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Effects of HIIT Associated with *Coutoubea spicata* Supplementation on Tissue and Oxidative damage Biomarkers in Wistar Rats

Efectos del HIIT Asociado a la Suplementación de *Coutoubea spicata* en los Biomarcadores del Tejido y Daño Oxidativo en Ratas Wistar

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SUMMARY: This study aimed to evaluate the effects of six weeks of HIIT on tissue and oxidative damage markers in rats supplemented with *Coutoubea spicata* fraction. Thirty-two male Wistar rats were divided into 4 groups: Baseline (GB); supplemented with 100 mg/kg of *Coutoubea spicata* fraction (GSCS); exercised for 6 weeks with the HIIT protocol (GH); supplemented with 100 mg/kg of *Coutoubea spicata* fraction + HIIT for 6 weeks (GHCS). Exercised animals performed the HIIT protocol (2 x 2). Tissue damage CK, LDH, ALT and AST markers in plasma were analyzed, as well as oxidative stress MDA and SH biomarkers in plasma and in cardiac, hepatic and muscle tissues. The results showed that CK, LDH, AST and ALT enzymes showed increase in GH when compared to GB ($p<0.0001$). However, CK, AST and ALT markers reduced their concentrations in GHCS when compared to GH ($p<0.0001$), indicating that *Coutoubea spicata* supplementation attenuated the damage in muscle and liver tissues induced by HIIT. Plasma, liver and muscle MDA showed increase in GH after HIIT sessions; however, when compared to GHCS, it showed reduced levels ($p<0.0001$). SH was elevated in the GH group when compared to GB in plasma and liver tissues ($p<0.0001$); in contrast, reduction in GHCS when compared to GH was observed in plasma, liver and cardiac tissues, demonstrating the redox effect of HIIT on some tissues. Thus, our findings showed that *Coutoubea spicata* has antioxidant activity, reducing oxidative damage markers and consequently tissue damage in healthy Wistar rats after HIIT protocol, but it also demonstrated redox balance after analyzing oxidative stress markers.

KEY WORDS: HIIT; ***Coutoubea spicata*****; Oxidative stress; Tissue damage.**

INTRODUCTION

High Intensity Interval Training (HIIT) has gained popularity due to its physiological benefits in a short period of time, unlike other types of training (Cipryan *et al*., 2017). Due to the high metabolic demand of this type of exercise, it can lead to increase in the production of reactive oxygen species (ROS) (Powers *et al*., 2016), which are synthesized during exercise, mainly in muscle. This increase, when not neutralized or resynthesized by the antioxidant system, triggers oxidative stress, leading to damage to molecules and tissues (Bogdanis *et al*., 2013).

In this context, some supplements have been studied in literature, especially antioxidants, as a way to improve the redox profile (Ammar *et al*., 2018). Thus, Coutobea spicata known in Brazil as bitter root is used as a medicinal herb, in addition to presenting a range of substances from the flavonoid class (Dos Santos *et al*., 2020). Therefore, the aim of this study was to evaluate the effects of six weeks of HIIT on tissue and oxidative damage biomarkers in Wistar rats supplemented with *Coutoubea spicata* fraction.

MATERIAL AND METHOD

Animals and experimental protocol. For this study, 32 male Wistar rats were selected, weighing between 250 and 300 g, which were obtained from the Animal Facility of the

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Intracellular Signaling Research Nucleus (NUPESIN, Federal University of Sergipe (UFS)), randomly housed, kept at controlled temperature of 22 ± 3 °C with 12-h light-dark cycle and with free access to feed (Labina®) and water.

Animals were divided into four groups (n=8): Baseline group (GB): control group of animal's basal state, euthanized before the experimental period; Group supplemented with *Coutoubea spicata* fraction (GSCS): animals that received treatment of 100 mg/kg of body weight of *Coutoubea spicata* fraction; HIIT Group (GH): animals that performed the HIIT protocol 3 times a week for 6 weeks; HIIT group supplemented with *Coutoubea spicata* (GHCS): animals that received treatment of 100 mg/kg body weight of *Coutoubea spicata* fraction + HIIT protocol.

This study was carried out in compliance with recommendations and standards established by the National Council for the Control of Animal Experiments (CONCEA). The research protocol with experimentation was approved by the Ethics Committee in Research with Animals (CEPA) of UFS (Protocol number 03/2019). Animals were euthanized after being anesthetized with intraperitoneal injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), followed by exsanguination, and blood and organs, gastrocnemius muscles, liver and heart were removed. Every effort was made to minimize suffering.

Before the performance of the HIIT protocol, animals were submitted to adaptation to the liquid medium for 15 uninterrupted days. GH and GHCS rats were familiarized according to the swimming protocol adapted from Manchado-Gobatto (2010). Thus, from the 1st to the 3rd day, animals were immersed in shallow water (10 cm) for 15 min; from the 4th to the 10th day, they were adapted in deep water (60 cm), lasting 2 min, adding another 2 min each day until the 10th day; and from the 11th to the 15th day, they were submitted to deep water again, with weights of 3 % of body weight attached to the back for 5 minutes, adding 5 min each day, until the 15th and last day.

GSCS animals followed the same adaptation protocol to the liquid environment as GH and GHCS animals, with the last 5 days of adaptation being changed, where from the 11th to the 15th day, they were submitted to adaptation in deep water for 25 min each day, until the last adaptation day. GB animals were adapted for 15 days in shallow water (10 cm), and then euthanized. These animals performed the adaptation process without minimum stress, with changes being evaluated based on the animals' basal state.

The anaerobic threshold (AT) was used as a way to determine the load used in the HIIT training, with protocol adapted from Manchado-Gobatto (2010). In each of these loads, a drop of blood (2μL) was extracted from the tail of animals through a needle puncture using lancets for blood glucose testing to determine blood glucose using Accu-Check Go glucometer (Roche Diagnostics GmbH, D-68298 Mannheim, Germany). After performing AT, bisegmentation of the glycemic curve was performed in individual graphs with the blood glucose vs. exercise intensity value (Dos Santos *et al*., 2021). The estimated load for the beginning of training was 6 % of body weight.

After AT, the maximal repetition test (MRT) was performed to determine the number of repetitions for the execution of the long HIIT protocol. MRT was performed with protocol proposed by Pimenta *et al*. (2015), which consists of series of 20 s for 10 s of passive recovery using overload of 10 % of body weight, being repeated until exhaustion, and the exhaustion time was determined when the animal was more than 10 cm submerged in water and unable to return to the water surface to breathe after 10 s. The long HIIT protocol was performed 3 times a week for 6 weeks, with sets of 2 min of activity for 2 min of passive recovery, repeating 7 times, with initial body load of 6 %, adding 1 % each week.

***Coutobea spicata* collection, processing, extraction and administration.** *Coutoubea spicata* parts were dried at room temperature, reduced to powder and submitted to maceration with 95 % ethanol for 5 days. Then, the material was filtered and concentrated on a rotary evaporator under reduced pressure at 45 °C to give 424 g of the ethanol extract (yield of 9.53 %). A portion of ethanol extract (252 g) was dissolved in methanol: water (2: 3) solution and submitted to liquid-liquid extraction with organic solvents to obtain hexane (28.5 g, yield of 11.31 %), chloroform (51.2 g, yield of 20.32 %), ethyl acetate (45.8 g, yield of 18.17 %), and hydromethanol (123.4 g, yield of 48.97 %) fractions. The ethyl acetate fraction (EAF) was used in the in vivo assay. Thus, GHCS animals were supplemented 1 hour before training with the *Coutoubea spicata* fraction administered at 100 mg/kg of body weight (Dos Santos *et al*, 2020), intragastrically (by gavage) using metallic cannula specific for rodents, with rounded end so that animals suffered no harm. GSCS animals received the same application and returned to housing.

After the last training session and euthanasia, blood was collected in heparinized EDTA tube and immediately centrifuged at 4000 x g for 15 min at ± 4 °C, and the serum was stored at ± -80 °C. Organs were washed 3 times with 1.15 % potassium chloride (KCl) solution. Subsequently, they were homogenized, where each gram of tissue was mixed with 5 mL of KCl + 10 μL of phenylmethylsulfonyl fluoride (PMSF –100 mmol. L-1) + 15 μL of 10 % Triton

solution and centrifuged at 3000 xg for 10 min at ± -70 °C for further analysis of oxidative stress markers.

Biochemical analyses. Using plasma, analyses of muscle and liver damage biochemical markers were performed: creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST), alanine aminotransferase (ALT), using commercial kit (Labtest®, Brazil) according to manufacturer's recommendations.

Lipid oxidation was determined by measuring thiobarbituric acid reactive substances (TBARS) according to method described by Lapenna *et al*. (2001). For analysis of sulfhydryl groups (SH), they were quantified to identify the antioxidant level of tissues and plasma, according to methodology described by Faure & Lafond (1995).

Statistical analysis. For data analysis, they were represented as mean ± standard deviation, using the Shapiro-Wilk normality test. For post-test comparison between groups, one-way ANOVA and Post-Hoc Bonferroni were used. Considering the statistically significant difference among samples at $p<0.05$, the Graph Pad Prism statistical software version 7.0 was used.

RESULTS

It was demonstrated after analyzing CK results (Fig. 1A) that GH increased by 173.50 % compared to the baseline group (GB) ($p<0.0001$). GHCS showed increase of 86.59 % compared to GB ($p<0.001$). GH showed increase of 46.58 % when compared to GSCS ($p<0.0001$). GHCS showed increase of 80.13 % when compared to GSCS ($p<0.0001$). The group supplemented with *Coutobea spicata* reduced 31.78 % compared to the GH group ($p<0.001$).

After analyzing LDH tissue damage marker (Fig. 1B), the results demonstrate that GSCS obtained increase of 174.87 % compared to the GB group ($p<0.0001$). GH increased by 180.43 % compared to GB ($p<0.0001$). GHCS increased 143.3 % when compared to GB ($p<0.0001$). There were no significant differences between GH, GHCS and GSCS ($p>0.05$).

AST liver damage marker (Fig. 1C) was elevated in GH by 176.44 % compared to GB ($p<0.0001$). GH increased 148.23 % compared to GSCS ($p<0.0001$). GHCS reduced 62.73 % compared to GH ($p<0.0001$). ALT marker (Fig. 1D) showed increase in GH of 214.47 % compared to GB ($p<0.0001$), and showed increase of 122.48 % when compared to GSCS ($p<0.0001$). GHCS reduced 66.65 % compared to GH ($p<0.0001$).

Plasma MDA was analyzed (Fig. 2A), where it was demonstrated that GH increased 39.75 % compared to GB ($p<0.05$). GHCS reduced 34.3 % compared to the GB group ($p<0.05$). GHCS reduced 42.34 % compared to GSCS ($p<0.01$) and GHCS reduced 53.01 % compared to GH (<0.0001).

Organ MDAs were measured from the serum. Liver MDA (Fig. 2B) showed increase in GSCS of 54.25 % compared to GB ($p<0.001$). GH showed increase of 102.39 % compared to GB ($p<0.0001$). GHCS increased 68.39 % compared to GB ($p<0.0001$). GH increased 31.21 % compared to GSCS ($p<0.01$). In the case of cardiac MDA

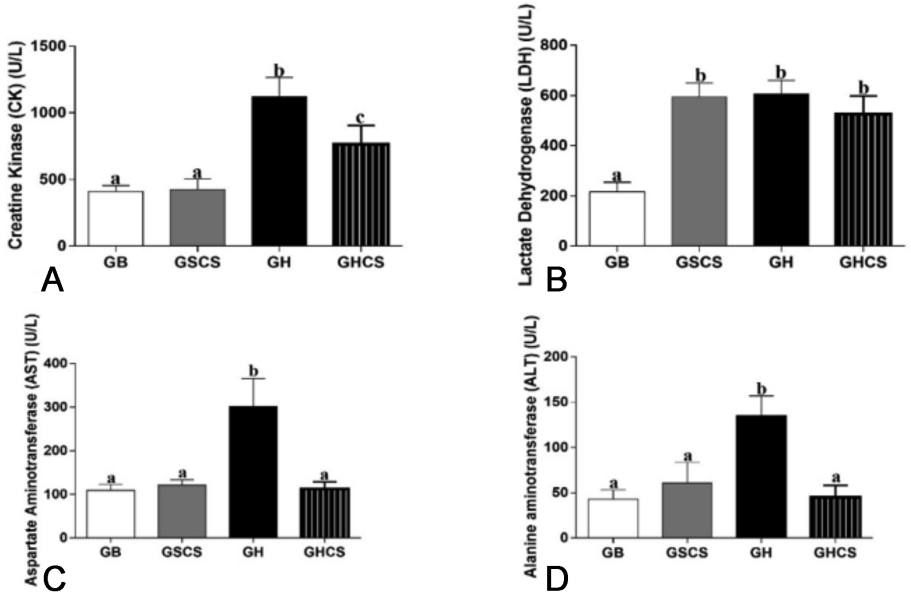

Fig.. 1. Plasma analysis of biochemical markers indicative of tissue damage. Different letters represent significant differences between groups and equal letters do not represent significant differences.

(Fig. 2C), the results showed increase in GSCS of 49.63 % compared to GB ($p<0.0001$). GH reduced 38.49 % compared to GSCS ($p<0.0001$). GHCS reduced 37.43 % compared to GSCS ($p<0.0001$). Finally, muscle MDA (Fig. 2D) showed increase of 25.35 % in GSCS compared to GB ($p<0.01$). GH increased 35.78 % compared to GB ($p<0.0001$) and GHCS reduced 19.41 % compared to GH ($p<0.01$).

Sulfhydryls were analyzed, demonstrating that in plasma (Fig. 3A), GSCS reduced 21.77 % compared to GB ($p<0.05$). GH increased 21.89 % compared to GB ($p<0.05$). GH also increased 55.81 % compared to GSCS ($p<0.0001$). GH increased 28.40 % compared to GHCS ($p<0.05$). In the liver (Fig. 3B), it increased 45.23 % in GH compared to the GB group ($p<0.05$). GH also increased by 80.58 % compared to GSCS ($p<0.001$). GH increased by 42.29 % compared to GHCS. In the cardiac tissue (Fig. 3C), it showed increase in GH of 309.54 % compared to GB ($p<0.0001$). GHCS increased 202.48 % compared to GB ($p<0.0001$). GH increased 242.82 % compared to GSCS ($p<0.0001$). GHCS increased 152.18 % compared to GSCS ($p<0.0001$). GH increased 35.41 % compared to GHCS ($p<0.01$). Finally, in muscle (Fig. 3D), it showed increase in GSCS of 46.15 % compared to GB ($p<0.01$), GH reduced 42.72 % compared to GSCS ($p<0.001$). GHCS reduced 55.52 % compared to GSCS ($p<0.001$).

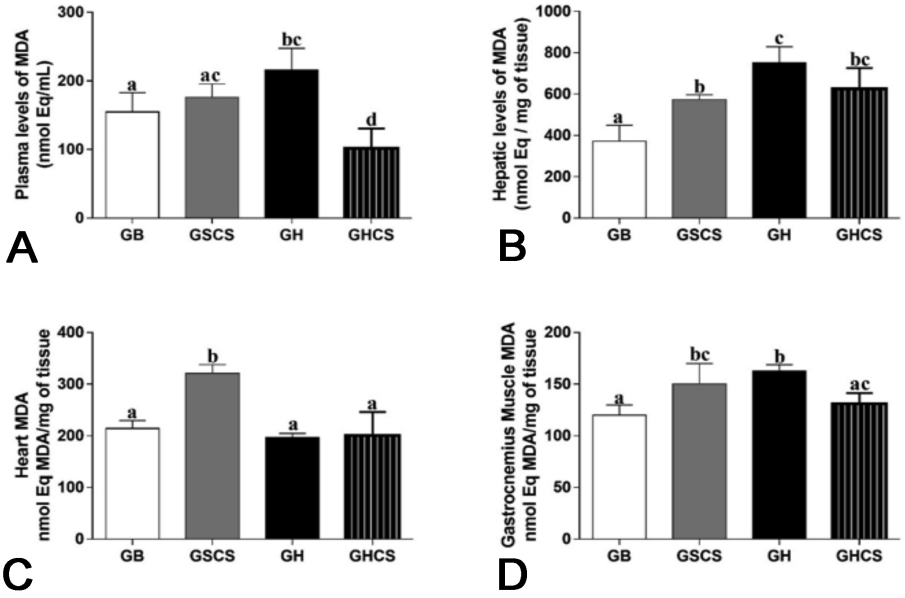

Fig. 2. Analysis of the MDA biomarker in blood plasma and in liver, cardiac and muscle tissues. Different letters represent significant differences between groups and equal letters do not represent significant differences.

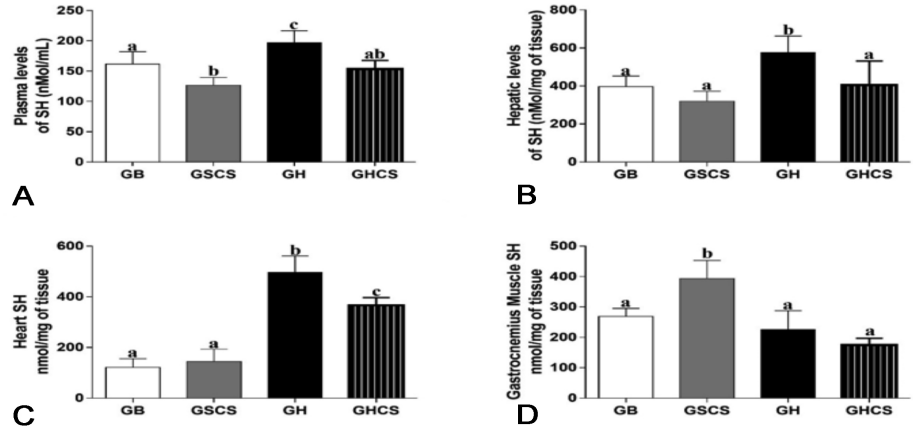

Fig. 3. Analysis of the SH biomarker in blood plasma and in liver, cardiac and muscle tissues. Different letters represent significant differences between groups and equal letters do not represent significant differences.

DISCUSSION

The present study was aimed at investigating the effects of high intensity interval training on oxidative and tissue damage in healthy rats supplemented or not with *Coutoubea spicata* fraction. Therefore, it was shown that the HIIT protocol induced oxidative stress in some tissues, as well as muscle and liver damage; however, *Coutoubea spicata* supplementation associated with HIIT showed attenuation of oxidative and tissue damage.

Data showed that the exercise groups (GH and GHCS) when compared to sedentary ones (GB and GSCS) showed increase in CK and LDH enzymes, indicating muscle damage after HIIT sessions. Other studies also reported increase in CK and LDH enzymes after strenuous exercise, both in rats and in humans, showing damage to muscle tissue after high-intensity exercise (Dos Santos *et al*., 2014; Cipryan *et al*.).

However, when GH and GHCS are compared, attenuation in CK levels in group supplemented with CS can be

observed, showing that the CS fraction reduced the muscle tissue damage induced by HIIT. Dos Santos *et al.* (2020) carried out a study with diabetic rats and identified that CK levels reduced after moderate-intensity physical exercise sessions associated with *Coutoubea spicata* supplementation. In addition, other studies carried out with supplementation rich in polyphenols observed reduction in muscle damage after high-intensity exercise (Bazzucchi *et al*., 2019; Massaro *et al*., 2019; Tung *et al*., 2019).

ALT and AST liver damage biomarkers also showed significant changes between groups. Exercised animals showed increase in AST and ALT after training sessions, corroborating studies by Suarsana *et al*. (2020) and Ramos *et al*. (2013), who used high-intensity training in rats and found increase in ALT and AST concentrations. However, it was evidenced through data that *Coutoubea spicata* supplementation associated with exercise promoted reduction in ALT and AST markers, indicating greater protection against liver tissue damage.

MDA is used as a lipid peroxidation biomarker, being a marker that reflects the increase of this substance causing the appearance of oxidative stress. In the present study, it was shown that HIIT induced oxidative stress in plasma and in muscle and liver tissues. Pillon Barcelos *et al*. (2017), emphasized that chronic exercise models increase liver-related stress. Dos Santos *et al*. (2014), Delwing-de Lima *et al*. (2018) and Souza *et al*. (2020) used a high-intensity exercise protocol in animals and identified that it induced oxidative stress, corroborating the findings of this study.

However, it was evidenced in results that after HIIT sessions, there was a significant increase in SH levels in plasma and in liver and cardiac tissues, demonstrating the antioxidant action of HIIT, providing redox balance. Thus, a review study carried out by de Sousa *et al*. (2017), indicated that physical training, regardless of type, intensity and volume, can promote redox balance. In this sense, a study by Oliveira *et al*. (2020) also showed increase in the redox status in trained rats.

Furthermore, in the present study, it was shown that *Coutoubea spicata* supplementation associated with HIIT provided reduction in oxidative stress markers in plasma and tissues. Studies have reported that chemical compounds present in *Coutoubea spicata* such as glycosides and flavonoids are effective in reducing oxidative stress (Dos Santos *et al*., 2020). The benefits of supplementation with flavonoid compounds in the reduction/attenuation of oxidative stress induced by high-intensity exercise are well reported in literature, being considered excellent natural antioxidants (Malaguti *et al*., 2013; Myburgh, 2014; Neves *et al*., 2018).

In conclusion, the findings of this study have shown that long HIIT sessions caused tissue, muscle and liver damage that could have been triggered by oxidative damage, indicated by oxidative stress markers. The association of the *Coutoubea spicata* fraction with HIIT considerably improved concentrations, demonstrating that the plant fraction would act as antioxidant, attenuating oxidative stress in Wistar rats. It is necessary to emphasize that our findings also indicate that the long HIIT protocol contributed to redox balance, showing that exercise provides positive adaptations in the redox metabolism.

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RESUMEN: Este estudio tuvo como objetivo evaluar los efectos de HIIT en los marcadores de daño tisular y oxidativo en ratas suplementadas con *Coutoubea spicata* durante seis semanas. Treinta y dos ratas Wistar macho se dividieron en 4 grupos: línea de base (GB); suplementados con 100 mg/kg de fracción de *Coutoubea spicata* (GSCS); ejercitados durante 6 semanas con el protocolo HIIT (GH); suplementado con 100 mg/kg de fracción de *Coutoubea spicata* + HIIT durante 6 semanas (GHCS). Los animales ejercitados realizaron el protocolo HIIT (2x2). Se analizaron los marcadores de daño tisular CK, LDH, ALT y AST en plasma, así como los biomarcadores de estrés oxidativo MDA y SH en plasma y en tejidos cardiaco, hepático y muscular. Los resultados indicaron que las enzimas CK, LDH, AST y ALT mostraron aumento en GH en comparación con GB ($p<0,0001$). Sin embargo, los marcadores CK, AST y ALT redujeron sus concentraciones en GHCS en comparación con GH ($p<0,0001$), lo que indica que la suplementación con *Coutoubea spicata* atenuó el daño en los tejidos musculares y hepáticos inducido por HIIT. La MDA de plasma, hígado y músculo mostró un aumento en la GH después de las sesiones de HIIT; sin embargo, en comparación con GHCS, mostró niveles reducidos ($p<0,0001$). Se observó SH elevado en el grupo de GH en comparación con GB en plasma y tejidos hepáticos ($p<0,0001$); en contraste, se observó una reducción en GHCS en comparación con GH en plasma, hígado y tejidos cardíacos, lo que demuestra el efecto redox de HIIT en algunos tejidos. Por lo tanto, nuestros hallazgos mostraron que *Coutoubea spicata* tiene actividad antioxidante, con reducción de los marcadores de daño oxidativo y, en consecuencia, el daño tisular en ratas Wistar sanas después del protocolo HIIT, pero además demostró el equilibrio redox después de analizar los marcadores de estrés oxidativo.

PALABRAS CLAVE: HIIT; ***Coutoubea spicata*****; Estrés oxidativo; Daño tisular.**

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