Leucine supplementation affects metabolic parameters and redox balance in a Cushing's syndrome model in rats

Francisco Sávio Martins Borges
DOMEN (Metabolic Diseases, Exercise and Nutrition) Research Group, Department of Biophysical and Physiology, Federal University of Piaui, Teresina, Brazil.

Letícia Sousa de Sá
DOMEN (Metabolic Diseases, Exercise and Nutrition) Research Group, Department of Biophysical and Physiology, Federal University of Piaui, Teresina, Brazil.

Railson de Sousa Santos
DOMEN (Metabolic Diseases, Exercise and Nutrition) Research Group, Department of Biophysical and Physiology, Federal University of Piaui, Teresina, Brazil.

Brenda Caroline Rodrigues Miranda
DOMEN (Metabolic Diseases, Exercise and Nutrition) Research Group, Department of Biophysical and Physiology, Federal University of Piaui, Teresina, Brazil.

João Orlando Piauilino Ferreira Lima
DOMEN (Metabolic Diseases, Exercise and Nutrition) Research Group, Department of Biophysical and Physiology, Federal University of Piaui, Teresina, Brazil.

Johan Jardel Mesquita Prado
DOMEN (Metabolic Diseases, Exercise and Nutrition) Research Group, Department of Biophysical and Physiology, Federal University of Piaui, Teresina, Brazil.

Amanda Ferraz Braz
DOMEN (Metabolic Diseases, Exercise and Nutrition) Research Group, Department of Biophysical and Physiology, Federal University of Piaui, Teresina, Brazil.

Amanda Marreiro Barbosa
DOMEN (Metabolic Diseases, Exercise and Nutrition) Research Group, Department of Biophysical and Physiology, Federal University of Piaui, Teresina, Brazil.

Renato Sampaio Mello-Neto
DOMEN (Metabolic Diseases, Exercise and Nutrition) Research Group, Department of Biophysical and Physiology, Federal University of Piaui, Teresina, Brazil.

Luiz Henrique César Vasconcelos
Departament of Physiology and Pathology, Health Sciences Center, Federal University of Paraiba, João Pessoa/PB, Brazil

Aline de Freitas Brito (alineebritoo@gmail.com)

School of Physical Education, University of Pernambuco, Recife/PE, Brazil

Francisco Leonardo Torres-Leal

DOMEN (Metabolic Diseases, Exercise and Nutrition) Research Group, Department of Biophysical and Physiology, Federal University of Piaui, Teresina, Brazil.

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Abstract

Cushing's syndrome (CS) is a disease that promotes several metabolic disorders and redox imbalance. Thus, in this paper, we standardized an experimental model of CS in rats and evaluated the effects of supplementation with leucine (LEU). Rats were injected intraperitoneally with dexamethasone (0.4; 0.6; 0.8; and 1.0 mg/kg) 4x/wk. for four weeks. The effects of LEU supplementation (0.5, 1.0, and 1.5% v/v in water to drink) were also evaluated. In vitro and in vivo evaluation of metabolic and redox parameters by biochemical and molecular assays defined the severity of the disease. Dexamethasone 1.0 mg/kg promoted the most severe symptoms of CS: such as weight loss, adiposity increase, glucose intolerance, and insulin resistance, as well as an increase in oxidative status in the liver. Moreover, LEU promoted a pattern of mixed dyslipidemia, adiposity increase, and raised serum levels of aspartate aminotransferase (AST). Likewise, it increased myeloperoxidase (MPO) activities and decreased the CAT mRNA in the liver, while it reduced NADPH oxidase (NOX) mRNA. Supplementation with LEU worsens metabolic status and liver damage in rats with CS without an evident antioxidant potential, suggesting that the administration of LEU represents a risky strategy to patients suffering from CS.

1. Introduction

In recent years, glucocorticoids (GCs) have been among the most used drugs to treat acute and chronic inflammatory diseases because of their anti-inflammatory and immunosuppressive effects [1]. That has led to more efficient synthetic GC analogs than endogenous cortisol, presenting plasma half-life and greater affinity with the GC receptor [1, 2].

Among the synthetic GC analogs, dexamethasone is the most common due to its fifty-fold higher affinity to GC receptors than cortisol, since it is not inactivated by the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), conversing cortisol to inactive metabolites and protecting mineralocorticoid receptors from GC binding, and its bioavailability [1, 2]. However, despite their therapeutic efficiency, when administered in long-term treatments, elevated blood GC levels trigger several side effects: such as hypothalamic-pituitary-adrenal axis suppression, peptic ulcers, osteoporosis, diabetes mellitus, cutaneous atrophy, and Cushing's syndrome (CS) [3, 4].

In recent years, CS has aroused concern among researchers, as the reported incidence was estimated at 1.2 to 2.4 per million per year in one population-based study, according to UpToDate Database [5]. Besides, individuals with CS are twice more likely to die than those who do not have this condition [6]. This syndrome may be caused by endogenous, such as hypercortisolism due to ATH-independent cortisol secreting adrenocortical tumor, or exogenous factors, due to long-term use of GC-based drugs [7, 8].

GCs have catabolic effects on carbohydrates, lipids, and proteins, promoting the release of substrates for energy production in stressful situations, such as glucose, glycerol, free fatty acids, and amino acids [9]. Under normal physiological conditions, the cutaneous adipose tissue functions as a fat stock that prevents its deposition in the organs due to its greater sensitivity to the actions of insulin than the visceral

adipose tissue, thus preventing metabolic disorders [10]. In contrast, literature reports that the excess of GCs promotes a centripetal redistribution of adipose tissue [11], as well as other dysfunctions, such as insulin resistance, glucose intolerance, hypertriglyceridemia, hepatic steatosis, decreased bone mineral density, and muscle atrophy [4, 12].

The known mechanisms for metabolic changes in energy regulation of the cell on CS are centered around inhibiting AMP-activated protein kinase (AMPK), which triggers an increase in lipogenesis and fat storage in visceral adipose tissue [13]. Moreover, there is evidence of the influence of oxidative stress, because of the overproduction of oxidants by enzymes, like NADPH oxidase (NOX), and the impairment of antioxidant defenses, such as glutathione peroxidase (GPx) and superoxide dismutase (SOD), that have led to extensive cell damage, endothelial dysfunction and, thus, the development of other diseases, such as atherosclerosis and hypertension [14, 15].

Besides understanding the consequences and mechanisms related to SC, identifying possible therapeutic alternatives is extremely clinically relevant. In this context, branched-chain amino acids (BCAA), such as leucine, have shown importance as nutrients for preventing the development of obesity and improving insulin sensitivity [16, 17]. In the context of obesity, studies report that when orally administered, LEU, in addition to increasing insulin secretion and improving metabolism [18], activates hypothalamic neuronal circuits to control food intake [19]. Likewise, other studies have shown that leucine supplementation helps to improve glucose and cholesterol metabolism, in addition to reducing insulin resistance [16] and total cholesterol concentrations [20] and having antioxidant activity [20]. Therefore, the influence of leucine on the effects promoted by CS is of noteworthy investigation.

Thus, this work aimed to develop an experimental model of CS in rats through prolonged exposure to exogenous synthetic GC and to characterize it by measuring anthropometric and biochemical parameters. In addition, another goal was to evaluate the influence of leucine supplementation on metabolic and oxidative stress parameters of animals with SC.

2. Materials And Methods

2.1 Animals and procedures

Male adult Wistar rats (Rattus norvegicus) weighing between 250–300 g were obtained from the Biotherium of the Center of Agricultural Sciences of Federal University of Piauí. The animals were kept under controlled conditions of temperature (22 ± 2ºC), relative humidity of 55 ± 10% and a light-dark cycle (lights on from 6 a.m. to 6 p.m.), with free access to water and food (Presence®, Paulínea, SP, Brazil), following the guidelines for the ethical use of animals (21), which was previously approved by the Animal Ethics and Experimentation Committee of the Federal University of Piauí (protocol number 314/17).

2.2 Drugs and Reagents

Dexamethasone acetate of prolonged-release and immediate onset (Decadronal®) was obtained from Aché Pharmaceutical Laboratories S. A (Guarulhos, SP, Brazil). Ajinomoto Inter American Industry and Trade Ltd. (São Paulo, SP, Brazil) kindly donated the amino acid L-leucine. Ethylenediaminetetraacetic acid (EDTA) and sodium thiopental (Thiopentax®) were obtained from Cristália Pharmaceutical Chemicals Ltd. (Itapira, SP, Brazil) and Lidocaine hydrochloride from Novafarma Pharmaceutical Industry Ltd (Anápolis, GO, Brazil). Insulin (Humulin®) was obtained from Eli Lilly Ltd (São Paulo, SP, Brazil) and the glucose 50% from Equiplex Pharmaceutical Industry Ltd (Aparecida de Goiania, GO, Brazil).

2.3 Groups and treatments

The animals were initially randomly divided into five groups (n = 5–10 animals per group): Control Group (CG), which received 0.9% NaCl, and DEXA Groups, which received dexamethasone intraperitoneally in doses of 0.4, 0.6, 0.8, and 1.0 mg/kg of body weight, respectively. These doses were chosen from literature data using dexamethasone to induce CS [8].

After choosing the dose needed for CS inducing, the animals were randomly divided, once again, into three new groups: DEXA + leucine (diluted in water for drinking) in three concentrations (0.5, 1.0, and 1.5%) (22). Leucine and dexamethasone administration occurred at the same time.

For all groups, the treatment lasted for four weeks with a frequency of saline or dexamethasone application four times a week. Body mass, food, and water consumption were monitored three times a week.

For the groups that received leucine, the substance was reintroduced on the days of measuring water intake.

2.4 Organs isolation and measurements

After the four weeks, the animals were euthanized by administration of an excessive dose of thiopental after an 8-hour fast. Then, a median laparotomy was performed, followed by dissection and weighing of the liver, adipose tissues (epididymal, retroperitoneal, and mesenteric), and adrenal gland. The organ weight was normalized accordingly to the weight of the animal.

2.5 Biochemical analysis on serum and liver

2.5.1 Obtaining of serum and liver samples

After euthanasia, the blood was collected by the trunk in a tube without anticoagulant, centrifuged 1,000g for 15 minutes to obtain the serum. And a 1.5 mL aliquot was stored in microtubes to analyze glucose, triacylglycerol (TAG), high-density lipoprotein (cHDL), very low-density lipoprotein (cVLDL), low-density lipoprotein (cLDL), total cholesterol, albumin, alanine (ALT), and aspartate aminotransferases (AST).

Likewise, after the median laparotomy followed by weighing the liver, an organ sample was separated to TAG measurement.

2.5.2 Serum biochemical analysis

Serum concentrations of glucose, TAG, cHDL, cLDL, total cholesterol, albumin, ALT, and AST were analyzed using commercial kits (Labtest Diagnostica, Lagoa Santa, MG, Brazil) in a LabMax Plenno automated equipment (Centerlab, Belo Horizonte, MG, Brazil). All concentrations were expressed in mg/dL [23].

2.5.3 Assessment of hepatic TAG levels

A liver sample (100 mg) was transferred to 10 mL tubes containing NaCl (1 M) and homogenized in Ultra-Turrax. Then, chloroform/methanol (2:1) was added and centrifuged at 5,000 rpm for 5 minutes. The lower phase (methanolic) was removed to dry in a boiling water bath. Then, the sample was suspended in TritonX100/Methanol (2:1) and stirred. After that, we followed a colorimetric enzymatic method, according to the manufacturer's instructions (Triglycerides Liquiform, Labtest Diagnostica, Lagoa Santa, MG, Brazil), in a LabMax Plenno automated equipment (Centerlab, Belo Horizonte, MG, Brazil). All concentrations were expressed in mg/dL [23].

2.5.4 Analysis of intraperitoneal glucose and insulin tolerance

The animals fasted for 8 hours, then they were submitted to intraperitoneal glucose tolerance test (ipGTT) [24]. Immediately after blood collection from the tail vein, to measure blood glucose (baseline 0), a 50% glucose solution (200 mg/100g body weight) was administered intraperitoneally. After glucose administration, blood samples were collected from the tail vein at 15, 30, 60, and 120 minutes to assess blood glucose, which was determined using reactive strips and a glucometer (On Call Plus, Acon Laboratories Inc.). From these values, the area under the curve (tAUC) was calculated [25].

Intraperitoneal insulin tolerance test (ITTip) was performed 72 hours after ipGTT. The animals fasted for 8 hours [24], starting at 6 a.m. Reactive strips and a glucometer (On Call Plus, Acon Laboratories Inc.) determined the basal blood glucose, followed by intraperitoneal administration of regular insulin solution (2 U/kg body weight, diluted in saline), and collection of blood samples from the tail vein after 15, 30, 45, and 60 minutes for blood glucose assessment [26]. The glucose decay constant (kitt) was calculated by the formula 0.693/t1/2, in which t1/2 is the half-life of plasma glucose calculated by the slope of the curve obtained during the linear phase of plasma glucose decay detected at 5 to 60 minutes after insulin infusion [26].

2.6 Evaluation of oxidative stress markers in liver tissue

2.6.1 Measurement of MDA levels

The concentrations of MDA were determined by the adapted method based on the production of reactive substances of thiobarbituric acid (TBARS) [27]. Briefly, 200 µL of liver homogenate in 0.1 M sodium phosphate buffer pH 7.0 was added to 350 µL of 20% acetic acid (pH 3.5) and 600 µL of thiobarbituric

acid 0.5%. Then, the mixture was incubated for 45 minutes at 100°C in a water bath and then cooled in an ice bath for 15 minutes. After, 50 µL of sodium dodecyl sulfate (SDS) 8.1% was added to the mixture, centrifuging for 15 minutes at 12,000 rpm at 25°C.

The supernatant was read at wavelengths of 532, 510, and 560 nm in the spectrophotometer, and the corrected absorbance was calculated using the proposed formula to minimize the interference of the heme pigments and hemoglobin: Abs = 1.22 x [A532 - (0.56 x A510) + (0.44 x A560)] [28]. An analytical calibration curve was prepared using MDA as a standard, in concentrations of 1, 5, 10, 25, and 50 nmol/mL. The results were expressed in $mM.L^{-1}$ of homogenate.

2.6.2 Evaluation of superoxide dismutase (SOD) activity

The evaluation of SOD activity required preparation of the reaction medium with 4.96 mg of cytochrome C, 2 mL of MilliQ water, 2 mL of 500 µM xanthine, 2 mL of 1 mM EDTA, and 14 mL of 0.05 M potassium phosphate buffer solution pH 7.8. Subsequently, 10 µL of the liver homogenate, 7.5 µL of xanthine oxidase, and 250 µL of the reaction medium were added to an Elisa plate. The blank was prepared with 7.5 µL of xanthine oxidase and 250 µL of the reaction medium. The preparation was read every minute for 6 minutes, using an automatic plate reader (BioTek Synergy Mx, Winooski, VT, USA) at a wavelength of 550 nm at 25ºC. The determination was made in duplicate and the results were expressed in $mM.L^{-1}$ of protein [29].

2.6.3 Evaluation of catalase (CAT) activity

CAT activity was determined by quantifying the rate of decomposition of hydrogen peroxide (H_2O_2) by decreasing the optical density at 230 nm at 37°C [30]. The reaction medium was prepared using 9 mL of 10 nM H_2O_2, 0.5 mL of Tris HCl 1 M, EDTA 5 mM pH 8.0, and 0.4 mL of Milli-Q water. To start the reaction, it was added 7.5 µL of the homogenate diluted in potassium phosphate buffer and 250 µL of the reaction medium. Then, the samples were incubated at 37°C. The absorbance readings were taken every minute for 6 minutes and were performed using an automatic plate reader at 230 nm (BioTek Synergy Mx, Winooski, VT, USA). One unit (U) of the catalase corresponded to the enzyme activity that allowed the hydrolysis of 1 µmol of H_2O_2 per minute at 37°C at pH 8.0.

2.6.4 Evaluation of reduced glutathione (GSH) activity

We used a 10% organ homogenate in 0.02 M EDTA for the analysis of reduced glutathione activity. The sample consisted of an addition of 2 mL of the homogenate to 2 mL of 10% trichloroacetic acid (TCA), which was stirred for 30 seconds and centrifuged at 3.000 rpm for 15 minutes. After that, 1 mL of the supernatant was transferred to another tube and was added to 2 mL of 0.4 M Tris, 0.2 M EDTA at pH 8.9, and 50 µL of 0.01 M Ellman's Reagent. Then, the sample was homogenized by inversion. The reading was done using an automatic plate reader (BioTek Synergy Mx, Winooski, VT, USA) at a wavelength of 412 nm. The blank was established with 1 mL of water, 2 mL of Triscom buffer, and 50 µL of DTNB. The results were expressed in $mM.g^{-1}$ of protein [31].

2.6.5 Evaluation of myeloperoxidase (MPO) activity

Myeloperoxidase activity was measured based on the formation rate of the oxidation product of o-dianisidine in the presence of H_2O_2 [32]. The reading was performed on a microplate by adding 10 µL of liver tissue solution in 200 µL of the reading solution, consisting of 27 mL of distilled water, 3 mL of pH 6.0 phosphate buffer, 15 mL of 1% H_2O_2, and 5 mg of o-dianisidine. The MPO activity was calculated from the maximum reaction speed, where the monitoring of the formation rate of the oxidation product of o-dianisidine was carried out by observing the increase in the absorbance of the mixture at 450 nm, obtaining reading at zero time and after a minute. The reading was done using an automatic plate reader (BioTek Synergy Mx, Winooski, VT, USA). The results were expressed in U/µL of the sample, considering a unit of MPO defined as the amount in µmol of hydrogen peroxide (H_2O_2).

2.7 Quantification of mRNA by RT-qPCR

2.7.1 Extraction of mRNA

Total RNA was extracted from aliquots of 100 mg of liver tissue for the analysis of mRNA expression of the SOD, CAT, NOX4, and FATP2 genes using the Trizol reagent (Invitrogen™, Waltham, MA, USA). The total RNA extracted from the liver was stored at − 80 ºC for later analysis by RT qPCR with deoxyribonuclease I for the genomic DNA removal. For mRNA concentrations analysis, the absorbance was determined at a wavelength equivalent to 260/280 nm in a Synergy H1 Multi-Mode Reader spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.7.2 Reverse transcription (RT) and RT-qPCR

The total RNA samples were transcribed to complementary DNA (cDNA) in a thermocycler (Veriti®). For cDNA synthesis, 2 µg of total RNA was used in the reaction of each sample with Randon primer, using High-Capacity cDNA Reverse Transcription Kits (Invitrogen number 4375575) in a final volume of 20 µL.

In a single cycle, reverse transcription was performed in several stages as described: I) 10 minutes at 25°C; II) 120 minutes at 37°C; III) 5 seconds at 85ºC; IV) ending at 4°C. For the real-time polymerase chain reaction, the concentrations mRNA of genes of interest were estimated by comparing the samples and the internal control (GPDH) in duplicates, based on the real-time detection of PCR products measured by fluorescence with quantification with ABI ABI Prism 7300 sequence detector (Applied Biosystems, Waltham, MA, USA), following the methodology described by [33].

Under pre-determined cycling conditions, the reaction took place in two stages: first, for 2 minutes at 50 ºC and 95 ºC for 10 minutes, and the second with amplification in 40 cycles: denaturation for 15 seconds at 95 ºC and 63 ºC for 60 seconds; annealing with extension for 2 minutes at 72 ºC. Using the Genbank database, primers were designed (Table 1). The primers (200–800 nM) and samples (50 ng cDNA) concentrations were standardized.

Table 1
List of primer sequences for RT-PCR analysis

Genes	Forward	Reverse
SOD	TCAAGCGTGACTTTGGGTCT	TGATTAGAGCAGGCGGCAAT
CAT	CTCAGGTGCGGACATTCTATAC	GACTCCATCCAGCGATGATTAC
NOX4	TTCTGGACCTTTGTGCCTATA	CCATGACATCTGAGGGATGATT
FATP2	CTCTTTCAGCACATCTCGGA	CCTCTTCCATCAGGGTCACT
SOD: superoxide dismutase; CAT: catalase; NOX: NADPH oxidase; FATP: fatty acid transporter.		

2.8 Statistical analysis

The data were expressed as mean ± standard error of the mean (SEM). The first step of the statistical analysis was a normality test to determine whether the samples followed a Gaussian distribution. The Kolgomorov-Smirnof test was used ($n \leq 6$). When the analyzed samples showed normal distribution, parametric statistical tests were applied and, when they did not pass the normality test, non-parametric statistical tests. For comparisons of two populations, an unpaired t-test (two-tailed) was used, as indicated in the legends of the figures. For the multiple comparison test, one-way ANOVA was performed, followed by Tukey post-test. In all analyzes, a statistically significant difference was considered when the p-value was $p < 0.05$. Statistical analysis software used was GraphPad Prism software version 7.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1 Evaluation of accumulated weekly food intake, water ingestion, and body weight evolution along the four weeks

The treatment with DEXA (0.4; 0.6; 0.8, and 1.0 $mg.kg^{-1}$), alone (Fig. 1A) or combined with administration of LEU (0.5, 1.0, and 1.5%) (Fig. 1B), did not alter the animals' food consumption, compared to the control, on the first week. However, from the second week until the end of treatment (fourth week), all groups treated with different doses of DEXA showed a significant reduction in food intake (Fig. 1A). When associated with the administration of DEXA 1.0 mg.kg-1 and LEU (0.5, 1.0, and 1.5%), the animals' food intake remained higher than the control; however, it did not differ significantly from the DEXA group (Fig. 1B).

Regarding the water ingestion, we found no difference between the control, DEXA (1.0 $mg.kg^{-1}$) and DEXA/LEU (0.5, 1.0, and 1.5%) groups (Fig. 2).

The treatment with DEXA (0.4; 0.6; 0.8, and 1.0 $mg.kg^{-1}$) promoted a significant reduction in body weight after the second week of treatment (Fig. 3A).

The LEU administration (1.0 and 1.5%) accentuated the weight loss in animals that received DEXA (Fig. 3A). Otherwise, the administration of 0.5% LEU did not significantly potentiate the weight loss compared to the DEXA group (Fig. 3B).

3.2 Analysis of the adrenals and adipose tissue weight and adiposity index

We only observed a significant reduction in adrenal weight in the group DEXA (1.0 $mg.kg^{-1}$), while the doses of 0.4; 0.6, and 0.8 $mg.kg^{-1}$ did not change it. Moreover, the combined treatment with DEXA 1.0 $mg.kg^{-1}$ and LEU (0.5, 1.0, and 1.5%) maintained the reduction in adrenal mass, compared to the control group (Table 2).

Table 2
Measurements of adrenal and adipose tissues weight and adiposity index on rats with CS supplemented with leucine.

	Control	Dexa 1.0 mg/kg	Dexa + leu 0.5%	Dexa + leu 1.0%	Dexa + leu 1.5%
Adrenal (mg/g)	0.007 ± 0.0@	0.0024 ± 0.0***	0.0027 ± 0.0***	0.0037 ± 0.0**	0.0035 ± 0.0**
Retroperitoneal WAT (mg/g)	0.76 ± 0.13	0.73 ± 0.22	0.84 ± 0.35	0.69 ± 0.37	0.61 ± 0.35
Epididymis WAT (mg/g)	1.36 ± 0.15	1.86 ± 0.12**	1.95 ± 0.15*@	1.96 ± 0.44	2.02 ± 0.34
Mesenteric WAT (mg/g)	1.00 ± 0.04	1.37 ± 0.14*	1.67 ± 0.25*@	1.75 ± 0.32	1.77 ± 0.23
Adiposity index	2.95 ± 0.34	3.60 ± 0.76*	4.38 ± 0.58*	5.24 ± 0.89*	6.11 ± 0.85*

WAT: White Adipose Tissue. Data expressed as mean ± standard error of the mean (n = 5). One–way ANOVA followed by Tukey's post–test: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Statistically different from the control group); @$p < 0.05$ (Statistically different from DEXA 1.0 mg/kg group).

When evaluating the adipose tissue mass, we observed no change in the mass of retroperitoneal deposits between groups; however, epididymal and mesenteric deposits increased in DEXA 1.0mg/kg and DEXA/LEU 0.5% groups. Otherwise, The adiposity index based on total fat deposit increased in animals from DEXA 1.0mg/kg and DEXA/LEU (0.5, 1.0, and 1.5%) groups (Table 2).

3.3 Serum biochemical assessment

The administration of DEXA 1.0 $mg.kg^{-1}$ promoted a significant elevation on albumin levels, despite not changing the levels of glucose, triglycerides, total cholesterol, HDL, VLDL, and LDL lipoproteins (Table 3).

Table 3
Plasma biochemical assessments on rats with CS supplemented with leucine.

	Control	Dexa 1.0 mg/kg	Dexa + leu 0.5%	Dexa + leu 1.0%	Dexa + leu 1.5%
Albumin (g/dL)	1.99 ± 0.06	2.33 ± 0.10*	2.43 ± 0.11*	2.45 ± 0.15*	2.52 ± 0.04*
Triacylglycerol (mg/dL)	62.8 ± 14.2	102.4 ± 13.1	120.4 ± 13.8	169.0 ± 24.2*@	133.0 ± 10.35*
Cholesterol (mg/dL)	51.0 ± 2.9	63.6 ± 7.7	59.4 ± 9.4*@	84. ± 4.6*	78.0 ± 6.8
Glucose (mg/dL)	126.8 ± 1.8	140.4 ± 10.5	136.4 ± 2.3	142.8 ± 13.2	131.8 ± 11.2
HDL (mg/dL)	16.4 ± 1.0	26.4 ± 2.4	26.0 ± 3.8	30.5 ± 2.2*	36.0 ± 4.8*
VLDL (mg/dL)	12.6 ± 2.8	20.5 ± 2.6	24.1 ± 2.7	33.8 ± 4.8*	26.6 ± 2.0*
LDL (mg/dL)	22.0 ± 2.7	19.8 ± 6.3	12.7 ± 4.5	19.7 ± 5.7	15.4 ± 4.3

HDL: High–Density Lipoprotein. VLDL: Very Low–Density Lipoprotein. LDL: Low–Density Lipoprotein. Data expressed as mean ± standard error of the mean (n = 5). One–way ANOVA followed by Tukey post–test: *$p < 0.05$ (Statistically different from the control group); @$p < 0.05$ (Statistically different from DEXA 1.0 mg/kg group).

Furthermore, on the animals from the group DEXA/LEU, we observed the maintenance of elevated albumin levels and a significant increase in serum triglycerides and total cholesterol compared to the control and DEXA group. Besides, in DEXA/LEU 1.5%, we observed elevated HDL and VLDL levels (Table 3).

3.4 Assessment of triacylglycerol levels and liver damage

The levels of hepatic triacylglycerol and the serum levels of aspartate and alanine aminotransferases (AST and ALT, respectively) did not significantly change in the group administrated with DEXA 1.0 $mg.kg^{-1}$, compared to the control. However, the concomitant administration of DEXA and 0.5% LEU promoted an increase in hepatic triacylglycerol and the serum levels of ALT and AST (Fig. 4).

3.5 Analysis of glucose and insulin tolerance

We observed a difference in the total area under the curve (tAUC) between the control and DEXA 1.0 $mg.kg^{-1}$ in the ipGTT (Figs. 5A and C). Otherwise, in the ipITT, there was no significant difference between the two groups during the sixty minutes (Fig. 5B). However, the assessed glucose decay constant (KITT) showed a delay in the serum glucose levels reduction in the group administered with DEXA 1.0 $mg.kg^{-1}$ compared to the control (Fig. 5D).

3.6 Evaluation of hepatic malondialdehyde levels and the activity of superoxide dismutase (SOD), reduced glutathione (GSH), catalase (CAT), and myeloperoxidase (MPO)

The increase in MDA levels in liver tissue homogenate was significant in the animals administrated with DEXA 1.0 $mg.kg^{-1}$. However, the treatment with 0.5% LEU did not differ for either the control or DEXA group (Fig. 6A).

Regarding the activities of oxidant and antioxidant enzymes, we found that the treatment with DEXA 1.0 mg.kg-1 alone did not significantly modify the activity of none of the targeted enzymes (Fig. 6BE); except for the activity of the oxidative enzyme MPO that had its activity increased in animals from the DEXA/LEU 0.5% group (Fig. 6E). Meanwhile, we did not observe a significant alteration in the activities of the antioxidant enzymes SOD, CAT, and GSH for this group compared to the control and DEXA groups (Fig. 6BD, respectively).

3.7 Measurement of hepatic mRNA expression of SOD, CAT, NOX, and fatty acid transporter 2 (FATP2)

We observed no significant change in the mRNA expression for SOD, CAT, and FATP2, in the hepatic homogenate of all groups, compared to the control; otherwise, the NOX4 mRNA was decreased in the DEXA group, compared to control (Fig. 7AD).

Furthermore, CAT mRNA was reduced in animals of the DEXA/LEU 0.5% group, compared to the DEXA group (Fig. 7B).

4. Discussion

Dexamethasone, a potent synthetic glucocorticoid, is known to lead to changes in the metabolisms of carbohydrates, lipids, and proteins and to act in other tissues, in a time and dose-dependent manner [3]. Additionally, chronic glucocorticoid excess disrupts the internal milieu, resulting in central obesity, muscle atrophy, and fatty liver [21]. Given the severity of this disease, we sought to standardize a rat model of Cushing's syndrome induced by chronic glucocorticoid over levels and then to assess the potential effects of the administration of the branched-chain amino acid leucine on the pathophysiological process since there are few studies regarding the systemic effects of this amino acid.

In the present study, we observed the development of characteristic metabolic patterns of CS, such as visceral obesity, adrenal atrophy, and biochemical alterations. These effects accompanied lower body mass and food intake. In addition, the administration of leucine potentiated some of these effects and promoted liver damage associated with reduced antioxidant defenses.

A neurohumoral system-mediated primarily by the hypothalamus is in charge of hunger and satiety control by increasing the number of circulating hormones, such as leptin and insulin, which are responsible for initiating the signs of satiety in the central nervous system [34, 35]. Furthermore, the inhibition of ghrelin activity by glucocorticoids is directly related to the reduction in body mass [36].

Another important aspect is the reduction in body mass and muscle atrophy, both known characteristics of chronic use of dexamethasone, due to the increase in muscle proteolysis and the inhibition of protein synthesis, which is an additional effect resulting from the increase in lipolysis [37, 38].

In this context, the administration of dexamethasone, in all doses tested, promoted a reduction in food intake but not water from the second week of treatment. In addition, there was a reduction in body weight evolution in these animals, which is directly related to lower food intake.

It has been shownm, in a literature review [39], that obesity and weight gain were present in 95% of patients with CS; however, despite this apparent discrepancy, the data obtained in the present work demonstrate a decrease in body mass in animal models of CS, and for these models weight gain is not common. These results agree with previous data, in which the CS model showed significant body mass loss in the first weeks, while in the control group, there was an increase in body mass. In subsequent weeks of this study, the group with CS showed a gradual increase in body weight [8]. The lipogenic effects on the visceral adipose tissue and the catabolic effects on the skeletal muscle may explain those results [39].

The effects of the supplementation of branched-chain amino acids (BCAA), such as L-leucine effects on metabolic syndromes and body composition, are diverse. Several studies have shown that their use can result in a decrease in total body weight in obese rats, with decreased adiposity, in addition to improving insulin sensitivity and reducing plasma cholesterol concentrations [16, 20].

When assessing the influence of leucine supplementation in the three tested doses, we found an increase in the anorexigenic effect without changes in water intake despite the weight loss remaining similar to what we observed on the treatment with dexamethasone alone.

Some previous research study an association of leucine supplementation and carbohydrates, proteins, or other amino acids. However, when supplemented in the absence of other BCAAs (isoleucine and valine) or with a low protein and carbohydrate diet, animals show a decreased food consumption, body mass, and growth [40].

These effects may be associated with reduced concentrations of ghrelin and increased concentrations of leptin, which are responsible for the perception of hunger and satiety, respectively, thus contributing to the reduction in body mass [41, 42], as it has been previously described for dexamethasone [36]. Likewise, The literature has shown that leucine promotes protein anabolism and lipolysis (43), contributing to these observed results.

Adrenal atrophy is a classic effect in CS that results from chronic treatment with glucocorticoids [8]. Glucocorticoid treatment inhibits the hypothalamus-pituitary-adrenal (HPA) axis, thus, the hypothalamus stops releasing the corticotrophin-releasing hormone, responsible for synthesizing the adrenocorticotropic hormone (ACTH), so it does not act by promoting cell tropism on the adrenals, resulting in the atrophy of this gland [3]. Furthermore, the chronic use of glucocorticoids favors greater visceral adiposity [44].

Likewise, glucocorticoid acts on pre-adipocytes inducing differentiation and increase in adipose mass, in addition to increasing the activity of lipoprotein lipase (LPL), which induces fatty acids storage on tissues [45].

As expected for elevated glucocorticoid levels, we observed that the administration of dexamethasone 1.0 $mg.kg^{-1}$ promoted a reduction in the adrenal mass, indicating inhibition of the HPA axis. Furthermore, there was a greater deposition of visceral fat, a result confirmed by a higher adiposity index. Moreover, the administration of leucine (0.5, 1.0, and 1.5%) potentiated these effects, indicating a non-beneficial action of this amino acid in the context of CS.

The glucocorticoid excess, either endogenous (Cushing's Syndrome) or exogenous (long-term glucocorticoid treatment), results in visceral adipose tissue deposition, muscle atrophy, fatty liver, hypertension, hyperglycemia, dyslipidemia, and insulin resistance [2, 46]. In this study, we observed that administration alone of dexamethasone 1.0 $mg.kg^{-1}$ promoted hyperalbuminemia and that, when associated with leucine supplementation, the rats developed mixed dyslipidemia, illustrated by the elevation of TAG, cholesterol, and VLDL levels. Moreover, leucine supplementation maintained hyperalbuminemia.

In CS, the secretion of the hormones insulin and glucagon is compromised. These hormones are responsible for controlling, in addition to glycemic levels, the intracellular concentration of cholesterol, which if altered could lead to an increase in cholesterol levels and trigger a reduction in the activity of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, a limiting enzyme in cholesterol biosynthesis [47].

Furthermore, hyperalbuminemia can also be explained by the inhibition of the HPA axis, leading to a reduction in aldosterone production, which is responsible for regulating sodium homeostasis [8]. The increase in albumin levels may result from the decrease in circulating volume, leading to a higher concentration of plasma proteins.

Fat deposition and liver damage are characteristics of excess glucocorticoids in the body, as previous research has shown [2, 46]. In this study, the administration of leucine, together with dexamethasone, led to an increase in liver TAG deposition (Fig. 4A). In addition, we observed an increase in the levels of the AST enzyme, but not ALT, in this association (Fig. 4B-C). This result is a highly sensitive indicator of hepatocellular damage and, to a certain extent, indicates liver damage [48].

Some studies have shown that glucocorticoids promote the decrease of glucose transport stimulated by insulin [49, 50]. When in excess, they lead to a decreased uptake and oxidation of this substrate in skeletal muscle [51], which are likely responsible for promoting glucose intolerance and insulin resistance. When administered for a prolonged period or even in high doses, glucocorticoids promote glucose intolerance, as the high synthesis and secretion by pancreatic β-cells of insulin cannot compensate for the metabolic demand [52]. In this work, dexamethasone 1.0 mg/kg promoted a reduction in glucose tolerance (Figs. 5A and C), which may be associated with a morphological

adaptation of the pancreas, as glucocorticoid favors hypertrophy of pancreatic β cells and consequently an increase in insulin secretion [24, 53]. Furthermore, these effects accompany a pattern of insulin resistance in the animals in the group that received the highest dose of DEXA (Figs. 5B and D).

Those data agree with the expected results for CS models, as it is well established that this disease promotes metabolic disorders associated with lower glucose uptake due to insulin resistance [30, 5].

Based on the parameters described so far, we observed that the administration of dexamethasone 1.0 $mg.kg^{-1}$ proved to be the most effective in promoting the development of CS in rats and that supplementation with leucine promoted a worsening effect in the observed parameters, including reduced body and muscle mass and increased visceral adiposity, hyperalbuminemia, and dyslipidemia.

The antioxidant defense system works by reducing the damage caused by free radicals and these reactive species, and when there is lipid peroxidation resulting from increased fat, it causes an imbalance in the oxidative and antioxidant system [54]. With the increase in fat, there may be progressive and cumulative cell damage because of pressure due to the large body mass, where cell injury, which releases pro-inflammatory cytokines, generating reactive oxygen species [55].

MDA is a biomarker and one of the secondary products of lipid peroxidation, derived from the β-rupture of endo-cyclization of polyunsaturated fatty acids, such as linoleic, arachidonic, and docosahexaenoic acid [56]. The triggering of inflammatory processes in CS is often associated with myeloperoxidase (MPO), an important inflammatory indicator, considering that the release of this enzyme is due to macrophages activated during inflammation and that the reactive species produced by them can activate factors of transcription, including the NF-kB signaling pathway (transcription factor involved in the synthesis of pro-inflammatory cytokines) increasing the inflammatory process [57].

In this work, the concentrations of MDA in the liver increased in the DEXA group but did not change in the group that received leucine, indicating a reduction in lipid peroxidation mediated by the action of this amino acid. However, these values should be observed with caution, as the group that received leucine had significantly higher values of MPO. Therefore, these data cannot indicate whether leucine in isolation could control the entire cellular antioxidant system.

Oxidative stress can trigger cell adaptation or injury. When adapted, the cells tolerate oxidative stress by regulating the synthesis of antioxidant defenses until the balance is restored [58]. This picture of oxidative stress can cause damage to the most diverse types of biomolecules – including DNA, proteins, and lipids – since the target of oxidative stress varies depending on the cell, type of exposure, or even the intensity of the stress [59].

Several enzymes are part of the defense mechanism, including SOD, CAT, and glutathione peroxidase (GPx), in addition to others that are not directly related to the process, such as glucose-6-phosphate dehydrogenase (G6DP), and non-enzymatic antioxidants such as vitamin E, vitamin C and flavonoids [60].

SOD and CAT act mainly in hydrophilic regions, SOD with specificity for O_2 – dismutation, generating hydrogen peroxide, while CAT decomposes H_2O_2. Since SOD and CAT are two enzymes that cooperate, the activities of these two enzymes should change synchronously and simultaneously. GSH is important for acting in thiolic homeostasis, maintaining the cell redox balance, and defending against electrophilic agents. This antioxidant benefit occurs through the reactive thiol group (SH) of its cysteine [61].

We observed that the activity of these antioxidant enzymes did not change by dexamethasone or leucine administration, indicating no counterbalancing antioxidant response induced by oxidative stress indicated by the high levels of MDA and MPO.

However, we observed that, although SOD mRNA was unaffected by glucocorticoid and leucine, the mRNA transcript for CAT decreased with leucine supplementation and, controversially, NADPH oxidase 4 (NOX4), oxidative enzyme-producing superoxide anion, was also reduced. In addition, the fatty acid transporter protein type 2 (FATP2), important in the transport of fatty acids and metabolism, did not show statistically significant differences between the groups, although this marker is commonly associated with fatty liver [62].

5. Conclusions

The CS induction model using dexamethasone in rats is rarely in the literature. Thus, this study presented a model of CS induction in rats, in addition to showing the effects of leucine supplementation against CS. The latter should be cautiously considered, as it presents results that do not indicate whether the use of leucine, isolated, is beneficial for the treatment of CS.

Declarations

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Author contribution

FSMB participated in the conceptualization, was the main executor, and obtained, analyzed, and discussed all data. LSS, RSS, BCRM, JOPFL, JJMP, AFB, AMB, and RSMN participated in the acquisition and analysis of data. LHCV, AFB, and FLTL participated in the conceptualization, analysis, writing, review, and editing of the article.

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Availability of data and material:

Available on request.

Conflict of Interest

The authors declare that there is no conflict of interest.

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Figures

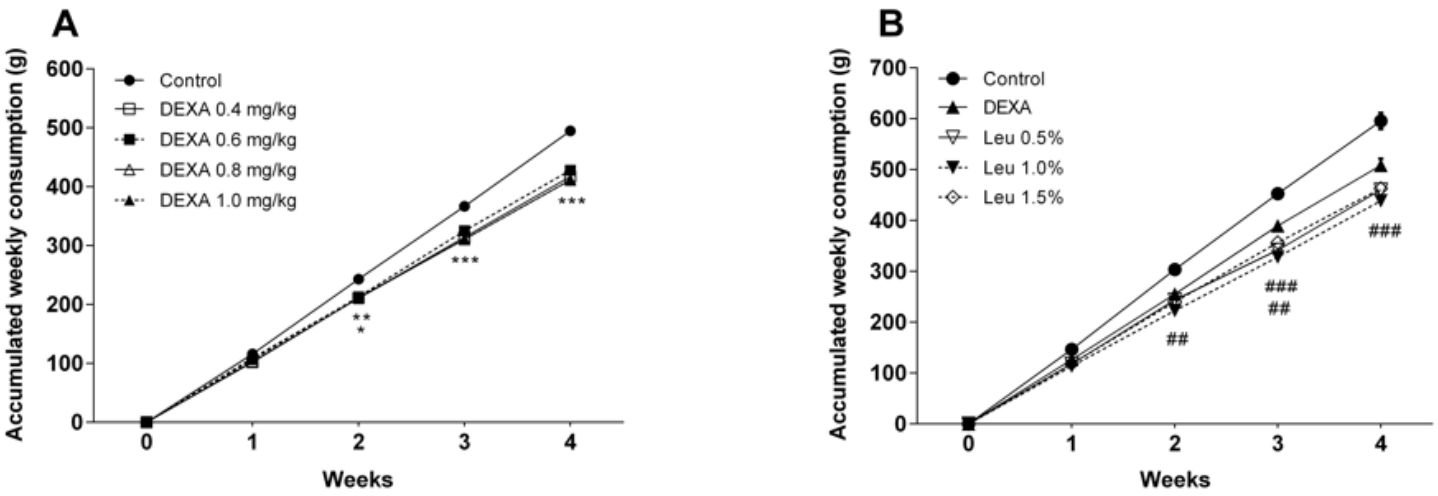

Figure 1

Measurement of cumulative food consumption in rats with CS supplemented with leucine.

Data expressed as mean ± standard error of the mean (n = 5). One-way ANOVA followed by Tukey's post-test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Statistically different from the control group). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ (Statistically different from the DEXA 1.0 mg/kg group).

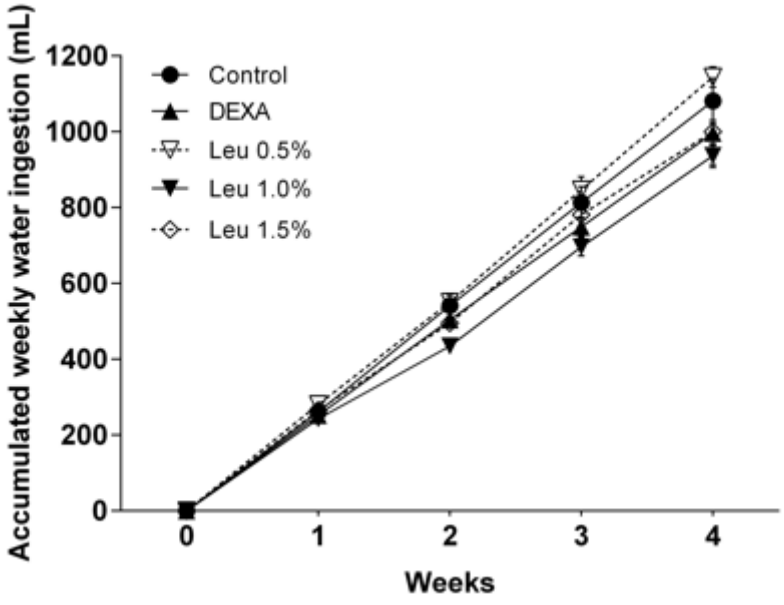

Figure 2

Measurement of cumulative water ingestion in rats with CS supplemented with leucine.

Data expressed as mean ± standard error of the mean (n = 5).

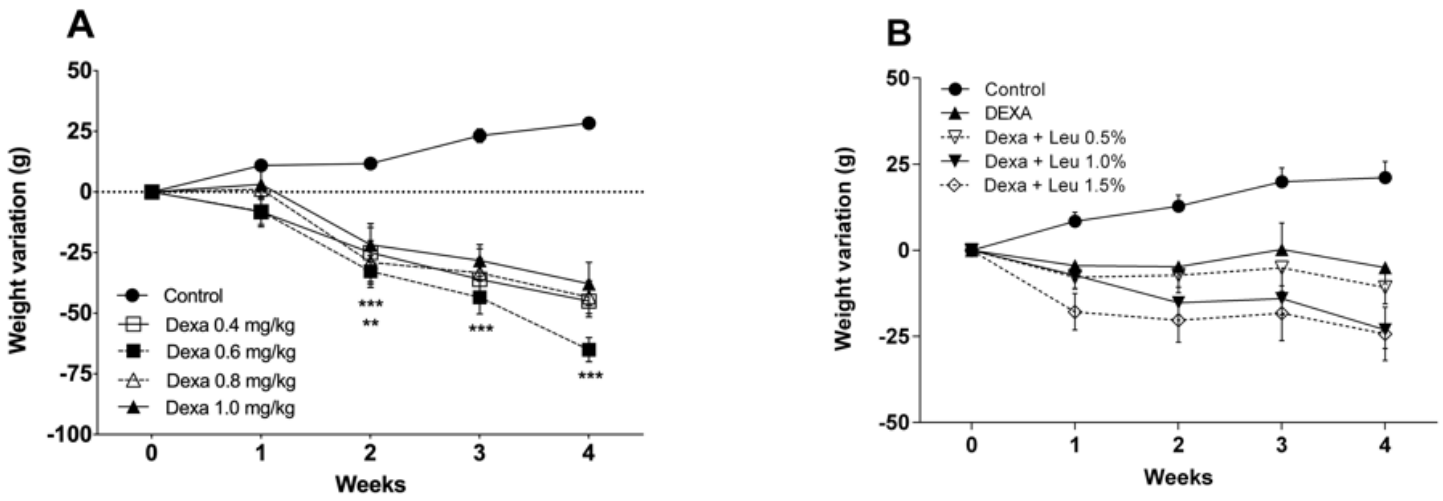

Figure 3

Accumulated weight variation in rats treated with dexamethasone and leucine.

Data expressed as mean ± standard error of the mean (n = 5). One-way ANOVA followed by Tukey's post-test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ (Statistically different from the control group).

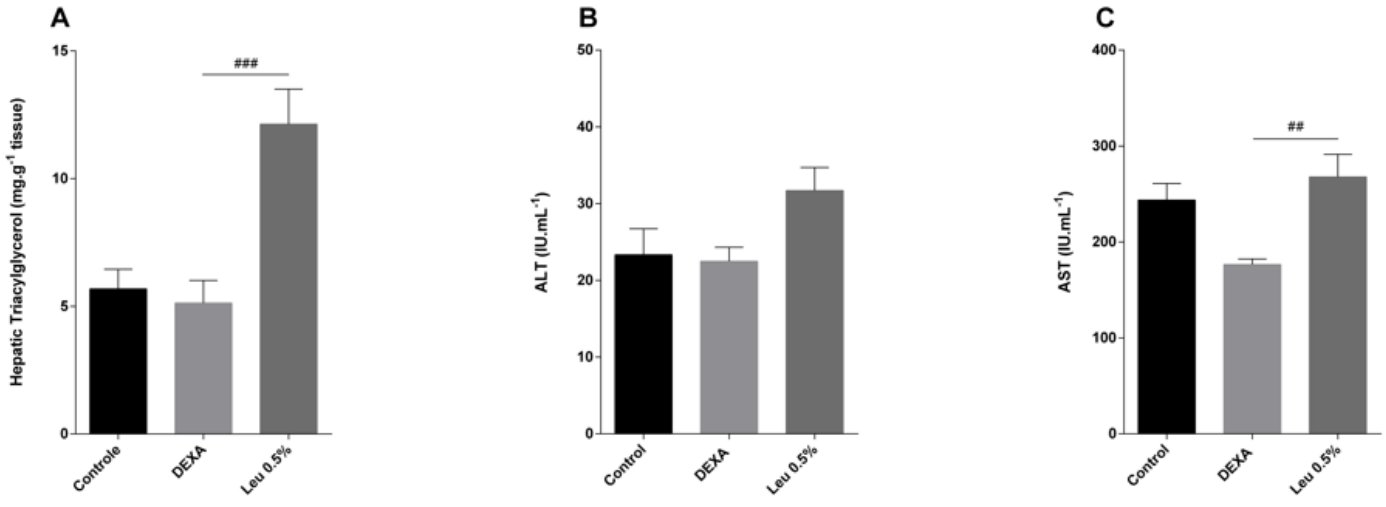

Figure 4

Evaluation of hepatic triacylglycerol concentrations (A), ALT (B), and AST (C) levels in rats with CS treated with leucine.

Data expressed as mean ± standard error of the mean (n = 5). One-way ANOVA followed by Tukey's post-test: $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ (Statistically different from the DEXA group).

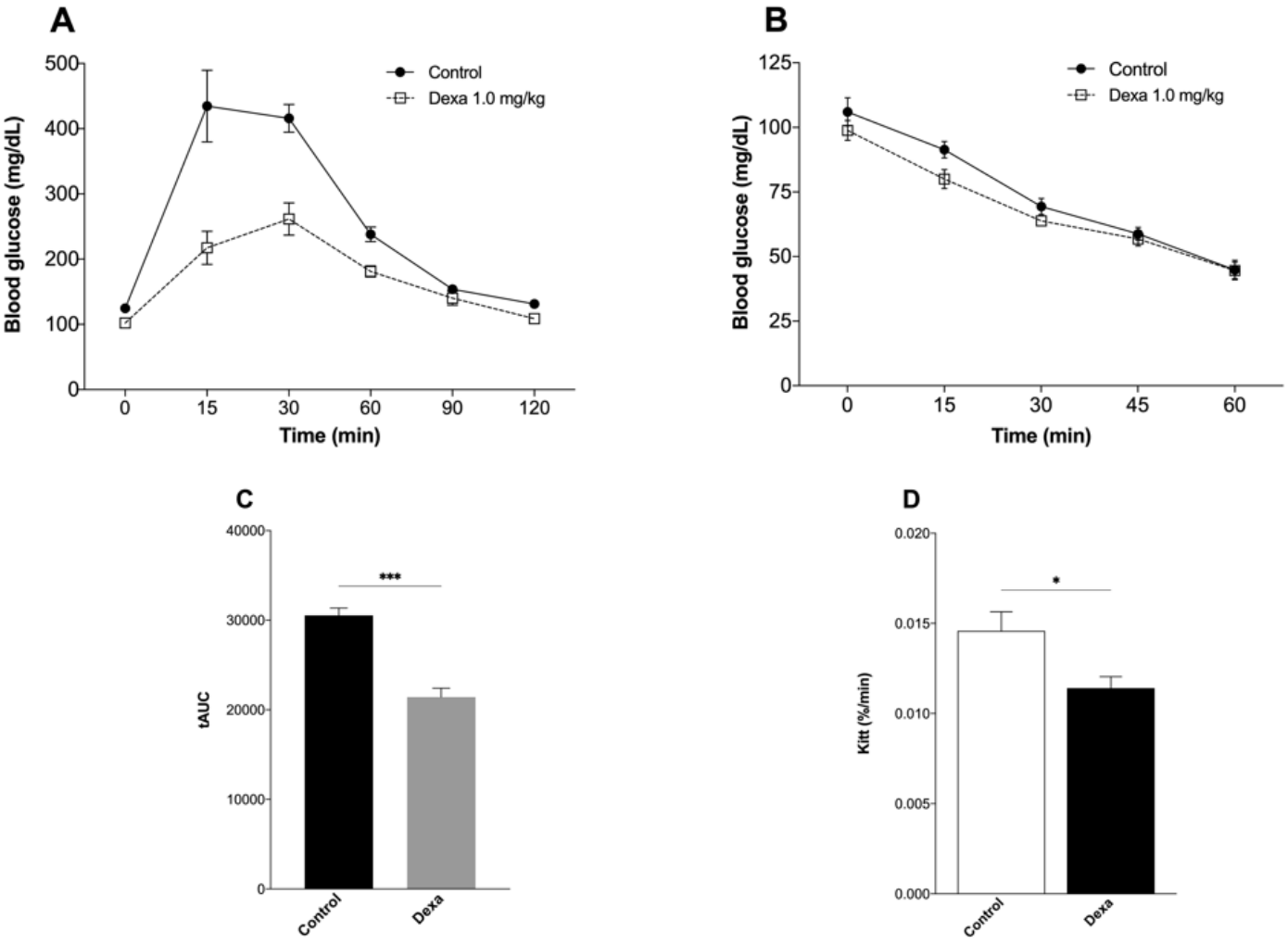

Figure 5

Glycemic curve of the intraperitoneal glucose (A) and insulin tolerance test (B), total area under the curve (tAUC) for glycemic curve (C) and glucose decay rate (D).

Data expressed as mean ± standard error of the mean (n = 5). T-test: $^{*}p < 0.05$ and $^{***}p < 0.001$ (Statistically different from the control group).

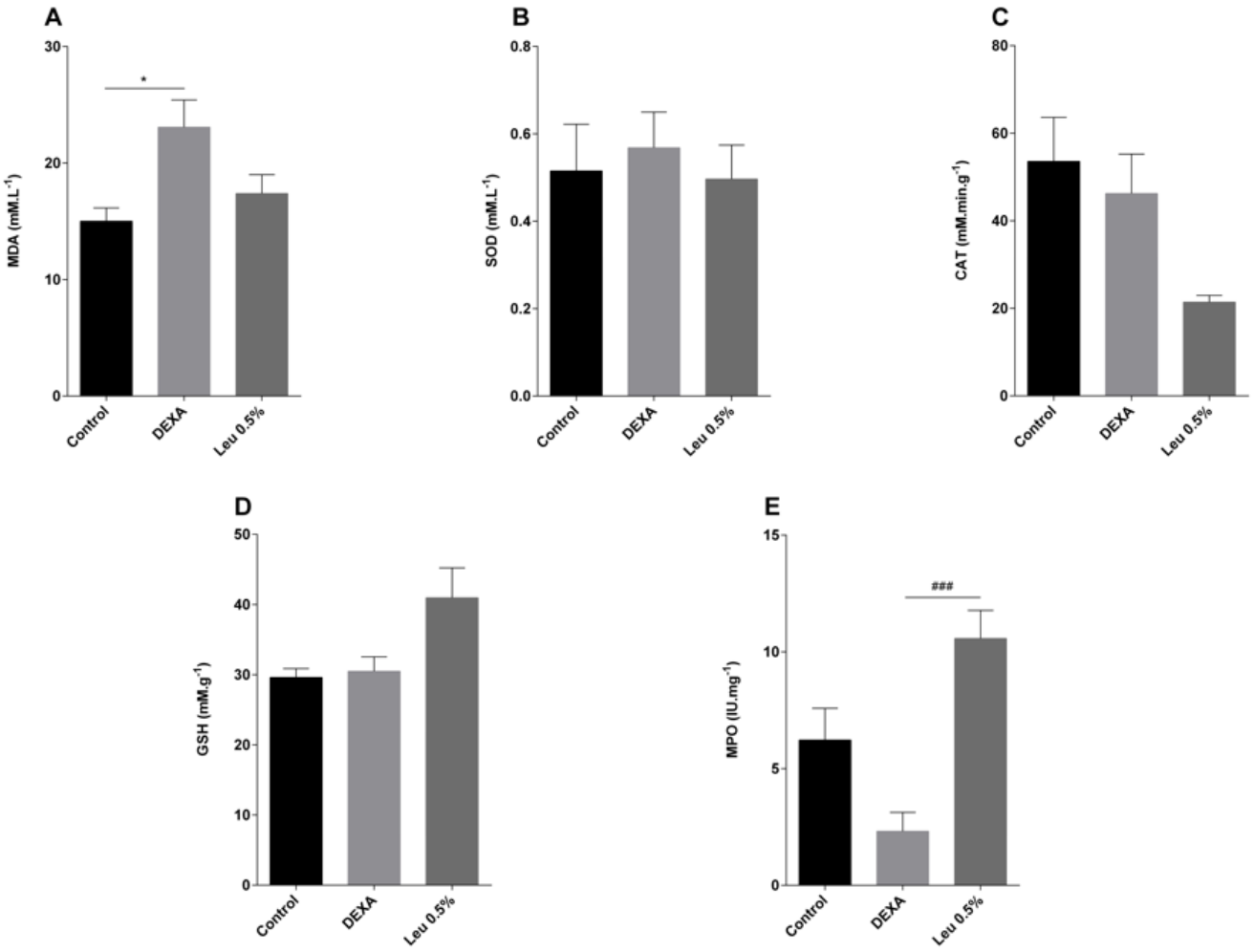

Figure 6

Evaluation of the levels of MDA (A) and the activities of SOD (B), CAT (C), GSH (D), and MPO (E) on hepatic tissue in rats with CS treated with leucine.

Data expressed as mean ± standard error of the mean (n = 5). One-way ANOVA followed by Tukey's post-test: $^{*}p < 0.05$ (Statistically different from the control group); $^{###}p < 0.001$ (Statistically different from the DEXA group).

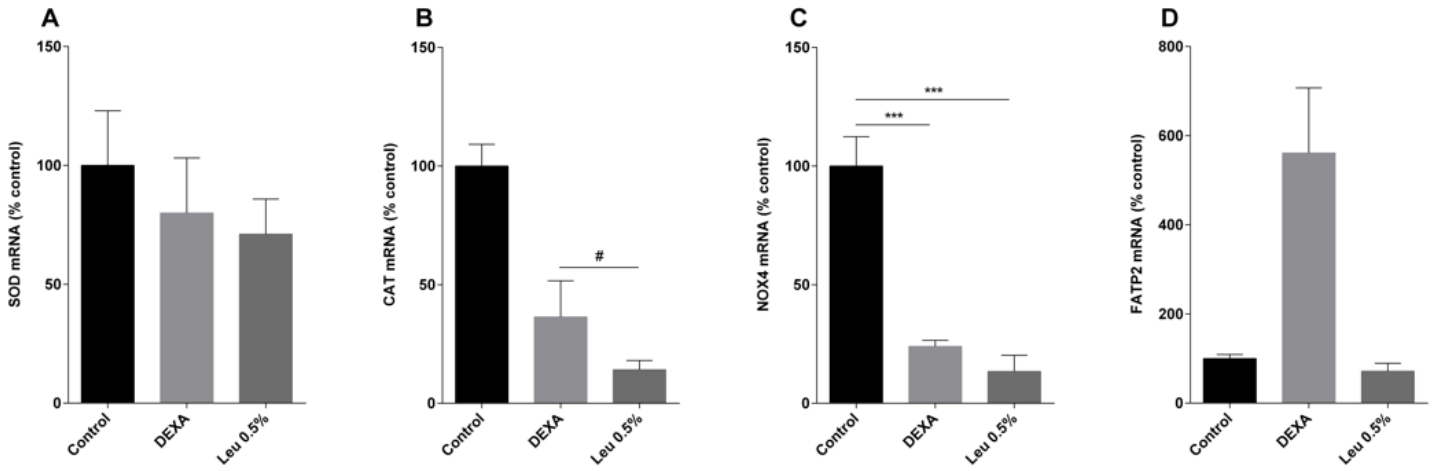

Figure 7

Analysis of mRNA expression of SOD (A), CAT (B), NOX4 (C), and FATP2 (D) on liver tissue in rats with CS treated with leucine.

Data expressed as mean ± standard error of the mean (n = 3-5). One-way ANOVA followed by Tukey's post-test: $^{***}p < 0.001$ (Statistically different from the control group); $^{\#}p < 0.05$ (Statistically different from the DEXA group).