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Preventive Effect of Gamma-Oryzanol on Physiopathological Process Related to Nonalcoholic Fatty Liver Disease in Animals Submitted to High Sugar/Fat Diet

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Abstract: Nonalcoholic fatty liver disease (NAFLD) is the main cause of liver disease. The physiopathological processes involved in the disease are metabolic syndrome (MetS) components (central obesity, dyslipidemia, insulin resistance/type 2 diabetes, hypertension), genetic, and dietary factors, including unsaturated fats and sweetened beverages, which are able to lead to inflammation and oxidative stress, conditions associated with progression and severity of NAFLD. Gamma-oryzanol (γOz) is a nutraceutical obtained from rice brain oil with many benefits to health, from immunological to metabolic. The aim of this study is to test the preventive effect of γOz on the physiopathological process related to nonalcoholic fatty liver disease in animals submitted to high sugar/fat diet. Male Wistar rats (±187 g) were randomly divided into four experimental groups to receive: control diet (C, $n = 6$), control diet plus γOz (C + γOz, $n = 6$), high sugar/fat diet (HSF, $n = 6$), or high sugar/fat diet plus γOz (HSF + γOz, $n = 6$) during 30 weeks. HSF groups also received water plus sucrose (25%). γOz was added to diets to reach 0.5% of final concentration. The HSF group presented MetS, liver inflammation and oxidative stress, and micro and macrovesicular steatosis. HSF plus γOz was protected against these changes. It is possible to conclude that gamma-oryzanol was effective in modulating the physiopathological process related to nonalcoholic fatty liver disease in animals submitted to a high sugar/fat diet.

Keywords: nonalcoholic fatty liver disease; gamma-oryzanol; metabolic syndrome; bioactive compounds

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1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of liver disease. It refers to a spectrum that comprehends from simple steatosis to nonalcoholic steatohepatitis (NASH), which may evolve to cirrhosis and hepatocellular carcinoma (HCC) [1–3]. NAFLD is currently considered the hepatic manifestation of metabolic syndrome and affects around 25% of the worldwide population [1].

The literature reports a major association between NAFLD and metabolic syndrome (MetS) components, especially insulin resistance. Therefore, together with MetS, inflammation and oxidative stress are considered physiopathological processes involved in NAFLD progression, associated with worse hepatic lesion [4,5]. Some data from the literature demonstrate that the western dietary pattern, characterized by unsaturated fats and sweetened beverages [2], leads to molecular alterations that favor hepatic inflammation, oxidative stress, and lipogenesis, such as upregulation of sterol regulatory element-binding protein 1c (SREBP-1c) and factor nuclear kappa B (NF-κB) and downregulation of peroxi-

some proliferator-activated receptor-α (PPAR-α) and nuclear erythroid 2-related factor 2 (Nrf2) [6].

In this way, the interest in natural compounds and their effect in preventing or treating diseases has emerged. Gamma-oryzanol (γOz) is a nutraceutical obtained from rice bran oil and composed of a mixture of ferulic acid esters of phytosterols and triterpenoids, cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate [7]. The positive effects of γOz include antioxidant and anti-inflammatory action, effects on the immune system, and treatment or attenuation of dyslipidemia, diabetes, obesity, and neurological disorders, as demonstrated in preclinical experiments, models, and observations [8–11]. However, there is a lack of studies about the effects of γOz as a possible modulator of the physiopathological process involved in NAFLD. Thus, the aim of this study is to test the preventive effect of γOz on the physiopathological process related to nonalcoholic fatty liver disease in animals submitted to a high sugar/fat diet.

2. Methods

2.1. Experimental Protocol

Male Wistar rats (±187 g) were randomly distributed into 4 experimental groups to receive: control diet (C, n = 6), control diet + γOz (C + γOz, n = 6), high sugar/fat diet (HSF, n = 6), or high sugar/fat diet + γOz (HSF + γOz, n = 6) during 30 weeks. HSF diet groups also received water + sucrose (25%). Figure 1 presents the experimental protocol of this study. The animals were kept in an environmental controlled room in accordance with the National Institute of Health's Guide for the Care and Use of Laboratory Animals. The experiment was approved by the Animal Ethics Committee of Botucatu Medical School (1310/2019). At the end of the of 30 weeks, the animals were fasted for 8 h, anesthetized (thiopental 120 mg/kg/i.p.), and euthanized. Then, blood samples were collected, and plasma was obtained. The adipose tissue was dissected and weighed for nutritional parameters assessment. The liver was excised and fractionated in aliquots for histological analysis or stored in a freezer at −80 °C until further analysis.

The consumption of diets and water was ad libitum. Both diets used in this study were previously published by our research group [12]. Table 1 presents the nutritional values of each diet.

Table 1. Nutritional values of control and HSF diets.

Nutritional Values		
Protein (% of ingredients)	20.0	18.0
Carbohydrate (% of ingredients)	60.0	53.5
Fat (% of ingredients)	4.00	16.5
% of unsaturated	69.0	47.0
% of saturated	31.0	53.0
% Energy from protein	22.9	16.6
% Energy from carbohydrate	66.8	49.2
% Energy from fat	10.4	34.2
Energy (kcal/g)	3.59	4.35

HSF: high sugar/fat diet.

2.2. Gamma-Oryzanol

The isolated compound was purchased from Tokyo Chemical Industry Co., Ltd. (Toshima, Kita-ku, Tokyo, Japan) (lot.5ZZYLPJ) and added to the diets at 0.5% of final concentration (w/w) based on previous works from our research group [12]. This dose mimics the daily rice consumption of an adult individual in Brazil (100 g/day), according to data from the Family Budget Survey (POF) 2008–2009 [13].

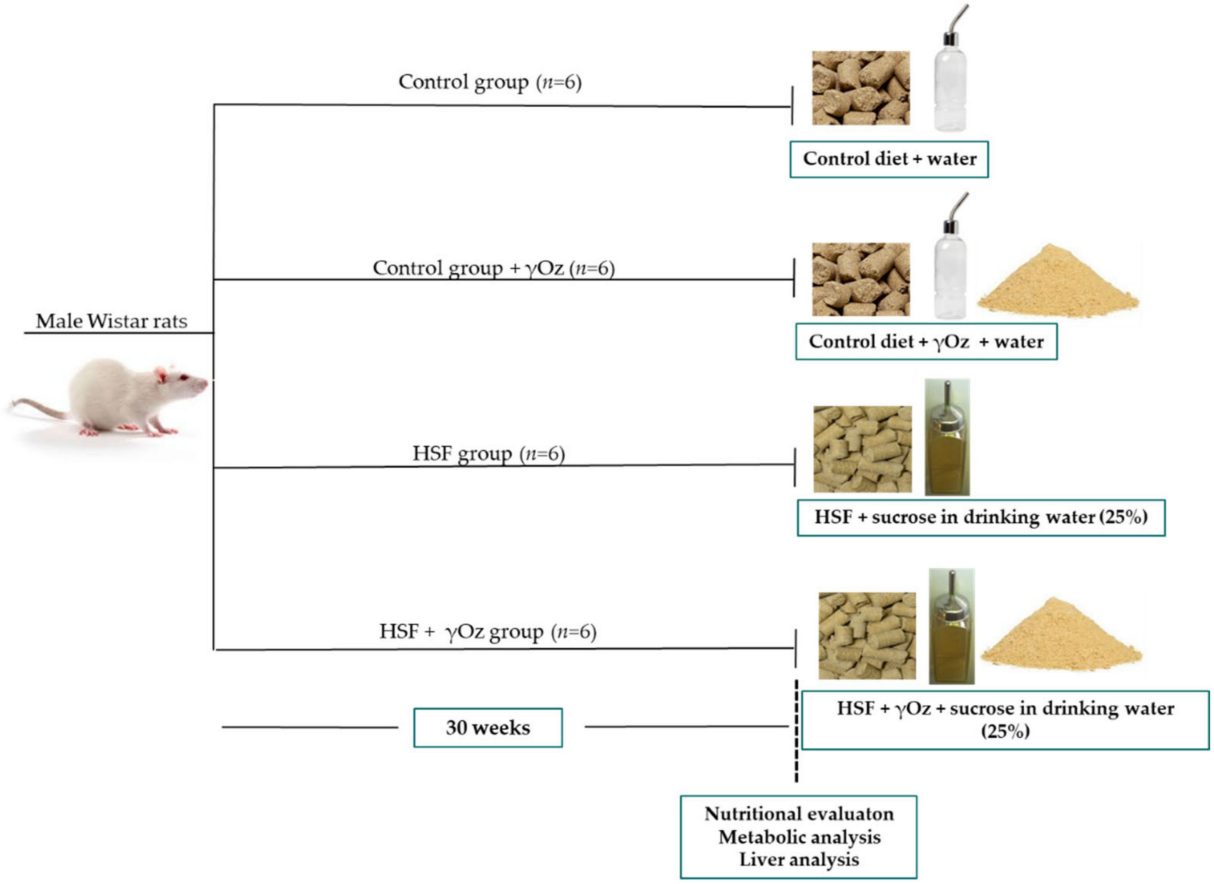


Figure 1. Experimental protocol. Animals were fed during 30 weeks. Control groups received control diet plus water; HSF groups received high sugar/fat diet plus sucrose in drinking water (25%). Nutritional evaluation and metabolic and liver analysis occurred at the end of 30 weeks. HSF—high sugar/fat diet; γOz—gamma-oryzanol.

2.3. Nutritional Parameters

The nutritional evaluation performed in this study considered: chow fed, water intake, caloric intake, final body weight, and adiposity index. Chow and water intake considered the animals' daily leftovers. Caloric intake was determined by multiplying the energy value of each diet (g × Kcal) by the daily food consumption. For the HSF group, caloric intake also considered the calories from water with sucrose (0.25 × 4 × mL consumed). Body weight was measured weekly. To estimate animal body fat, the adiposity index (AI) was used and obtained by the formula: [(VAT + EAT + RAT)/FBW] × 100 [10].

2.4. Metabolic and Hormonal Analysis

Glycemic profile was evaluated by glycemia, insulin plasmatic level, and by the homeostatic model of insulin resistance (HOMA-IR). Glycemia was determined in a blood drop using a glucometer (Accu-Chek Performa; Roche Diagnostics Brazil Limited, São Paulo, Brazil). The insulin level was measured using the enzyme-linked immunosorbent assay (ELISA) method (EMD Millipore Corporation, Billerica, MA, USA) and the HOMA-IR was calculated by the following formula: HOMA-IR = (fasting glucose (mmol/L) × fasting insulin (μU/mL))/22.5 [10].

Triglycerides plasmatic level was measured by colorimetric method (BIOCLIN®, Belo Horizonte, MG, Brazil) analyzed by Chemistry Analyzer BS-200 (Mindray Medical International Limited, Shenzhen, China).

2.5. Liver Evaluation

2.5.1. Preparation of Liver for Analysis

One hundred milligrams of hepatic tissue were homogenized in 1.0 mL of Phosphate-Buffered Saline (PBS), cold solution, pH 7.4, with Sigmafast Protease Inhibitor cocktail tablets (Sigma, St. Louis, MO, USA) using an ULTRA-TURRAX T25 basic IKA Werke Staufen/Germany. After, the samples were centrifuged at 800× *g* at 4 °C for 10 min. The supernatant was used to analyze inflammatory markers, MDA, and protein carbonylation, as follows. All the results were normalized by the total protein content, which was determined using a colorimetric method (BioClin, Quibasa Química Básica Ltda., Belo Horizonte, MG, Brazil), and the readings were performed in a microplate reader.

2.5.2. Inflammatory Markers

Tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) levels were assessed by ELISA method (R&D System, Minneapolis, MN, USA).

2.6. Oxidative Stress Markers

Malondialdehyde Levels (MDA)

MDA level was used to estimate lipid peroxidation as previously described [10]. The reading was performed on Spectra Max 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 535 nm. The MDA concentration was obtained by the molar extinction coefficient (1.56×10^5 M^{-1} cm^{-1}) and expressed in nmol/g protein [14].

2.7. Protein Carbonylation

Carbonylated proteins were measured by DNPH (2,4-dinitrophenylhydrazine) method [15]. The absorbance was set at 450 nm. The results were calculated with molar extinction coefficient (22,000 M^{-1} cm^{-1}) of DNPH and expressed as nmol/mg protein. Carbonylated proteins levels are expressed in nmol of DNPH/mg of protein.

2.8. Histological Analysis

Hepatic tissue was stored in 4% paraformaldehyde with 0.1 M PBS (pH 7.4) during the first 24 h. After, tissue was transferred to 70% ethyl alcohol until paraffin waxing. Histological sections obtained from the paraffin block were laid on slides and stained with hematoxylin and eosin (H&E). Macrovesicular and microvesicular steatosis were both separately scored, and the severity was graded based on the percentage of the total area affected. The scores were: 0 (<5%), 1 (≥5–25%), 2 (≥25–50%), and 3 (>50%). The difference between macrovesicular and microvesicular steatosis was defined by whether the vacuoles displaced the nucleus to the side (macrovesicular) or not (microvesicular) [16]. Ten fields were analyzed per slide per animal. For the statistical analysis, it was considered the sum of the scores obtained in the 10 analyzed fields.

2.9. Hepatic Triglycerides Levels

Intracellular hepatic triglyceride levels were determined by colorimetric method (Triglyceride Colorimetric Assay Kit; Cayman Chemical, Ann Arbor, MI, USA) [17]. The results were corrected by the amount of protein.

2.10. Statistical Analysis

Results are expressed as mean ± standard deviation or median and interquartile range. Differences among the groups were determined by two-way analysis of variance. Parametric variables were subjected to the Tukey post-hoc test to compare all the groups. Non-parametric variables were compared by Kruskal–Wallis post-hoc test. For the histological parameters, the Poisson distribution or the binomial distribution followed by the post-hoc test Wald multi-comparison were used. These statistical analyses were performed using the software Statistical Analysis System (SAS) 9.4 (SAS Institute Inc., Campus

Drive Cary, NC, USA) by an experienced statistician. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Nutritional Intake

Figure 2 shows the parameters related to nutritional intake. It is possible to note that HSF and HSF + γOz groups presented lower chow fed compared to control and control plus γOz, respectively (Figure 2A). At the same time, it is possible to observe a higher water intake in both HSF and HSF plus γOz groups compared to respective control groups (Figure 2B). There was no difference for caloric intake among the groups (Figure 2C). In addition, there is no interference of γOz in chow, water, and calorie intake because these parameters were similar between HSF and HSF plus γOz groups (Figure 2A–C).

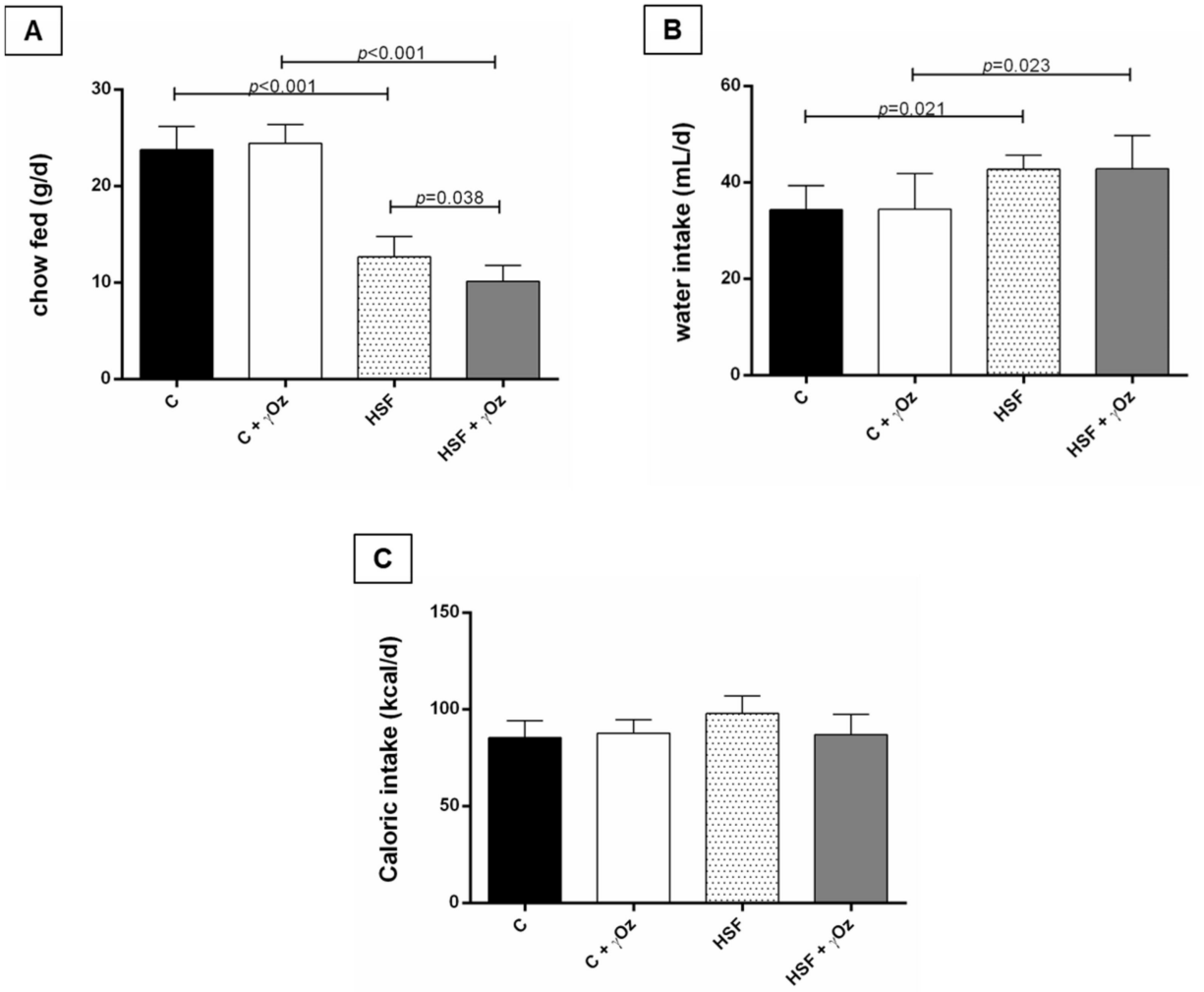


Figure 2. Nutritional intake. (**A**) Chow fed (g/d); (**B**) Water intake (mL/d); (**C**) Caloric intake (kcal/d). Comparison by two-way ANOVA followed by Tukey test. $p < 0.05$ as significant.

3.2. Metabolic Syndrome Parameters

The MetS parameters are presented in Figure 3. It notes that the HSF diet used in this study promoted obesity and significant metabolic and hormonal changes. The HSF group presented increased final body weight (Figure 3A) and adiposity index (Figure 3B) compared to the control group. In opposition, γOz treatment prevented these conditions in the HSF plus γOz group. Although plasma glucose (Figure 3D) had remained equal among the groups, it is possible to observe an increase in insulin levels (Figure 3E), characterizing

insulin resistance, which is confirmed by the HOMA-IR values (Figure 3F). Insulin resistance was prevented in the HSF plus γOz group. Dyslipidemia (Figure 3C) was another condition noted in the HSF group and attenuated in the HSF plus γOz group, demonstrating a positive effect of the compound. Systolic blood pressure was higher in HSF and HSF plus γOz groups compared to control and control plus γOz groups, respectively. No effect of the compound was noted in this parameter.

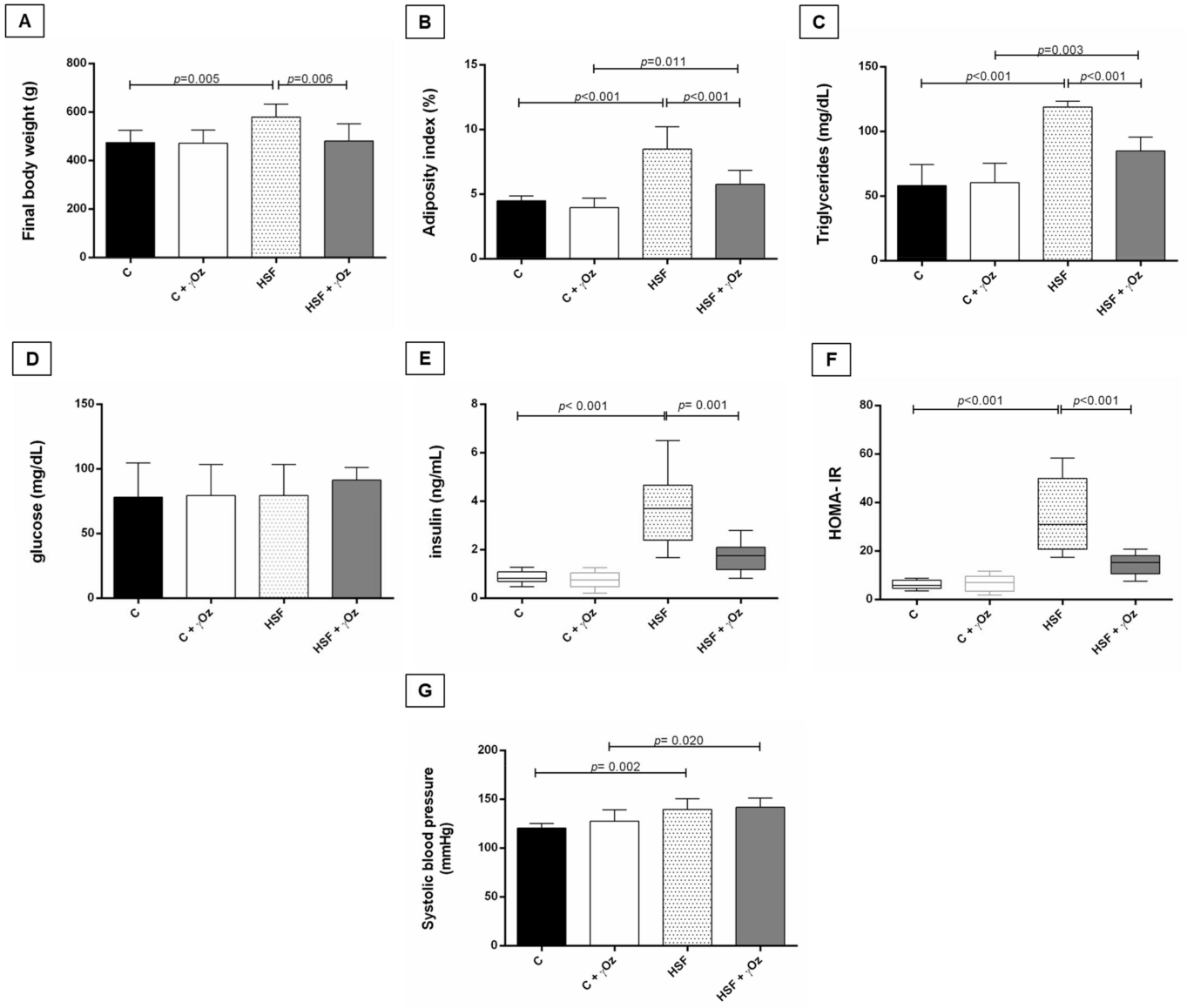


Figure 3. Metabolic syndrome parameters. (**A**) Final body weight (g); (**B**) Adiposity Index (%); (**C**) Triglycerides (mg/dL); (**D**) Glucose (md/dL); (**E**) Insulin (ng/mL); (**F**) HOMA-IR; (**G**) Systolic blood pressure (mmHg). Comparison by two-way ANOVA followed by Tukey or Kruskal–Wallis test. $p < 0.05$ as significant.

3.3. Oxidative Stress and Inflammatory Parameters

Inflammation and oxidative stress are important obesity-related conditions and associated with the worst hepatic consequences and the results can be verified in the Figure 4. Regarding hepatic inflammation, evaluated by TNF-α and IL-6, it is possible to observe that these cytokines were increased in the HSF group compared to the control group. Although IL-6 was higher in the HSF plus γOz group compared to the control γOz group, the compound was effective in attenuating the rise in relation to the HSF group, demonstrating

a positive effect of γOz mitigating the elevation of this cytokine. The compound used in this study was also effective to mitigating the elevation of TNF-α levels compared to the HSF group (Figure 4A,B).

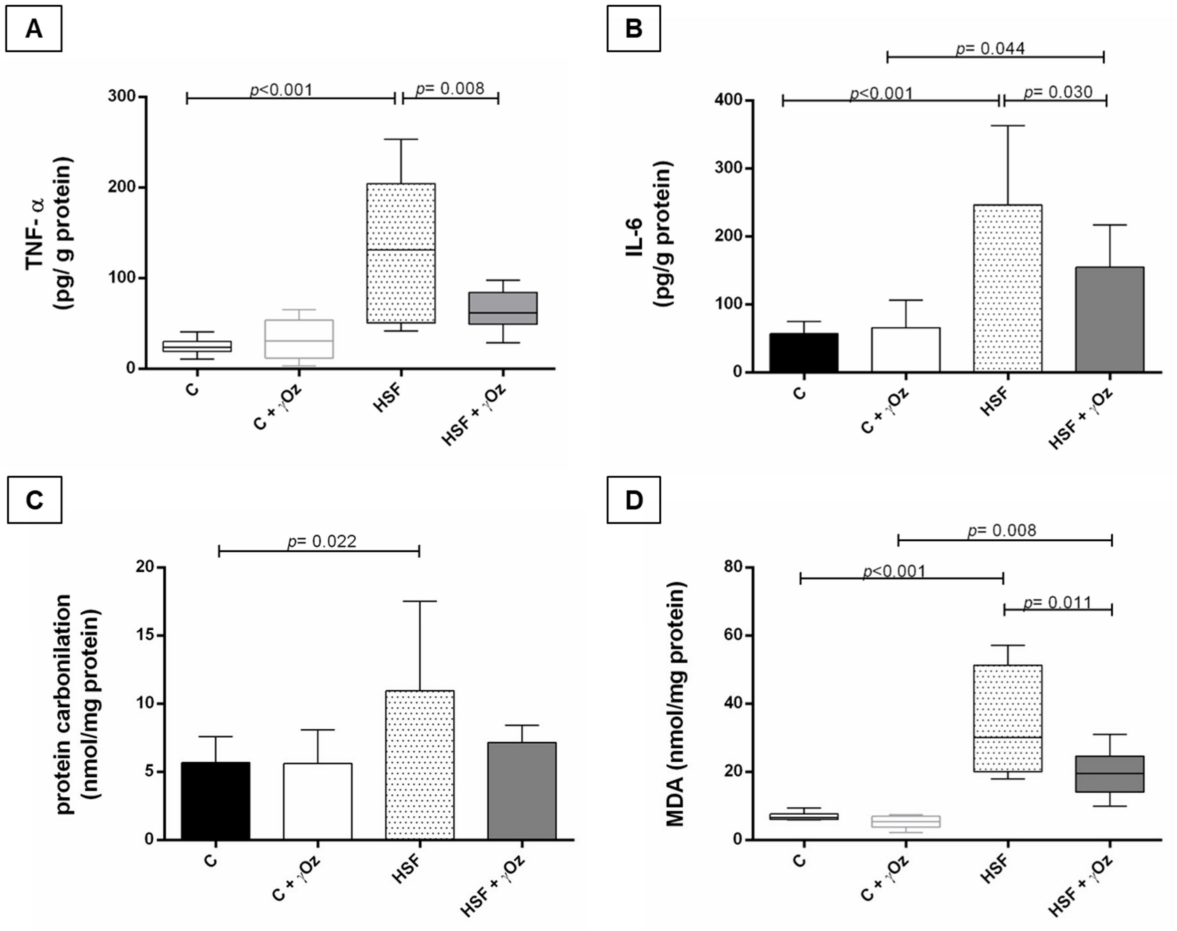


Figure 4. Oxidative stress and inflammatory parameters. (**A**) Tumoral necrosis factor alpha (TNF-α, pg/g protein); (**B**) Interleukin-6 (IL-6, pg/g protein); (**C**) Protein carbonylation (nmol/mg protein); (**D**) Malondialdehyde (MDA, nmol/mg protein). Comparison by two-way ANOVA followed by Tukey test. $p < 0.05$ as significant.

Oxidative stress was evaluated by MDA, a marker of lipid peroxidation, and by carbonylated proteins (Figure 4C,D). The HSF group presented increased oxidative stress, observed by higher levels of MDA and protein carbonylation compared to the control group. Although MDA was increased in the HSF plus γOz group compared to the control plus γOz group, the levels were reduced in comparison to the HSF group, demonstrating a positive effect of γOz to attenuate lipid peroxidation.

3.4. Hepatic Analysis

The hepatic histological analysis considered the presence of micro and macrovesicular steatosis and it is presented in Figure 5. It notes that the HSF presented an increased score for micro and macrovesicular steatosis compared to the control group. The increased score for macrovesicular steatosis was noted in the HSF plus γOz group compared to the control plus γOz group. The positive effect of γOz is evidenced in microvesicular steatosis, because the compound mitigated the fat deposit compared to the HSF group. The HSF group also presented increased hepatic triglycerides levels compared to the control group, whereas the levels in the HSF plus γOz group were lower in relation to HSF.

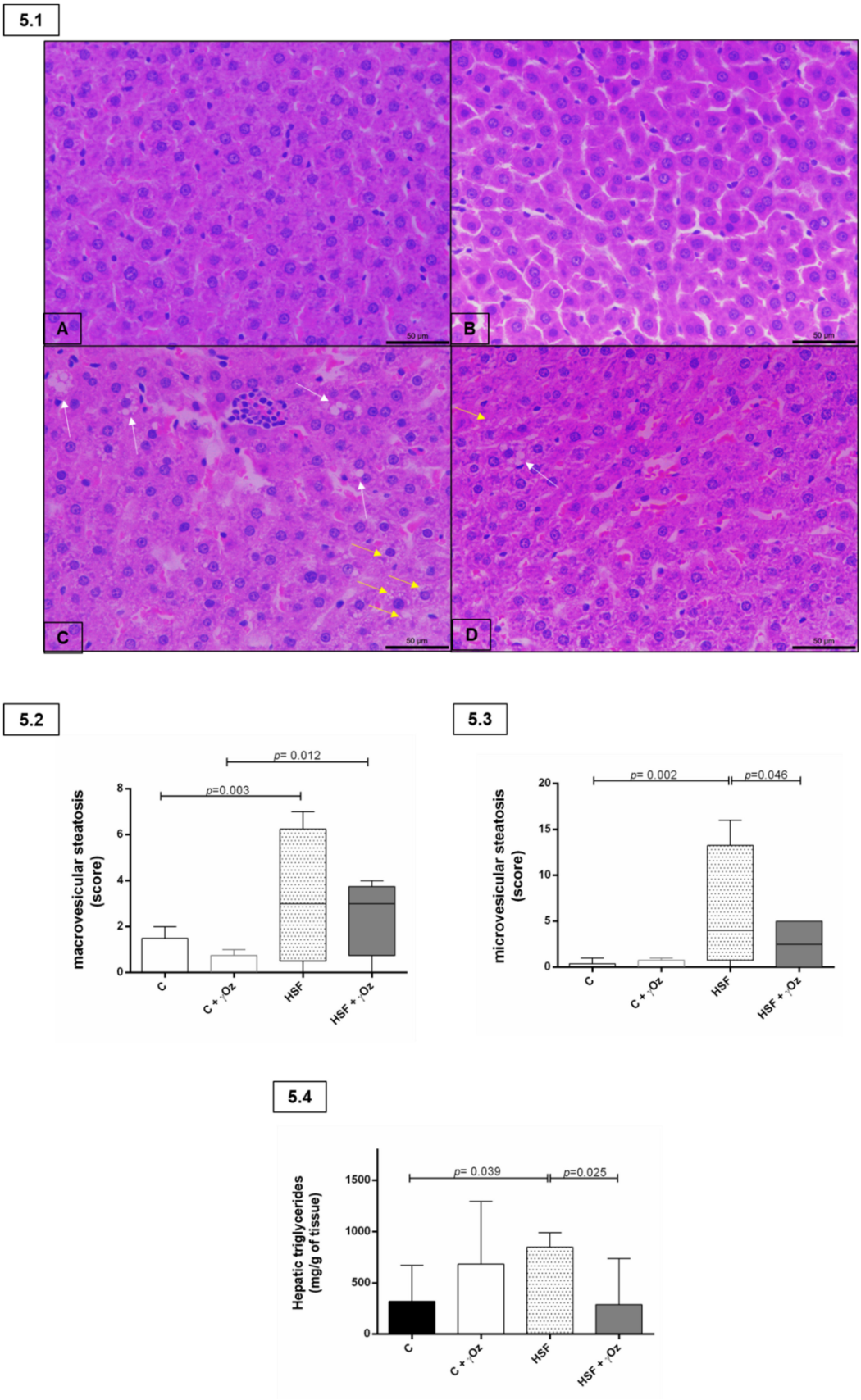


Figure 5. Hepatic steatosis analysis. (**5.1**) Histology of liver tissue using Hematoxylin and Eosin. (**A**)—Illustrative image (40× magnification) of control group. (**B**)—Illustrative image (40× magnification)

of control group + γOz. (**C**)—Illustrative image (40× magnification) of HSF group. (**D**)—Illustrative image (40× magnification) of the HSF + γOz group. (**5.2**) Microvesicular steatosis. (**5.3**) Macrovesicular steatosis. (**5.4**) Hepatic triglycerides levels (mg/g of tissue). Yellow arrows indicate microvesicular steatosis (**5.1** (**C**,**D**)), and white arrows indicate macrovesicular steatosis (**5.1** (**C**,**D**). γOz- gamma-oryzanol. Microvesicular and macrovesicular steatosis analyzed by Poisson distribution followed by Wald multiple comparison test.

4. Discussion

This study aimed to evaluate the preventive effect of γOz on the physiopathological process related to nonalcoholic fatty liver disease in animals submitted to a high sugar/fat diet. According to the literature, the main NAFLD physiopathological aspects include metabolic syndrome, inflammation, and oxidative stress [18]. Our results corroborate the literature because the group fed with a high sugar/fat diet presented obesity, dyslipidemia, insulin resistance, together with hepatic inflammation and oxidative stress, and micro and macrovesicular steatosis. In opposition, the HSF group treated with γOz was protected against obesity, dyslipidemia, insulin resistance, hepatic inflammation, and oxidative stress and microvesicular steatosis, demonstrating a positive effect of this compound.

According to the World Health Organization (WHO), obesity is defined as the abnormal or excessive fat accumulation that may compromise health. From 1975 to 2016, the number of obese adults in the world almost tripled, reaching more than 1.9 billion people. Among obesity causes, the literature reports that the positive energetic balance, which is the result of the association between sedentarism and higher consumption of high-caloric diets rich in sugars and fats, is the main one [19]. In this study, it was possible to note that the HSF diet developed by our research group, which mimics Western eating habits, promoted obesity. In contrast, the HSF plus γOz group was protected against obesity development. Although the compound action mechanism is not fully clarified, a recent study published by our group [20], around the same time that the study was published by Jung et al. [21], showed that γOz was able to increase proliferator-activated gamma receptor (PPAR-γ) gene expression in adipose tissue, which may explain obesity attenuation, because this nuclear receptor is involved in the expression of several genes associated with lipogenesis and adipocyte differentiation.

Under positive energy balance conditions, hypertrophy processes occur in adipose tissue in order to store excessive energy. However, when hypertrophied, adipocytes increase the production of chemotactic and inflammatory factors, resulting in a local inflammatory process that can later affect other organs [22]. In this sense, the literature has reported that the obesity-related inflammatory process seems to be the main condition associated with insulin resistance, hypertension, and dyslipidemia development [23–25]. However, because the animals of the HSF plus γOz group presented a lower adiposity index and final weight compared to HSF group, the antiobesogenic effect of γOz seems to have protected against metabolic syndrome manifestation. It is also noteworthy that the compound has anti-inflammatory effects, contributing beneficially to attenuating obesity-related disorders [26].

The liver is one of the several organs affected by obesity. This organ is responsible for several functions in the body, such as blood filtration and storage, bile secretion, vitamins and minerals storage, and metabolism of carbohydrates, proteins, and fats [27]. However, dietary factors and obesity-related disorders may contribute to inflammation in hepatic tissue, which may compromise its function. Some mechanisms are a trigger for this hepatic inflammatory condition, among them: increased lipolysis due to insulin resistance and adipokines and pro-inflammatory cytokines released from adipose tissue, among them, TNF-α and IL-6. These conditions in the liver collaborate for an increase in de novo lipogenesis, which can generate triglycerides, fatty acids, and other lipid metabolites accumulation, leading to liver oxidative stress due mitochondrial dysfunction and inflammation [28]. This inflammatory condition can also be the link between the manifestation of Metabolic Associated Fatty Liver Diseases and obesity. Thus, our data corroborate the literature, because the HSF group presented insulin resistance, obesity, and increased expression of IL-6 and

TNF-α. On the other hand, the HSF plus γOz group that received the compound along with the HSF diet presented, at the end of the experiment, insulin resistance attenuation, lower plasma insulin and triglycerides levels, and lower hepatic values of IL-6 and TNF-α. These results confirm the positive effect of gamma oryzanol on obesity-related disorders and the reduction in inflammation [11,20,29].

Oxidative stress plays a key role in NAFLD. Increased plasma free fat acids (FFAs) are taken up by the liver, increasing hepatic β-oxidation rate, which results in higher hepatic reactive oxygen species (ROS) production. Moreover, insulin resistance can also stimulate the release of ROS by upregulating microsomal lipid peroxidation and by downregulating mitochondrial β-oxidation. As a result, malondialdehyde (MDA) generation occurs, a lipid peroxidation by-product, able to activate inflammatory response and, consequently, cause cellular damage [30]. Protein carbonyl groups are considered early markers of proteins altered by oxidative stress because they may be introduced in proteins during lipid peroxidation by the reaction of nucleophilic side chains of cysteine, histidine, and lysine residues with aldehydes. The presence of oxidated proteins surrounding lipid droplets may be an important contributor to hepatocytes dysfunction in fatty liver disease [31]. Our data shows that oxidative stress was presented in the HSF group that presented higher hepatic levels of MDA and protein carbonyl. In opposition, HSF plus γOz presented lower MDA compared to HSF, showing a protector against lipid peroxidation. The free radical scavenging action of γOz as well as its preventative nature against lipoperoxidation offer it as a viable contender for natural use as an antioxidant [8].

The difference between macrovesicular and microvesicular steatosis is not limited to the histological characteristic. Macrovesicular steatosis is defined as large or small lipid droplets present in the cytoplasm with nucleus dislocation which is, alone, associated with a good prognosis with rare progression to fibrosis or cirrhosis. On the other hand, the microvesicular steatosis, which is characterized by the innumerable lipid droplets accumulation with a centrally placed nucleus, is a serious condition associated with fibrosis, cholestasis, necrosis, and impairment of the mitochondrial fatty acid oxidation [3]. Macrovesicular was noted in both HSF and HSF plus γOz with no effect of the compound. Even with no statistical effect of γOz, it is possible to note a numerical reduction in the HSF plus γOz score, indicating a possible beneficial effect on liver steatosis. Microvesicular steatosis was also presented in the HSF group; however, it was attenuated in the HSF plus γOz group compared to HSF, demonstrating an important protective effect of gamma-oryzanol.

In summary, this study showed that gamma-oryzanol mitigated obesity, insulin resistance, dyslipidemia, liver inflammation, and oxidative stress. It is possible to conclude that gamma-oryzanol was effective in modulating the physiopathological process related to nonalcoholic fatty liver disease in animals submitted to a high sugar/fat diet.

Author Contributions: Conceptualization: F.V.F.-F., J.P.d.C.S. and C.R.C.; Data curation: F.V.F.-F., J.P.d.C.S., A.J.T.F., F.M. and C.R.C.; Formal analysis: F.V.F.-F., A.J.T.F. and C.R.C.; Funding acquisition: C.R.C.; Methodology: F.V.F.-F., J.P.d.C.S., J.L.G., A.J.T.F., C.C.V.d.A.S., M.R.C., H.T.K., G.A.N. and F.M.; Project administration: C.R.C.; Supervision: F.V.F.-F. and C.R.C. Writing—original draft: F.V.F.-F., A.J.T.F. and C.R.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The experiment was approved by the Animal Ethics Committee of Botucatu Medical School (1310/2019).

Informed Consent Statement: Not applicable.

Data Availability Statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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