mL) contained: 100 mmol/L Tris-HCl pH 7.5, 10 mmol/L $MgCl_2$, 0.5 mmol/L NADP⁺ and sample, and it was started by the addition of 1 mmol/L glucose-6-phosphate values being determined in a spectrophotometer at 340 nm. One G6PDH unit corresponds to 1 mmol of substrate transformed per minute and the activity is expressed as units per mg protein. Measured by dinitrophenol-S-glutathione at 340 nm formation rate, glutathione-S-transferase (GST) activity was expressed as μ moles per milligram of protein. The reaction medium consisted of 19 mmol/L sodium phosphate buffer (pH 6.5), 1 mmol/L GSH and 1 mmol/L chloride dinitrobenzene [37].

2.5. Western Blotting

The skeletal muscle samples were homogenized (Ultra-Turrax) with a lysis buffer, pH 7.4, proportional to weight (20 mL/g of tissue). Electrophoresis and protein transfer were accomplished as described elsewhere [38, 39]. The membranes were refined for immunodetection using rabbit polyclonal antibodies for p-Akt (Ser-473) (60 kDa), Akt (60 kDa), (Santa Cruz Biotechnology) and GST (26 kDa) (Chemicon, Millipore). The bound primary antibodies were identified using goat anti-rabbit horseradish peroxidase-conjugate secondary antibody and membranes were disclosed by chemiluminescence. With an image densitometer (Imagemaster VDS CI, Amersham Biosciences Europe, IT), the autoradiographies produced were quantitatively analyzed for the protein levels. Using a standard molecular weight marker (Rainbow full range Bio-Rad, CA, USA), the molecular weights of the bands were identified. The results from each membrane were normalized to the Ponceau values

(5% in acetic acid) in place of applying tubulin or actin as control, since DHEA could modify cytoskeleton proteins [40]. To reduce interassay ranges, samples from all groups were treated in parallel (n=4 in each group). Protein expression significances were shown as arbitrary densitometric units.

2.6. Protein Determination

Protein was determined by the method of Lowry [41], BSA being applied as standard. The values were shown in mg of protein/mL. For the Western blot assay, the protein was quantified by the Bradford method [42].

2.7. Statistical Analysis

Two-way Analysis of Variance (ANOVA) followed by the post hoc Student-Newmann-Keuls (SNK) test was performed for the statistical analysis (SPSS 19.0). The interrelation between two variables was explored by the Pearson's correlation. Results are shown as the mean \pm SD. Values of $P < 0.05$ were considered significant.

3. RESULTS

Variations of glutathione system components, total GSH, reduced-GSH, GSSG and GSH/GSSG ratio, estimated in gastrocnemius muscles samples of different groups are reported in Table 1. There was a decrease in total (51%) and reduced (55%) GSH levels in skeletal muscle homogenates

of 24-months-old group treated with DHEA when compared to its control. Gastrocnemius samples of 24-months-old DHEA group exhibited augmented levels of GSSG when compared to its control (28%) and to 3 months-old DHEA group (41%). Consequently, a more oxidized redox status indicated by reduction in GSH/GSSG ratio was observed for the 24-months-old DHEA group when compared to its control (65%) and to the 3 months-old DHEA group (47%). Moreover, H_2O_2 concentration increased in the 3-months-old DHEA group (31.6 ± 4.8 nmol/mg of tissue) when compared to its control (8.4 ± 1.7 nmol/mg of tissue) and to the 24-month-old DHEA group (5.4 ± 0.8 nmol/mg of tissue) (Fig. 1). Regarding the older rats, DHEA treatment did not change the levels of H_2O_2 when compared to the non-treated control. Moreover, 24-months-old rats presented decreased H_2O_2 levels when compared to 3-months-old groups, independently of the treatment.

An increase in the G6PDH enzyme activity was observed in the skeletal muscle of 24-months-old groups (control = 6.03 ± 1.25 and DHEA = 4.91 ± 0.58 U/mg protein) when compared to 3-months-old groups (Control = 2.27 ± 0.52 and DHEA = 1.81 ± 0.27 U/mg protein), independently of treatment (Fig. 2A). The same result was observed regarding the GST enzyme activity when comparing 24-month-old groups (Control = 0.23 ± 0.06 and DHEA = 0.28 ± 0.04 $\mu\text{mol}/\text{min}/\text{mg}$ protein $\times 10^{-1}$) to 3-month-old groups (Control = 0.16 ± 0.02 and DHEA = 0.14 ± 0.01 $\mu\text{mol}/\text{min}/\text{mg}$ protein $\times 10^{-1}$) (Fig. 2B). No changes were found between the two 24-month-old groups regarding the G6PDH or GST activi-

Table 1. Glutathione components: GSH+GSSG (total glutathione), GSH (reduced glutathione), GSSG (oxidized glutathione), and GSH/GSSG ratio (status redox) evaluated in skeletal muscle homogenates from 3-month and 24-month-old rats after DHEA treatment (10 mg/kg, during 5 weeks) or Control.

Measurement/Group	3 Month-Old Control	3 Month-Old DHEA	24 Month-Old Control	24 Month-Old DHEA
GSH + GSSG (nmol/mg prot)	0.136 ± 0.036	0.105 ± 0.026	0.173 ± 0.031	0.084 ± 0.019^a
GSH (nmol/mg prot)	0.129 ± 0.036	0.098 ± 0.016	0.166 ± 0.031	0.075 ± 0.018^a
GSSG (nmol/mg prot $\times 10^{-1}$)	0.071 ± 0.006	0.068 ± 0.009	0.075 ± 0.010	$0.096 \pm 0.018^{a,b}$
GSH/GSSG ratio	18.24 ± 4.54	14.75 ± 3.85	22.74 ± 4.97	$7.86 \pm 1.18^{a,b}$

Values are expressed as mean \pm 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 3m, same treatment ($P < 0.05$).

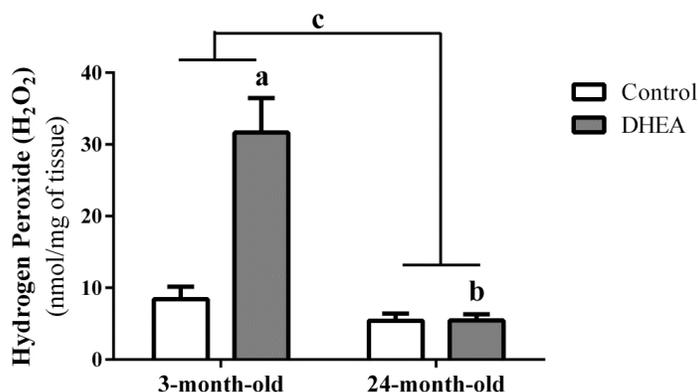


Fig. (1). Hydrogen peroxide (H_2O_2) levels in skeletal muscle homogenates from 3 and 24-month-old rats after DHEA treatment (10 mg/kg, per 5 weeks) or Control. Values are expressed as mean \pm SD (n=6 per group). Two-Way ANOVA followed by SNK. a: Significantly different from its control, same age ($P < 0.05$); b: Significantly different from 3-months, same treatment ($P < 0.05$); c: Significantly different from 3-months, independently of treatment ($P < 0.05$).

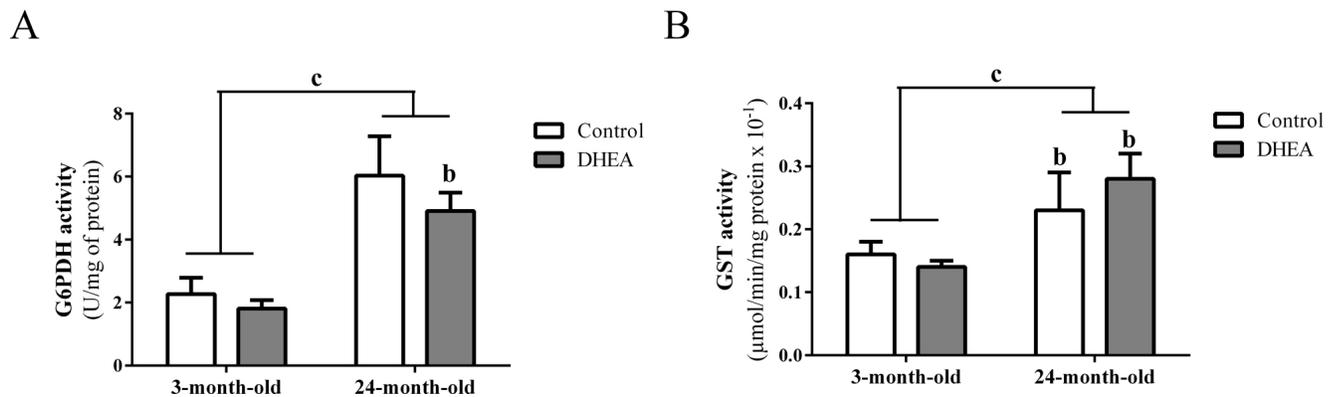


Fig. (2). Glucose-6-phosphate dehydrogenase (G6PDH) (A) and glutathione S-transferase (GST) (B) enzyme activities in skeletal muscle homogenates from 3 and 24-month-old rats after DHEA treatment (10 mg/kg, per 5 weeks) or control. Positive Pearson's correlation was observed between G6PDH and GST enzymes activities ($r = 0.664$; $P = 0.005$). Values are expressed as mean \pm SD ($n = 6$ per group). Two-Way ANOVA followed by SNK. b: Significantly different from 3-months, same treatment ($P < 0.001$); c: Significantly different from 3-months, independently of treatment ($P < 0.05$).

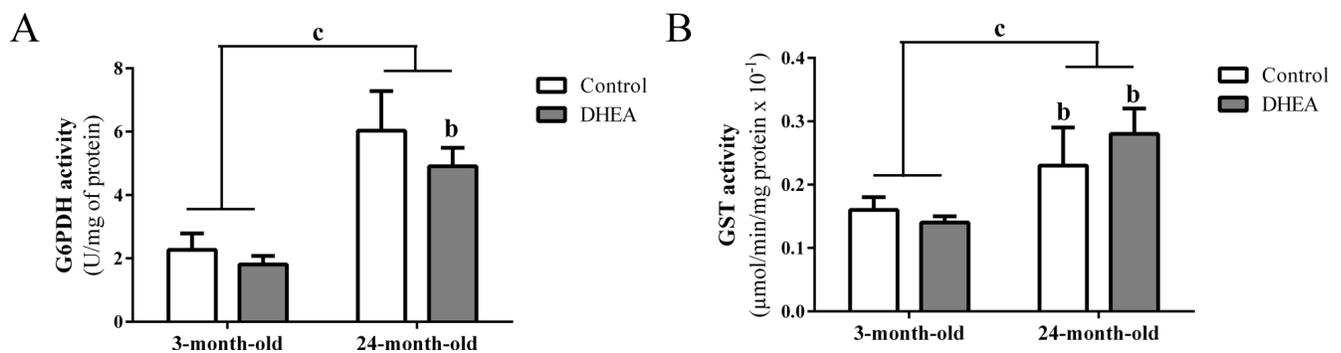


Fig. (3). Protein expression of glutathione S-transferase (GST) (A) and activity of Akt kinase, represented by ratio p-Akt/ total Akt (B) evaluated in skeletal muscle of the different groups after DHEA treatment (10 mg/kg) or control. Representative image of western blot (two for each group) Protein expression normalized by ponceau red. Values are expressed as mean \pm SD ($n = 6$ per group). Two-Way ANOVA followed by SNK. There were no statistical differences between groups ($P > 0.05$).

ties. Both enzymes activities were positively correlated ($r = 0.664$, $P = 0.005$).

Regarding the immunocontent of the GST protein, it did not present any significant difference in skeletal muscle of 3- and 24-month-old groups when considering age or DHEA treatment (Fig. 3A). In relation to the Akt protein expression, neither phosphorylated nor total isoforms did present differences between groups. Also, Akt kinase activity, commonly represented by the ratio between phosphorylated/total isoform, did not change regardless of treatment and age in the young and old rats (Fig. 3B).

4. DISCUSSION

The major outcome of this study was to reveal that chronic DHEA intervention favors a pro-oxidant condition in skeletal muscle of young and old rats as observed by augmented H_2O_2 levels and decreased GSH/GSSG ratio, respectively. Regarding the aging process, DHEA treatment markedly impacted on older rats, once it potentiates a more oxidized status. Besides, G6PDH and GST enzyme activities were increased in the older rats; DHEA did not modulate these antioxidant components, as well as the pro-survival and redox sensitive protein Akt.

Cellular GSH levels could be transformed by oxidative stress through variations in its biosynthesis or in the proportion of reduced to oxidized forms of glutathione, which might affect plural physiological reactions [43]. In the present study, for the 24-months-old rats, administration of DHEA induced a pro-oxidant redox status, indicated by a decrease in GSH/GSSG ratio, as a result, decreased GSH levels and increased GSSG levels. It is feasible that both increase in free radicals' generation and anti-oxidant defenses depletion might be implicated in the aging evolution process. Previous studies by our group observed that DHEA administration to aged rats (24-months-old) also decreased the GSH/GSSG ratio in heart tissue [27], thus supporting our findings. Increase in GSSG levels could also be due to the lower availability of NADPH, since less GSSG will be recycled to GSH. McCormick and cols (2006) have shown that in adipose tissue DHEA inhibits the oxidative pentose phosphate pathway, one of the main sources of cellular NADPH [44].

Although there were no differences in GSH, GSSG and GSH/GSSG ratios in 3 months-old rats, DHEA caused an important increase in H_2O_2 levels only in these rats, contributing also to a pro-oxidant status in the skeletal muscle. It is possible that the oldest group has normalized its defenses (e.g. higher GST activity) while the 3-months-old group was

not yet adapted. The increase in H_2O_2 levels in the younger rats could also result from increased muscle metabolic activity due to a synergistic effect between testosterone and DHEA, as well as by its increased DHEA-derived synthesis, since it was already shown that supplementation with DHEA (10 mg/kg, 6-weeks) duplicates testosterone levels in mice [45]. In addition, youngest rats present high levels of testosterone (400 pg/ml), declining significantly with aging, dropping more than 90% when the animal achieves 22 months of age [46]. Both DHEA and testosterone are known to increase the basal metabolism as demonstrated by the augmented cellular respiratory activity in young rats hepatocytes [47] and by the increase in glucose uptake in skeletal muscle in young rats [32]. Studies have also described that steroid hormones present a tissue-dependent effect in the rat muscle thus leading to a more pro-oxidant environment [48, 49]. Finally, treatment with DHEA caused a 2-fold decrease in thioredoxin antioxidant enzyme expression in skeletal muscle of young rats [32]. Taking all these evidence together, it is possible that the higher levels of H_2O_2 observed in 3-months-old rats could be a consequence of a set of factors, including an augmented basal metabolism and a not-fully adapted antioxidant defense system favoring a pro-oxidant environment. Moreover, age-related modifications in the antioxidant enzymatic system could be conflicting. Literature has documented that oxidative balance could be enhanced in aged animals when compared to other aging phases, relying on the tissue and the structure involved [22, 50-52].

Redox status preservation could be also a repercussion of the demanded G6PDH action. The reduced G6PDH activity has a beneficial effect on age-related diseases development and longevity [53]. Although DHEA was not able to modulate G6PDH in this study it was observed that age is a determinant factor in increasing G6PDH activity in skeletal muscle. In cardiac tissue of old rats, our group already demonstrated that DHEA increased G6PDH activity [27], evidencing that, in the skeletal muscle, chronic DHEA intervention may not present positive effects regarding the redox environment. In addition, it also shows that DHEA exhibits a tissue-dependent response. Although no differences were observed regarding GST immunocontent, there was a significant increase in GST activity of the 24-months-old groups when compared to 3-months-old groups. The above-cited enzymes also presented significant positive correlation, indicating that these enzymes are acting together in the modulation of physiological responses of the aging process, such as oxidative stress. The same result was previously observed in the heart tissue of the same age animals, however, the G6PDH activity in the muscle was twice bigger when compared to heart activity in older groups [27]. Also, a similar response was observed regarding GST in the cardiac tissue compared to the skeletal muscle, corroborating previous published results.

The redox-sensitive Akt protein, when activated, phosphorylates a range of intracellular signaling molecules that regulate cellular growth, survival and metabolism [54]. No significant differences were observed in phosphorylated and total Akt expression in skeletal muscle after chronic DHEA intervention. Similar results were observed in the hearts after DHEA intervention in rats of the same age [27]. Moreover, another study showed that a single dose of DHEA (10

mg/kg) did not modulate Akt in the skeletal muscle of healthy adult rats [29]. However, previous findings of our group showed that DHEA was able only to positively modulate Akt in the cardiac tissue of 18-month-old rats, and in the liver of adult and healthy male Wistar rats after 50 mg/kg DHEA intervention [22, 31]. An *in vitro* study with skeletal muscle cells, DHEA increased the p-Akt only at high concentrations (300 μ M) [24]. Additionally, the same group demonstrated that a single dose of DHEA administered to diabetic adult rats was able to increase Akt in skeletal muscle, which could act to improve the glucose metabolism [55]. Also, DHEA intervention in a rat Parkinson's disease model increased p-Akt and p-ERK in skeletal muscle [25]. A similar finding was reported by Ishizuka and cols (2007) [56] regarding DHEA activation of PI3K, among other kinases, in the adipose tissue and by Liu and cols (2008) regarding ERK 1/2 signaling pathway in bovine aortic endothelial cells [57]. Taken together, the present results demonstrate that chronic DHEA administration is not able to modulate Akt kinase in skeletal muscle of adult and old rats, reinforcing that DHEA acts over Akt signaling in a tissue- and dose-dependent manner. Corroborating this hypothesis, it was demonstrated by Yokokawa and colleagues (2015) that DHEA activated important metabolic-related proteins on C2C12 myotubes, such as AMPK and PGC-1 α , without modulation of Akt kinase [14]. Otherwise, as far as we know this is the first study showing that DHEA does not modulate this unique pro-survival protein, Akt, in the skeletal muscle of healthy elderly rats.

The present investigation brings findings that contribute to the better knowledge of DHEA effects over the skeletal muscle in a physiological process of muscular aging. In a double-blind placebo-controlled trial, Percheron and colleagues (2003) demonstrated that one-year oral administration of DHEA to healthy elderly people did not induce beneficial effects on muscle condition [13]. However, when DHEA is associated with exercise training, this hormone is able to contribute to the enhancement of muscle mass and strength in men [11], but not in post-menopausal women [10]. Also, exercise training has shown the capacity to normalize DHEA concentrations in aged subjects [58]. These data indicate that although DHEA is an important hormone to the aging process, its use from exogenous sources needs to be deeply investigated.

An important clinical interest in DHEA is based on many remarks, including: significantly impaired production since early adulthood, scientific evidence presenting changes in steroid levels related to multiple pathologies, and a prominent replacement therapy with DHEA which may relieve age-associated declines in an extension of functions [59].

CONCLUSION

In conclusion, the overall data presented in this study suggests that chronic DHEA administration to 3 and 24-month-old rats leads to a pro-oxidant condition without modulating the pro-survival Akt kinase in the skeletal muscle of healthy animals. Due to the large-scale self-administration of DHEA as an "anti-aging" dietary supplement, it is crucial to investigate its molecular mechanisms over oxidative stress- induced related diseases. In a transla-

tional approach, data presented here reinforce how necessary it is to evidence whether there are beneficial or harmful effects after the clinical use of DHEA as a therapeutic strategy for healthy individuals.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The experimental protocol was approved by the UFRGS Animal Care Committee (project number 2006535).

HUMAN AND ANIMAL RIGHTS

No humans were involved in this study. All animal procedures used in this study were in accordance with the Principles of Laboratory Animal Care (CONCEA - National Council of Animal Experimentation Control).

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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