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The effect of long-term DHEA treatment on glucose metabolism, hydrogen peroxide and thioredoxin levels in the skeletal muscle of diabetic rats

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ABSTRACT

Dehydroepiandrosterone (DHEA) is an endogenous steroid hormone involved in a number of biological actions. This study shows the effects of DHEA on glucose metabolism, hydrogen peroxide and thioredoxin levels in the skeletal muscle of control and diabetic rats. Control and diabetic rats were chronically treated with DHEA (10 mg/kg) diluted in oil. Plasma concentration of DHEA and glucose, glucose uptake and oxidation, hydrogen peroxide, GLUT4, Akt and thioredoxin (Trx) was measured in the muscle. Results showed that there was a decrease in blood glucose in diabetic rats, probably linked to an increase in the glucose oxidation by the muscle or glucose uptake by some tissues. Despite the increase in the expression of GLUT4 in DHEA-treated rats, the glucose uptake was only higher in the control rats, showing that the glucose transporter may be present but not functional in the diabetic rats. The low expression of Trx due to diabetes became even lower with DHEA treatment. Although the reduction in blood glucose may be favorable, the decrease in Akt and Trx displays an environment conducive to redox imbalance. Thus, further studies are needed to ascertain the effects of DHEA treatment in diabetic rats.

1. Introduction

Diabetes mellitus (DM) is characterized by a group of metabolic disturbances resulting from defects in insulin secretion, or insulin action, or both. Chronic diabetes hyperglycemia is associated with long-term damage, and with organ dysfunction and failure, especially in the eyes, kidneys, nerves, heart and blood vessels. Several different substances have been experimented with as promising strategies for the prevention and treatment of diabetes complications, especially substances with antioxidant potential, such as dehydroepiandrosterone (DHEA) [1–4].

DHEA is a steroid produced in the adrenal cortex, and can also be synthesized in the central and peripheral nervous system [5]. It is a multi-functional steroid involved in a wide range of biological effects in humans and rodents. Together with its sulfate ester (DHEA-S), it is the most abundant steroid in humans. It may act both directly or through its metabolites (including androstenediol and androstenedione), which can undergo further conversion to produce primarily testosterone and estradiol [6,7]. DHEA and testosterone are widely used as muscle-building or performance-enhancing drugs by athletes [8], and have been more recently promoted as anti-aging supplements, but the long-term benefits, as compared with the potential damage, are not known. Several beneficial effects have been observed in relation to the administration of DHEA (mainly in animals), including improvement in vascular function [9], cardioprotective action through anti-hypertrophic effects [10], neurotrophic and neuroprotective effects [11,12], and antioxidant effects on various organs, as well as on diabetes [13].

DHEA is often reported as having antidiabetic effects, but the pathophysiological explanation for this is unknown. DHEA treatment was found to be beneficial in obese Zucker rats suffering from diabetic nephropathy, and the effect was similar to the action of angiotensin-converting enzyme inhibitors [14]. Other metabolic effects were observed, such as decreased insulin resistance in dia-

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betic mice [15] and stimulated glucose uptake in fibroblasts [16], adipocytes [17,18] and hepatocytes [19].

Androgen hormones, such as DHEA and testosterone, may play important roles in glucose utilization in peripheral tissues. Numerous studies have demonstrated that these hormones influence glucose metabolism by inducing the acceleration of protein kinase B (Akt) phosphorylation via the activation of PI3-kinase [20,21]. The addition of DHEA to cultured skeletal muscle induced an elevation of glucose transporter GLUT4 expression and translocation, with increased Akt and protein kinase C *zeta/lambda* (PKC-ζ/λ) phosphorylation [22]. A single injection of DHEA in diabetic rats arrested the decline of the signaling pathway via Akt and PKC ζ/λ-GLUT4, as well as the main glycolytic enzymes, proving that DHEA can assist in improving and controlling blood glucose levels substantially, and in restoring impaired insulin transduction in the skeletal muscle of type 1 diabetes mellitus [23].

One possible mechanism of DHEA action is to modulate the oxidative balance [13,24]. DHEA has been considered as an antioxidant [13,25], but the effects of exogenous DHEA administration can be dual (antioxidant and/or pro-oxidant) depending on the dosage administered and the tissue specificity [26–29]. In addition, DHEA was considered to be a possible "fountain of youth" hormone by Baulieu [30], but despite initial enthusiasm, many questions concerning its efficacy remain unanswered, and their solution is a challenge for future research [31].

The aim of this study is, therefore, to investigate whether chronic DHEA administration in diabetic rats can bring about effects in terms of certain metabolic approaches, oxidative stress parameters, and survival proteins in the gastrocnemius muscle of diabetic rats.

2. Materials and methods

2.1. Animals

The experiments were performed on male Wistar rats (90 days old, 300 g). The animals were housed in plastic cages (three animals in each), and received water and pelleted food *ad libitum*. They were maintained under standard laboratory conditions (at a controlled temperature of 21 °C, 12 h light/dark cycle). Special care was taken to limit the number of animals used and their suffering. All animal procedures followed 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 the UFRGS Animal Care Committee.

2.2. Diabetes induction

Diabetes mellitus was induced by a single intraperitoneal injection of streptozotocin (Sigma, USA), in a 70 mg/kg dose [32]. After 3 days of streptozotocin injections, glucose concentration was verified, and those animals whose plasma glucose concentration was higher than 20 mmol/L were considered diabetic [13].

2.3. DHEA treatment

DHEA (dehydroepiandrosterone; Calbiochem, Germany) was dissolved in vegetable oil (the vehicle) and administered subcutaneously in a dose of 10 mg/kg once a week for 5 weeks. The control groups received only the vehicle. Four groups were formed: non-diabetic animals which received only oil (CTR-OIL), non-diabetic animals with DHEA (CTR-DHEA), diabetic animals with oil (DBT-OIL), and diabetic animals with DHEA (DBT-DHEA). The DHEA administration protocol was based on previous experiments in our laboratory, and on other studies [29,33].

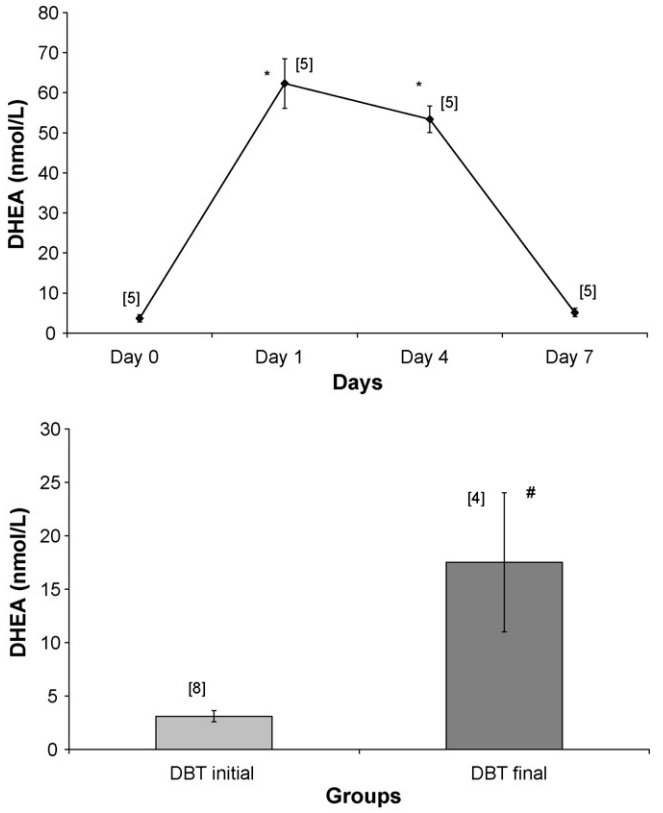

Fig. 1. Plasma DHEA concentration in CTR group before the first DHEA injection (Day 0) and 1, 4 and 7 days after the first DHEA injection (top); and plasma DHEA concentration in DBT group before and after the 5 weeks DHEA treatment (bottom). Data are given as mean ± SEM. Number of samples is shown in brackets. (*) Statistically different from Day 0 and Day 7 ($p < 0.05$, SNK). (#) Statistically different from DBT initial group ($p < 0.05$, SNK).

2.4. DHEA measurement

Free DHEA concentration in plasma was determined by using a specific Radioimmunoassay (RIA) kit for DHEA (DSL-9000; DSL, USA). Blood samples were collected from the tail vein before the first DHEA injection, and 1, 4 and 7 days after the first injection, to evaluate the efficiency of the treatment (Fig. 1).

The concentration of DHEA was also evaluated in the diabetic rats at the beginning and at the end of the experimental period (7 days after the last injection) in order to compare levels of concentration before and after treatment (Fig. 1).

2.5. Experimental procedure

At the end of the experimental period, the animals were weighed, and then killed by decapitation. Following this, trunk blood samples were collected for later analysis. The gastrocnemius muscles were excised, and a sample was placed in Petri dishes with cold Krebs–Ringer Bicarbonate (KRB) buffer pH 7.4 (118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 24.8 mM $NaHCO_3$). The muscles were sliced freehand with a slicer apparatus and placed in specific tubes for glucose uptake and oxidation. Another muscle sample was immediately frozen in liquid nitrogen for subsequent Western blot analysis, and for quantification of hydrogen peroxide.

2.6. Plasma glucose

Blood samples collected at the end of the experimental period were placed in heparinizaded tubes and centrifuged. Plasma glu-

cose was determined spectrophotometrically using the enzymatic method (Labtest, Brazil).

2.7. Glucose uptake

The gastrocnemius muscle samples (about 100 mg) were incubated in tubes containing 1 mL of KRB with 0.2 μCi [2-deoxyglucose-^{14}C] glucose (Amersham, USA). The gaseous phase was equilibrated using 5% CO_2:95% O_2. The tubes were placed in a Dubnoff incubator, and were constantly shaken for 30 min at 37 °C.

The tissue was disrupted to determine glucose uptake [34]. The uptake results are expressed as tissue/medium (T/M) ratio, which is the dpm/mL tissue fluid per dpm/mL incubation medium.

2.8. Glucose oxidation ($^{14}CO_2$ production)

Glucose oxidation was determined in accordance with Torres et al. [35]. Gastrocnemius muscle samples were incubated in flasks sealed with rubber caps at 37 °C in 1 mL of KRB, with 0.2 μCi [U-^{14}C] glucose (Amersham, USA), plus 5 mM glucose, for 60 min, and the gaseous phase was equilibrated by using 5% CO_2:95% O_2.

In these flasks, small glass wells were placed above the level of the incubation medium containing small strips of Whatman 3MM paper. Next, 1 M hyamine hydroxide (0.2 mL) was injected into the central wells to trap $^{14}CO_2$. Incubation was arrested by adding 0.2 mL of 50% trichloracetic acid through the rubber caps. The flasks were maintained overnight at room temperature in order to capture $^{14}CO_2$. The contents of the center well were transferred to vials containing scintillation liquid, and radioactivity was measured using an LKB counter with an automatic curve quench correction. Values of $^{14}CO_2$ production are expressed as nmol of ^{14}C glucose incorporated into CO_2 per mg of tissue per min.

2.9. Determination of hydrogen peroxide steady-state

Hydrogen peroxide was measured via its horseradish peroxidase (HRPO)-mediated oxidation of phenol red, leading to the formation of a compound measurable at 610 nm. Slices of fresh tissue from the muscle 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 0.28 mmol/L phenol red and 8.5 U/mL HRPO. After 5 min incubation, 1 mol/L NaOH was added and the solution's absorbance values were measured at 610 nm. The results were expressed in nmoles H_2O_2/g tissue [36].

2.10. Western blotting

Skeletal muscle samples were homogenized (Ultra-Turrax) with lysis buffer, pH 7.4. After protein measurement [37], sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (10%) was carried out using a miniprotean system (Bio-Rad, Hercules, USA) with broad range molecular weight standard (rainbow full range, Amersham, USA). Protein (90 μg) was loaded into each lane with loading buffer containing 0.375 mol/L Tris (pH 6.8), 50% glycerol, 10% SDS, 0.5 mol/L mercaptoethanol, and 0.002% bromophenol blue. Samples were heated at 90 °C for 2 min before gel loading. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Hybond; Amersham, USA) using an electrophoretic transfer system. The membranes were then incubated with 8% non-fat dry milk in TTBS (20 mmol/L Tris–HCl, pH 7.5; 150 mmol/L NaCl; 0.05% Tween-20, pH 7.4) for 60 min. The membranes were incubated overnight at 4 °C with the primary antibodies diluted in TTBS plus 2.5% bovine serum albumin (BSA). Rabbit polyclonal antibodies for GLUT4 (45Kda), p-Akt (60 kDa), Akt (60 kDa) and thioredoxin (55 kDa) (Santa Cruz, USA) were used. After washing with TTBS, the membranes were incubated for 2 h at room temperature with secondary antibody (goat anti-rabbit horse peroxidase-conjugate), and washed with TBS (20 mmol/L Tris–HCl; 150 mmol/L NaCl, pH 7.5). Following chemiluminescence ECL apposition of the membranes to autoradiographic films (Hyperfilm ECL; Amersham, USA), they were analyzed using an Image Master VDS Software version. The results for each membrane were normalized to total protein values from Ponceau stained (5% in acetic acid), instead of using tubulin as a control, since DHEA and diabetes could alter the cytoskeleton proteins [38]. Samples from all the experimental groups were processed in parallel ($n = 4$ in each group).

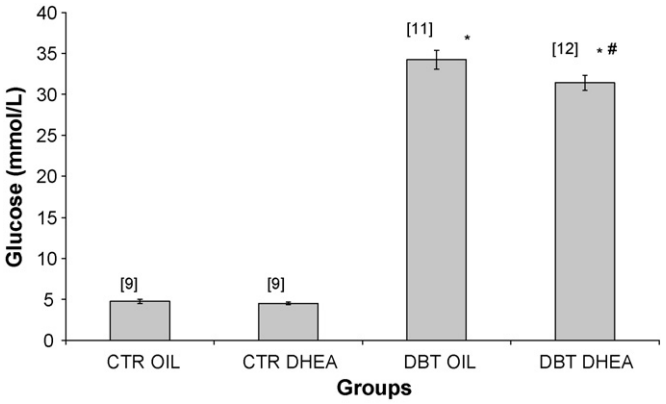

Fig. 2. Plasma glucose concentration in control and diabetic rats, treated with oil or DHEA. Control rats treated with oil (CTR-OIL); control rats treated with DHEA (CTR-DHEA); diabetic rats treated with oil (DBT-OIL); diabetic rats treated with DHEA (DBT-DHEA). Data are given as mean ± SEM. Number of samples is shown in brackets. (*) Statistically different from respective control groups ($p < 0.05$, SNK). (#) Statistically different from DBT-OIL ($p < 0.05$, SNK).

2.11. Statistical analysis

The results were expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used, followed by the Student–Newman–Keuls (SNK) test in order to compare parameters. $p < 0.05$ was taken as the level of significance. All the tests were performed with Jandel Sigma Stat for Windows, version 3.5.

3. Results

3.1. Effect of DHEA treatment on plasma DHEA concentration

In the middle of the first week of the experimental period, it was found that the values of plasma DHEA concentration were higher than before the first injection (Day 1) and the second injection (Day 7) ($p < 0.05$; Fig. 1). At the end of the experimental period (5 weeks, one injection per week) the DBT-DHEA group displayed higher values of DHEA than at the beginning of the experimental period ($p < 0.05$; Fig. 1).

3.2. The effect of DHEA treatment on plasma glucose

Diabetic animals displayed increased plasma glucose, while DHEA treatment decreased levels of glucose concentration ($p < 0.05$; Fig. 2).

3.3. The effect of DHEA treatment on glucose uptake, oxidation and GLUT4

To understand the effect of DHEA treatment on glucose metabolism, quantification of glucose uptake and oxidation (formation of CO_2 from [^{14}C] glucose) was performed. In the control animals DHEA treatment increased glucose uptake ($p < 0.05$), and

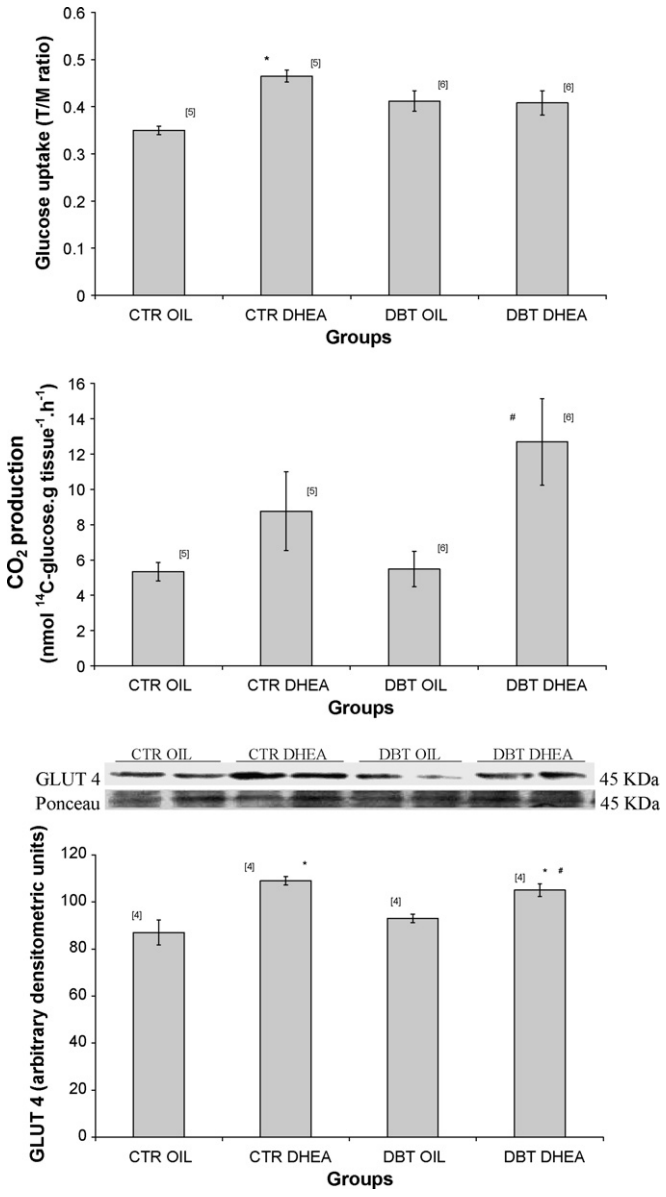

Fig. 3. Glucose metabolism in the gastrocnemius muscle in control and diabetic rats, treated with OIL or DHEA. Control rats treated with oil (CTR-OIL); control rats treated with DHEA (CTR-DHEA); diabetic rats treated with oil (DBT-OIL); diabetic rats treated with DHEA (DBT-DHEA). The [^{14}C] glucose uptake (top), the [^{14}C] glucose oxidation (middle) and Western blot analysis using GLUT 4 antibodies (bottom). The uptake is expressed in tissue/medium (T/M) ratio. The oxidation is expressed in CO_2 formation from [^{14}C] glucose. Data are given as mean ± SEM. Number of samples is shown in brackets. (*) Statistically different from CTR-OIL ($p < 0.05$, SNK). (#) Statistically different from DBT-OIL ($p < 0.05$, SNK).

in the diabetic animals no differences between the groups were observed (Fig. 3). Only in the DBT-DHEA group were higher oxidation values observed ($p < 0.05$; Fig. 3). Since changes were observed in the glucose uptake and oxidation, the level of expression of GLUT4 was ascertained. Both of the groups treated with DHEA increased their GLUT 4 expression as compared to the OIL groups ($p < 0.05$; Fig. 3).

3.4. The effect of DHEA treatment on hydrogen peroxide

Hydrogen peroxide was analyzed as a reactive oxygen species (ROS) which is stable and moderately representative in oxidative processes. DHEA treatment did not alter the amount of hydrogen peroxide in the gastrocnemius muscle. There were no significant differences between the four experimental groups (Fig. 4).

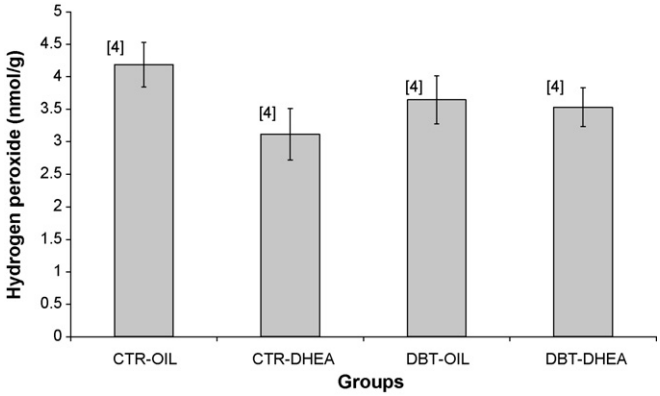

Fig. 4. Hydrogen peroxide quantification in gastrocnemius muscle slices in control and diabetic rats, treated with OIL or DHEA. Control rats treated with oil (CTR-OIL); control rats treated with DHEA (CTR-DHEA); diabetic rats treated with oil (DBT-OIL); diabetic rats treated with DHEA (DBT-DHEA). Data are given as mean ± SEM from 4 animals in each group. There is no statistical difference between the groups.

3.5. The effect of DHEA treatment on Akt ratio and Trx expression

Akt is central to the signaling pathway of GLUT4 and signaling events of cell survival, and our results show that the Akt ratio (p-Akt/total Akt) was significantly lower in the CTR-DHEA, DBT-OIL and DBT-DHEA groups as compared to the CTR-OIL group ($p < 0.05$; Fig. 5). The Trx levels are associated with susceptibility to oxidative stress, and in the CTR-DHEA, DBT-OIL and DBT-DHEA groups Trx expression is lower as compared to the CTR-OIL group, together with a further reduction in the DBT-DHEA group when compared to the DBT-OIL group ($p < 0.05$; Fig. 5).

4. Discussion

A number of studies have shown the effects of DHEA and its derivatives on both animal models and on humans in different scenarios, such as diabetes and obesity, and with a range of functions and tissues [4,13,14,39,40]. This paper has described the effects of chronic treatment with DHEA on diabetic animals, especially its effects on skeletal muscle glucose metabolism.

To confirm our experimental model, the supply of DHEA to tissues (plasma DHEA) during the first week of treatment is shown (Fig. 1), and demonstrates that DHEA levels increased during the week. When they fell (on Day 7) another injection was administered, thus ensuring the presence of chronic DHEA during the 5 weeks of treatment. As further validation of the model, the animals which had received 5 injections of DHEA by the end of the experiment displayed higher concentrations of DHEA than the animals which had received only oil (Fig. 1). Although we know that rats are not ideal models for studying the peripheral effects of DHEA, they are widely used in studies of diabetes and possible treatments [7,13,41].

DHEA acts as an antidiabetic agent by causing a significant decline of about 10% in the blood glucose of diabetic animals. Although statistically significant, this reduction does probably not represent a clinical improvement in these animals, since levels of glycemia remain very high. This decline may be due to a possible effect of DHEA stimulating glucose uptake by certain tissues, as has been observed in other tissues such as fibroblasts [16], adipocytes [17,18] and hepatocytes [19], or by stimulating oxidation [42]. Our results show an increase in glucose uptake in the muscle only in the control rats treated with DHEA, but not in the diabetic animals,

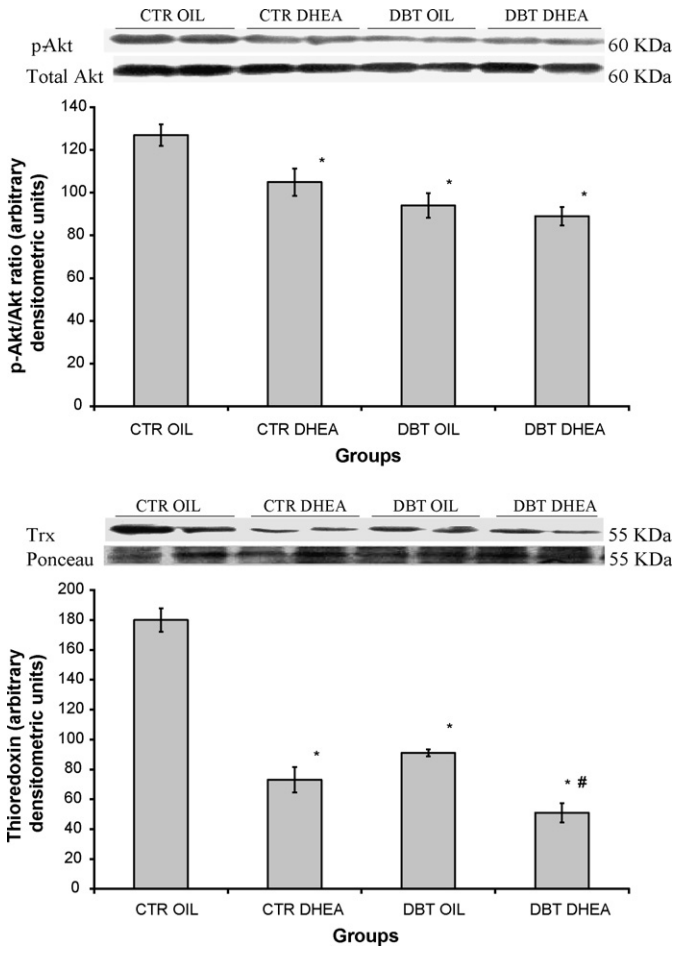

Fig. 5. Western blot analysis in gastrocnemius muscle homogenates in control and diabetic rats, treated with OIL or DHEA, using p-Akt and total Akt antibodies (top) and thioredoxin antibodies (bottom). Control rats treated with oil (CTR-OIL); control rats treated with DHEA (CTR-DHEA); diabetic rats treated with oil (DBT-OIL); diabetic rats treated with DHEA (DBT-DHEA). Data are given as mean ± SEM from 4 samples in each group. (*) Statistically different from CTR-OIL ($p < 0.05$, SNK). (#) Statistically different from DBT-OIL ($p < 0.05$, SNK).

despite the fact that the DHEA treatment increased the expression of the glucose transporter GLUT4 in both DHEA-treated groups, as was also observed in the studies already mentioned, where isolated cells or animals were treated acutely with DHEA [22,23]. DHEA or one of its metabolites generated an increase in the quantity of GLUT4 in the cells, probably by the route that involves the stimulation of AMP-activated protein kinase (AMPK) [43]. This route is also stimulated during increased energy demands (such as exercise) which are independent of the Akt/PI3-kinase. This is indicated by our results, since there was a decrease in Akt in both groups treated with DHEA [44,45]. The increased expression of GLUT4 does not necessarily mean that the carrier is active in the membrane. Its expression increased in both groups treated with DHEA, but its function may remain impaired in diabetics due to a defect in its translocation or activation [46–48].

We observed an increase in glucose oxidation in the muscle of about 40% in the CTR, and of 120% in the diabetic rats treated with DHEA. This corroborates the classic role of DHEA as a hormone that stimulates the use of energy by the cells, increasing the basal metabolism of the tissue [42]. Surprisingly, treatment with DHEA increased the oxidation of glucose in diabetics, even though it did not increase its uptake, as the DHEA may be stimulating glucose utilization via a metabolic pathway that does not do this directly. Once glucose is transported into the cells, it is phosphorylated to glucose-6-phosphate (G-6-P) and enters one of three major pathways: glycogen synthesis, glycolysis, or the pentose-phosphate pathway [49]. The relative contribution of muscle glycogen synthesis from the glucose taken up is very low when compared to glycolysis [50]. Furthermore, experimental *in vivo* and *in vitro* studies indicate that DHEA inhibits glucose-6-phosphate dehydrogenase (G6PDH) activity in mammalian cells (the first enzyme of the pentose-phosphate pathway [19,51]), and diverts more G-6-P to glycolysis, as was demonstrated in our study by the increase in oxidation, which resulted in an increased generation of ROS [52]. NADPH may prevent oxidative damage, and the inhibition of the pentose-phosphate pathway generates less NADPH, which may cause a redox imbalance.

Although our results demonstrated similar hydrogen peroxide levels in the groups studied, both the DHEA-treated rats and the diabetic rats showed diminished thioredoxin (Trx) protein expression. The Trx system is one of the most important mechanisms for the regulation of the redox balance [53], and its low levels may be associated with an environment which is more susceptible to oxidative stress. In addition, Trx regulates various intracellular signaling pathways for cellular growth, thus suppressing apoptosis [54]. Diabetes alone could result in decreased activation of Akt, resulting from defects in insulin signaling [33], as demonstrated by our results. The DHEA-treated groups also showed decreased Akt activation. This decrease was observed earlier, and it was also observed that DHEA may influence the activity of protein phosphatase 2A (PP2A), which consequently influences the phosphorylation of Akt [55,56]. Akt pathway induction is central to cell survival, and its decreased level of phosphorylation has been related to apoptotic events [57]. It has also been observed that Akt pathway activation increases the levels of antioxidant molecules (such as Trx) through the nuclear transcription factor (for example Nrf2) phosphorylation [58]. Thus the overall data suggest that the diabetic state and its association with DHEA treatment may provide an intracellular environment which is more susceptible to the redox imbalance. DHEA may generate in Akt and Trx almost the same effect as diabetes by reducing both of these. This means that in diabetics the decrease in Akt and Trx continues, and that Trx is reduced further in the DBT-DHEA group; the decrease caused by DHEA appears to be independent of the disease, since there was also a decrease in Akt and Trx in the animals treated with oil.

To sum up, these results showed that treatment with DHEA should be viewed with caution, although it is beneficial in certain respects, such as the partial reduction of blood glucose and increased glucose oxidation in diabetic rats. However, its effect can be harmful, since it reduces the levels of proteins essential for survival and also contributes to a redox imbalance in muscle tissue. That having been said, it is not appropriate here to conclude whether its overall effects are beneficial or harmful, as further studies are required to ascertain the efficacy of DHEA treatment.

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