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ZINC SUPPLEMENTATION REDUCES DIET-INDUCED OBESITY AND IMPROVES INSULIN SENSITIVITY IN RATS

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ABSTRACT

Rates of obesity have been growing at alarming rates, compromising the health of the world population. Thus, the search for interventions that address the metabolic repercussions of obesity are necessary. Here we evaluated the metabolic and antioxidant effects of zinc and branched-chain amino acid (BCAA) supplementation on obese rats. Male Wistar rats were fed with high-fat/high-fructose diet (HFD) or standard diet (SD) for 19 weeks. From the 15th week until the end of the experiment, HFD and SD-fed rats received zinc (6mg/kg) or BCAA (750mg/kg) supplementation. Body weight, abdominal fat, lipid profile, blood glucose, insulin, leptin, and hepatic transaminases were evaluated. In the liver, superoxide dismutase and catalase activities and lipid peroxidation were also analyzed. HFD-fed animals showed increased weight gain, abdominal fat pad, plasma insulin, leptin, and triglycerides levels in comparison to SD-fed rats. Zinc supplementation reduced all these parameters, suggesting a beneficial role for the treatment of obesity. BCAA, on the other hand, did not show any beneficial effect. Liver antioxidant enzymes and hepatic transaminases plasma levels did not change among groups. Lipid peroxidation was higher in HFD-fed rats and was not reverted by zinc or BCAA supplementation. In conclusion, zinc supplementation may be a useful strategy for the treatment of the metabolic dysfunction associated with obesity.

Key words: branched-chain amino acid, high-fat/high-fructose diet, insulin resistance, leptin, oxidative stress, zinc

INTRODUCTION

Obesity is a chronic multifactorial disease associated with several comorbidities and premature mortality in the population (Kyrgiou et al. 2017; Tchernof and Despres 2013). Overweight and obesity rates have reached epidemic proportions worldwide becoming a challenge to public health systems (Report 2017; Swinburn et al. 2011; Tappy and Le 2010; WHO and Organization 2016). Studies in this field are necessary to provide new strategies for treating obesity.

Consumption of a hypercaloric diet based on industrialized food products is the main cause of obesity nowadays (Perez-Escamilla et al. 2012; Rouhani et al. 2016). Moreover, the high amount of fructose contained in sweetened beverages, such as soft drinks, is also strongly associated with obesity (Hu 2013; Tappy and Le 2010).

The monosaccharide fructose favors the accumulation of triglycerides and free fatty acids in the liver, as well as in the blood, and it leads to insulin resistance. Fructose is also a regulator of hepatic glucose uptake and glycogen synthesis (Basciano et al. 2005; McGuinness and Cherrington 2003; Tappy and Le 2010). A high and chronic consumption of fructose (Basciano et al. 2005; McGuinness and Cherrington 2003) activates classical inflammatory pathways, such as nuclear factor kappa B (NFkB), which contribute to an overproduction of reactive oxygen species. Oxidative stress and inflammation converge, in turn, to outcomes associated with insulin resistance (Dekker et al. 2010).

Zinc (Zn) is the second most abundant essential trace micronutrient found in the human body. It is found in all human tissues playing catalytic or structural functions. Muscles and bones contribute with about 90% of zinc storage whilst 5% is stored in the liver (Mangray et al. 2015). As an enzymatic cofactor, zinc participates in the regulation of metabolism of carbohydrates, fats,

and proteins. It is also required for the activity of more than 200 metalloenzymes such as the antioxidant enzyme copper zinc superoxide dismutase (Haase and Rink 2009; Mangray et al. 2015).

Insulin secretion is dependent on the zinc influx through the zinc transporter ZnT8. Thus, dysfunction of zinc signaling may be involved in the pathogenesis of diabetes mellitus (Nicolson et al. 2009). On the other hand, zinc supplementation protected against apoptosis and reduced liver damage in patients with chronic hepatitis C or compensated cirrhosis (Fan et al. 2017; Matsuoka et al. 2009; Sun et al. 2015).

Valine, leucine, and isoleucine constitute the three branched-chain amino acids (BCAA). These are essential amino acids, which participate in the regulation of body protein balance and insulin secretion, modulation of the immune system, and reduction of muscle injury post-exercise (Fan et al. 2017; Holecek 2018). Elevated blood levels of BCAA are found in obesity and type 2 diabetes mellitus (Batch et al. 2014; Newgard et al. 2009), making them putative biomarkers of these metabolic diseases (Siomkajlo et al. 2017). In contrast, a population-based study showed that a higher BCAA intake was associated with a lower prevalence of overweight and obesity (Qin et al. 2011).

BCAA treatment also provides beneficial effects in non-alcoholic fatty liver disease, mainly in the resolution of insulin resistance associated with this condition (Lake et al. 2015). Using an animal model of non-alcoholic steatohepatitis, (Tanaka et al. 2016)) showed BCAA acts as an antioxidant, reducing oxidative stress and suppressing progression of this disease.

Despite advances in the understanding of the mechanisms involved in the pathophysiology of obesity, treatment of this condition is still undefined. Weight loss strategies are not always effective. Management of obesity involves changes in eating habits, physical activity, or even administration of some drugs and surgical intervention. However, a co-adjuvant

treatment is still necessary, as living habits are not easy to change. Zinc and BCAA are used in the treatment of metabolic dysfunction that occurs in cirrhosis, hepatitis, and nonalcoholic steatohepatitis (NASH). Hepatic steatosis caused by obesity is an initial stage of steatohepatitis and hepatic fibrosis, thus, zinc and BCAA treatments may be useful to prevent the evolution of steatohepatitis. Therefore, the aim of the present study was to investigate the effect of zinc or BCAA supplementation on metabolic and oxidative parameters of obese rats that consumed a high fructose/high fat diet (HFD).

METHODS

Animals and treatments

A total of 48 adult male Wistar rats, weighing 150-200g, were obtained from the Animal Facility of the Universidade Federal de Ciências da Saúde de Porto Alegre. The animals were kept in plastic boxes, with two animals per box, in an average temperature of 22 °C and a light and dark cycle of 12 hours. The study was approved by the Institutional Animal Care and Use Committee of the Universidade Federal de Ciências da Saúde de Porto Alegre (#359/16).

Rats were randomly assigned to each of eight experimental groups (n = 6/group): standard diet + vehicle (SD); SD + zinc (SD+Zn); SD+BCAA; HFD + vehicle (HFD); HFD+Zn and HFD+BCAA.

The standard diet had a caloric content of 3.4 kcal/g (63% carbohydrates, 11% lipids, and 26% proteins, NUVITAL, Brazil). The HFD had a caloric content of 4.5 kcal/g (35.7% carbohydrates, 19.2% proteins, and 45.1% lipids, Pragsoluções Biociências, Brazil). A solution containing 42g/L of fructose was prepared with 55% fructose and 45% sucrose (Synth, Brazil). Diets were administrated for 19 weeks and body weight was recorded weekly. Animals received Zn or BCAA supplementation daily from the 15th week until the end of the experiment (at the

19th week), and the SD+vehicle and HFD+vehicle groups received water by gavage for the same period. Zinc and BCAA were diluted in water and administered by gavage, at the doses of 6mg/kg and 750mg/kg, respectively.

Blood and plasma samples

The animals were fasted for 6 hours and the euthanasia occurred at 1 pm. Animals were anesthetized with 50 mg/kg of xilasin hydrochloride (Rompum®) and 100 mg/kg of ketamine hydrochloride (Ketalar®). Blood was collected through the retro-orbital plexus and placed in a heparin test tube (Liquemine®) immediately after the animals were anesthetized, right before the euthanasia. The blood was centrifuged at 2000 x g for 15 minutes at 4 °C and the plasma was stored at -20 ºC.

Tissue samples

Animals were euthanized by overdose of anesthetics. The final measurement of the naso-anal length was performed in order to calculate the Lee index (body weight$^{1/3}$/naso-anal length x 1000), which is correlated to the body mass index in humans (Bernardis and Patterson 1968). After ventral laparotomy, the liver and abdominal fat were removed. A liver sample was frozen in liquid nitrogen and stored at -80 °C for posterior analysis.

Histopathological analysis

A portion of the right lobe of liver was fixed in 10% formaldehyde, included in paraffin, and sectioned using a microtome (5 µm thickness). Slices were stained with hematoxylin and eosin and the histopathological analysis was performed by an experienced pathologist blind to the experimental groups.

Biochemical parameters

- Blood glucose and plasma lipid profile

Enzymatic colorimetric kits were used for measurements of glucose (Labtest, Brazil), triglycerides (Bioclin, Brazil), total cholesterol (Bioclin, Brazil), and high-density lipoproteins (HDL) (Biotécnica, Brazil). Low-density lipoproteins (LDL) were calculated by the equation: total cholesterol - high density lipoproteins - (triglycerides/5).

- Hepatic aminotransferases

Plasma alanine aminotransferase (ALT) and serum aspartate aminotransferase (AST) were analyzed by a kinetic colorimetric assay.

- Plasma leptin and insulin

Enzyme-linked immunosorbent assay (ELISA) Kits were used to determine leptin (Invitrogen) and insulin (Millipore) plasma levels according to manufacturer's instructions.

- Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) index

The HOMA-IR index was calculated using the following equation: [fasting insulin levels x fasting glucose levels] / 22.5 (Matthews et al. 1985).

Antioxidant enzymes activity and lipid peroxidation

Superoxide dismutase (SOD) activity was determined based on its ability to inhibit the reaction of superoxide radical with adrenaline. The oxidation of adrenaline leads to the formation of a colored product, adrenochrome. SOD activity is determined by measuring the rate of adrenochrome formation, detected at 480 nm using a spectrophotometer. One unit of SOD activity was defined as the amount of SOD capable of inhibiting 50% of the rate of adrenaline oxidation (Boveris et al. 1983).

Catalase (CAT) activity was evaluated by measuring the decomposition of hydrogen peroxide at 240 nm using a spectrophotometer (Boveris and Chance 1973).

Lipid peroxidation was measured by the method of thiobarbituric acid-reactive substances (TBARS) by the determination of malondialdehyde formation. The absorbance was determined at 535 nm using a spectrophotometer (Buege and Aust 1978).

Statistical analysis

Data were expressed as mean ± standard error of the mean. Statistical analysis was performed by two-way analysis of variance (ANOVA), followed by Bonferroni post-test. Treatment and diet were used as ANOVA factors. A significance level of 5% ($p < 0.05$) was set. GraphPad Prism® software package, version 5 was used to perform all statistical tests.

RESULTS

Weight gain was monitored over the 19 weeks of the study. At the end of the experiment, there was a significant increase in the weight gain of HFD-fed animals ($F_{1,25} = 12.18$ $p = 0.001$). However, in the HFD+Zn group there was a decrease in the weight gain compared to HFD+vehicle ($p < 0.001$). On the other hand, BCAA treatment was not able to revert the increased in weight gain following HFD (Figure 1A). As expected, HFD induced an increase in the abdominal fat pad ($F_{1,21}$=56.97 p<0.0001). This increase was less pronounced in the HFD+Zn group in comparison to HFD-fed rats treated with vehicle ($p < 0.001$, Figure 1B).

Glycemia of HFD-fed rats was significantly increased in comparison to SD-fed rats ($F_{1,29}$ = 32.10, $p < 0.0001$). Interestingly, in HFD-fed rats none of the treatments was able to revert this increase in blood glucose levels (Figure 2A). Plasma insulin was also increased in HFD-fed

animals ($F_{1,26}$ = 4.875, p = 0.03), but the HFD+Zn group showed decreased levels of insulin in comparison to HFD+vehicle group (p < 0.01), which reinforces the beneficial role of zinc treatment in HFD-fed rats (Figure 2B). Increased HOMA-IR following HFD corroborated the insulin plasma levels results ($F_{1,26}$ = 16.73, p = 0.0004). Despite the fact that zinc treatment did not reduce glucose levels in HFD-fed rats, the HFD+Zn group showed a lower HOMA-IR when compared to HFD+vehicle (p < 0.01). This finding indicates zinc ameliorates insulin sensitivity in HFD-fed animals (Figure 2C).

Leptin levels were higher in HFD+vehicle and HFD+BCAA compared to SD+vehicle (p < 0.001) and SD+BCAA (p < 0.05), respectively. However, HFD+Zn rats showed leptin levels similar to SD+Zn and lower than HFD+vehicle (p < 0.01), suggesting HFD causes hyperleptinemia, which is reversed by zinc supplementation ($F_{1,25}$ = 77.61, p = 0.0002) (Figure 3).

Triglycerides levels ($F_{1,26}$ = 56.93, p < 0.0001) were also increased in the plasma of HFD-fed rats. These levels were significantly higher in the HFD+vehicle (p < 0.001) and HFD+BCAA (p < 0.001) groups compared to their respective controls. However, HFD+Zn animals showed triglyceride levels intermediate between SD+Zn and HFD+vehicle, suggesting zinc treatment is able to reduce at least partially the triglycerides levels in HFD-fed rats (Figure 4A). There was also an effect of the diet on the total cholesterol levels ($F_{1,27}$ = 12.03, p,=,0.0002). However, the only significant difference was an increase in cholesterol levels found in HFD+BCAA as compared to HFD+vehicle groups (p,<,0.05, Figure 4B). No significant differences in LDL- and HDL-cholesterol plasma levels were found among groups (data not shown).

Oxidative stress and antioxidant defense in the liver were also evaluated. The activity of the antioxidant enzymes SOD and CAT did not show differences among the groups (Figure 5A and 5B). On the other hand, an increase in the thiobarbituric acid reactive substances (TBARS), a

marker of lipid peroxidation, was found following HFD ($F_{1,30} = 59.66$, $p < 0.0001$). TBARS were higher in the HFD+vehicle ($p < 0.01$), HFD+Zn ($p < 0.001$), and HFD+BCAA ($p < 0.01$) groups compared to their SD-groups (Figure 5C).

AST and ALT did not change among the groups (Figure 6A and 6B). Histological evaluation of the liver tissue did not show any histological features of structural damage or hepatic steatosis in HFD-fed animals (data not shown).

DISCUSSION

In the present study, we fed rats with a high-fat/high-fructose diet as a model of diet-induced obesity. We demonstrated that ingestion of this diet caused accumulation of adipose tissue and, consequently, triggered obesity, corroborating previous studies (Ishimoto et al. 2013; Marques et al. 2016; Toop and Gentili 2016). We also found hyperglycemia, hyperinsulinemia, insulin resistance, hyperleptinemia, hypertriglyceridemia, and increased oxidative stress following 19 weeks of HFD. Those findings were also reported elsewhere (Choi et al. 2008; Zhang et al. 2015). However, we show for the first time that zinc supplementation decreased visceral adiposity, improved insulin sensitivity, and decreased leptin levels in obese animals. On the other hand, BCAA supplementation did not affect any of these parameters.

In diabetes mellitus, zinc supplementation promotes reduction in glycemia, improvement of lipid profile, and a decrease in lipid peroxidation (Bao et al. 2010; Jayawardena et al. 2012). These positive effects are related to the amelioration of insulin sensitivity, promoting an improvement of glucose uptake (Jayawardena et al. 2012; Tang and Shay 2001). Adequate levels of zinc are essential not only to ensure appropriate synthesis, storage, and structural stability of insulin but also to protect against oxidative stress in type 1 and type 2 diabetes mellitus (Miao et al. 2013). In addition, ZnT8, a zinc transporter expressed in pancreatic beta and alpha cells, is

important for the regulation of insulin secretion (Rutter 2010; Rutter et al. 2016). Autoantibodies against ZnT8 (ZnT8A) were detected in 50%–60% of Japanese patients with type 1 diabetes and are a relevant prognostic feature of the disease, and a ZnT8 single nucleotide polymorphism has been reported in type 2 diabetes (Miao et al. 2013). It is also suggested that zinc displays an insulin-like action by promoting the phosphorylation of one or more components of the insulin pathway (Jayawardena et al. 2012; Tang and Shay 2001). Therefore, intake of adequate amounts of zinc is related to cellular homeostasis, playing an important role in the release and proper action of insulin (Rutter et al. 2016). It has been shown that zinc-deficient diets cause insulin resistance in animal models and there is also evidence of insulin resistance and hepatic steatosis due to zinc deficiency in patients with chronic liver disease (Himoto et al. 2015; Miao et al. 2013). On the other hand, zinc supplementation reverses alcohol-induced hepatic steatosis in mice (Kang et al. 2009). Our findings support the hypothesis that zinc supplementation provides beneficial effects on the regulation of insulin sensitivity in obesity. It should also be noted that zinc treatment not only prevents but also reverts the metabolic dysfunction associated with obesity, since supplementation was started at the 16th week following diet intervention. It is already known that 16 weeks of a HFD are sufficient to induce obesity and metabolic disorders (Ishimoto et al. 2013; Marques et al. 2016).

Zinc is also a structural component of antioxidant enzymes such as SOD. Thus, zinc deficiency impairs SOD synthesis, predisposing to oxidative stress. Increased levels of reactive oxygen species and decreased activity of antioxidant enzymes are well documented in obesity (Matsuda and Shimomura 2013), as are zinc-reduced plasma levels (Torkanlou et al. 2016). In diabetic rats, zinc supplementation increased SOD activity, decreased lipid peroxidation, and increased glutathione concentration in pancreas (Zhu et al. 2013).

In our diet-induced obesity model, the higher TBARS levels found in the liver of obese animals indicates lipid peroxidation. However, zinc supplementation did not increase SOD activity and was unable to prevent oxidative stress in the liver following a HFD.

In patients with type 2 diabetes mellitus, supplementation with 30 mg of zinc for 6 months did not increase SOD activity but caused a reduction in lipid peroxidation (Cruz et al. 2015). In the present study, it is likely that zinc supplementation for 4 weeks was not sufficient to reverse oxidative damage triggered by the 19 weeks of diet. However positive effects of zinc supplementation as an antioxidant are best seen in preventive treatments (Zhu et al. 2013).

Unlike zinc, BCAA supplementation was not effective in controlling or reducing obesity and its comorbidities in the present study. Date in the literature data are controversial concerning the relationship between BCAA and obesity. While the study of Wang et al. (2018) showed that obese individuals present increased serum concentration of BCAA, suggesting no deficiency of these amino acids in obesity (Heimerl et al. 2014; Takashina et al. 2016; Wang et al. 2018), other studies associated a higher intake of BCAA with a lower prevalence of overweight (Nagata et al. 2013; Qin et al. 2011). BCAA supplementation has been thought to activate anabolic pathways and stimulate insulin production (Bifari and Nisoli 2017). There is a close association between BCAA levels and blood glucose levels. The fact that BCAA upregulates glucose transporters and activates insulin release has been widely demonstrated (Holecek 2018). However, it has been suggested that excessive intake of amino acids in association with a reduction in the BCAA catabolism could lead to inhibition of insulin signaling (Zhang et al. 2017). Thus, the effects of BCAA supplementation seem to be controversial. It shows advantages in obese individuals when it is associated with physical activity, which activates fatty acid oxidation (Holecek 2018). On the other hand, the increase in BCAA plasma levels related to a reduction in amino acid catabolism may indicate type 2 diabetes mellitus (Siomkajlo et al. 2017; Wang et al. 2011). In this context,

BCAA supplementation could be a useful tool in the prevention of obesity or in association with a weight loss diet or a physical exercise program.

Nonalcoholic fatty liver disease (NAFLD) is currently the most prevalent liver disease worldwide, characterized by the accumulation of triglycerides in the liver. It is usually associated with obesity and is not related with excessive alcohol consumption. Lower oral intake of zinc was observed in patients with NAFLD. Zinc deficiency results in mitochondrial oxidative stress and subsequently iron overload, insulin resistance, and hepatic steatosis in patients with NASH. (Himoto 2018). In addition, in patients with hepatitis C virus, zinc supplementation showed anti-inflammatory effects in the liver (Himoto and Masaki 2018; Sugino et al. 2008). BCAA supplementation is also used for the treatment of NASH and cirrhosis. In these conditions, BCAA improves hypoalbuminemia and glucose tolerance, due to their effect in the glucose uptake in the skeletal muscle (Miyake et al. 2012). In this context, zinc or BCAA supplementation may have beneficial roles by protecting the liver and other metabolic tissues from oxidative damage and inflammation, which may help to prevent the progression and worsening of the pathological conditions.

In summary, this study reveals that zinc supplementation is beneficial for the treatment of metabolic dysfunction caused by obesity. On the other hand, BCAA supplementation was not effective in reversing this dysfunction. Despite the finding that zinc supplementation did not reduce diet-induced oxidative stress in our animal model, the enhancement in insulin sensitivity in obese rats receiving zinc supplementation suggests this micronutrient may be an important alternative for the treatment of obesity.

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FIGURES CAPTIONS

Figure 1: Changes in weight gain throughout 19 weeks of experiment (percentage relative to SD+vehicle animals) (A). Visceral fat deposition (g) at the end of the experimental period (B). Data are expressed as mean ± SEM. *p < 0.05 between SD and HFD with the same treatment; #p < 0.001 as compared to HFD-vehicle. SD, standard diet; HFD, high-fat/high fructose diet.

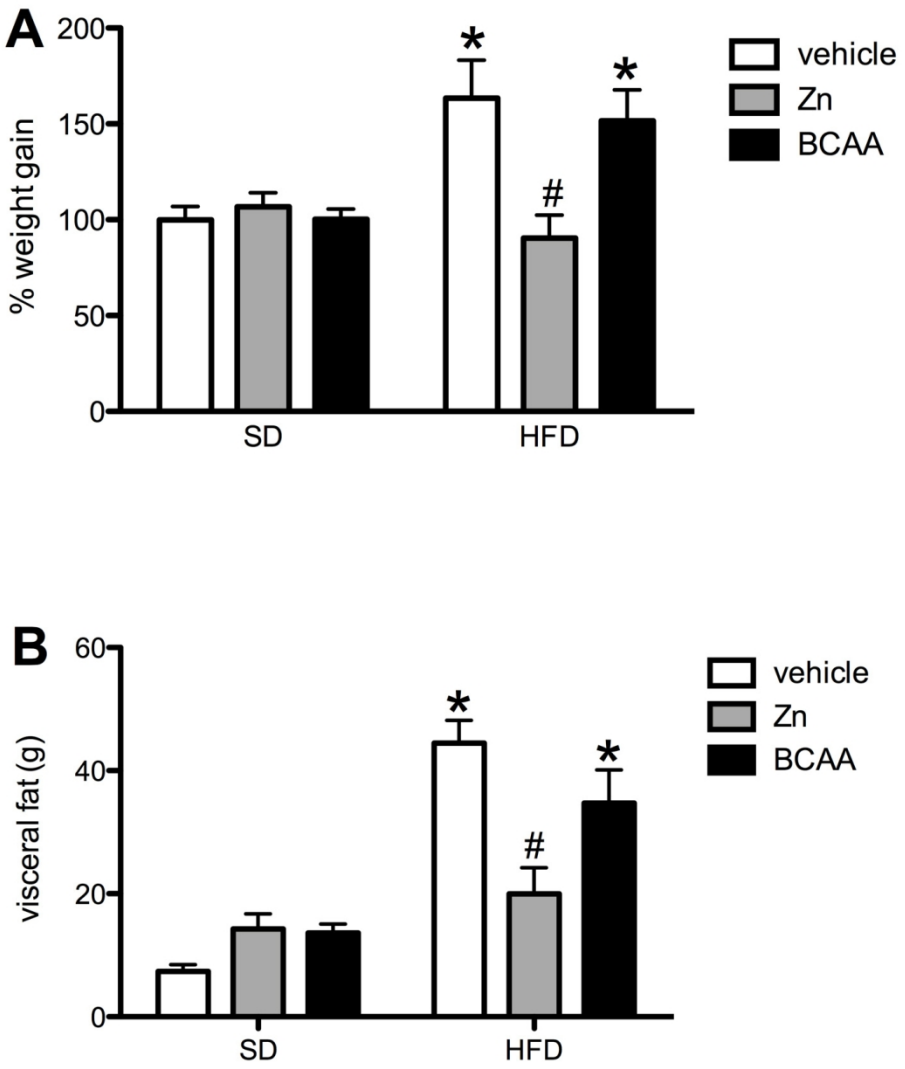
Figure 2: Fasting glucose plasma levels (A), insulin plasma levels (B) and HOMA-IR index (C) of SD and HFD-fed rats treated with vehicle, zinc or BCAA. Data are expressed as mean ± SEM. *p < 0.05 between SD and HFD with the same treatment; #p < 0.01 as compared to HFD-vehicle. SD, standard diet; HFD, high-fat/high fructose diet; HOMA-IR, homeostatic model assessment of insulin resistance.

Figure 3: Leptin plasma levels of SD and HFD-fed rats treated with vehicle, zinc or BCAA. Data are expressed as mean ± SEM. *p < 0.05 between SD and HFD with the same treatment; #p < 0.01 as compared to HFD-vehicle. SD, standard diet; HFD, high-fat/high fructose diet.

Figure 4: Plasma levels of triglycerides (A) and total cholesterol (B) of SD and HFD-fed rats treated with vehicle, zinc or BCAA. Data are expressed as mean ± SEM. *p<0.001 between SD and HFD with the same treatment; #p < 0.05 as compared to HFD-vehicle. SD, standard diet; HFD, high-fat/high fructose diet.

Figure 5: SOD activity (A), CAT activity (B) and TBARS (C) in the liver of SD and HFD-fed rats treated with vehicle, zinc or BCAA. Data are expressed as mean ± SEM. *p < 0.01 between SD and HFD with the same treatment. SOD, superoxide dismutase; CAT, catalase; TBARS, thiobarbituric acid-reactive substances; SD, standard diet; HFD, high-fat/high fructose diet.

Figure 6: Plasma levels of the aminotransferases AST (A) and ALT (B) of SD and HFD-fed rats treated with vehicle, zinc or BCAA. Data are expressed as mean ± SEM. AST, aspartate transaminase; ALT, alanine transaminase; SD, standard diet; HFD, high-fat/high fructose diet.



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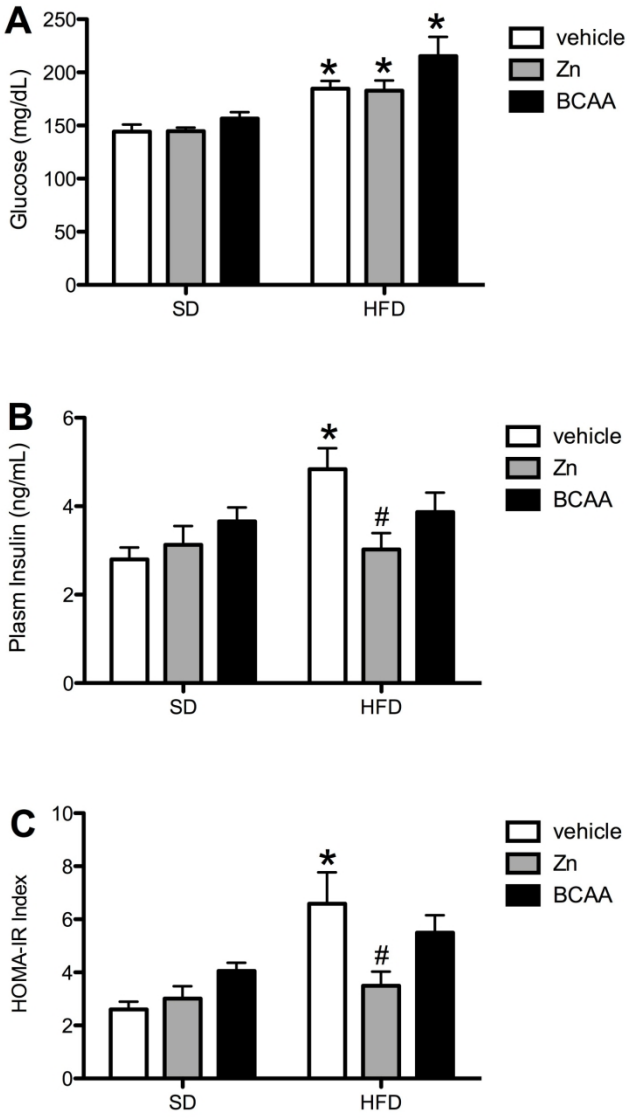

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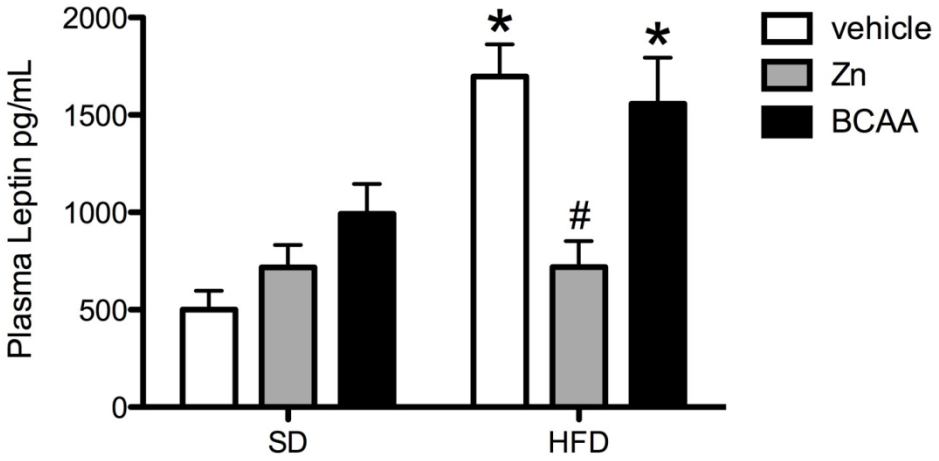

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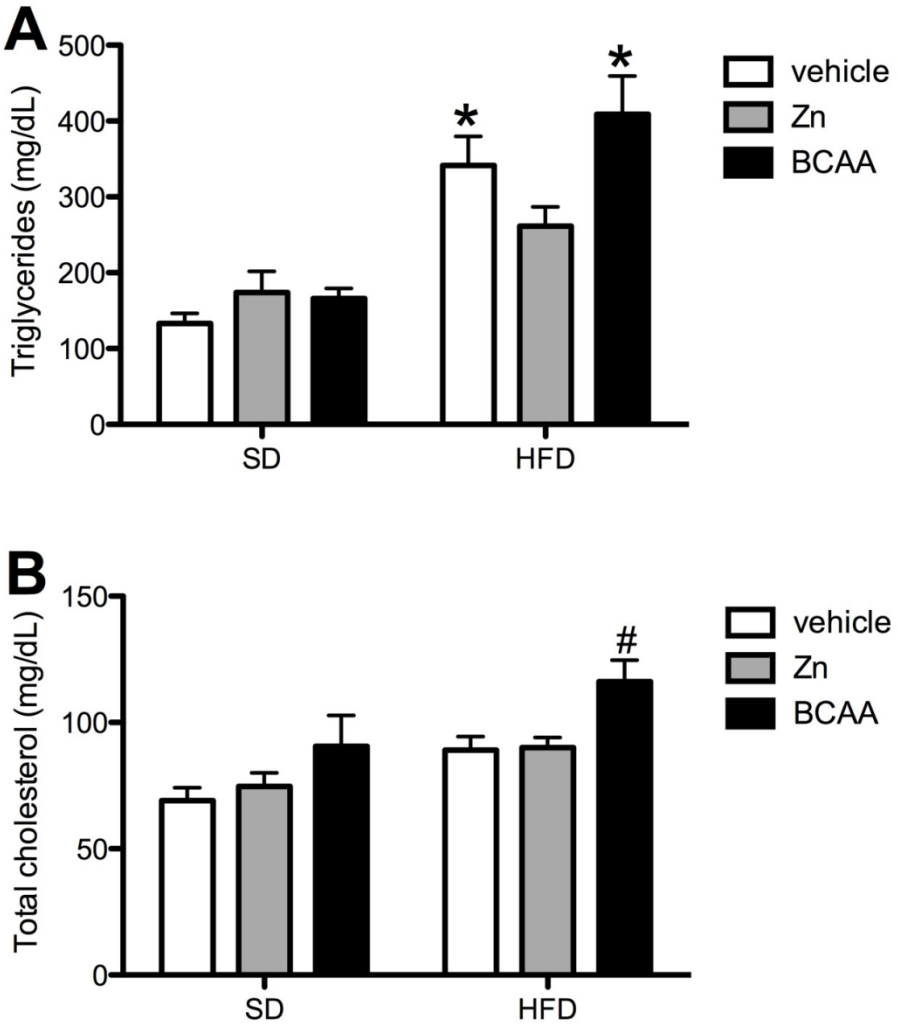

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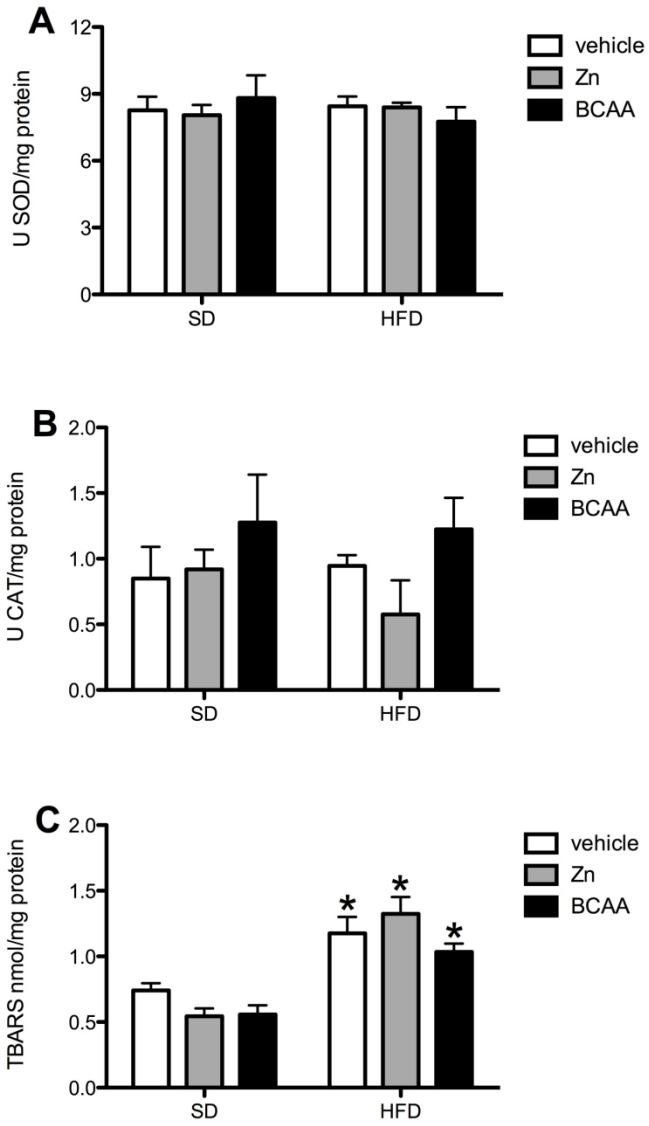

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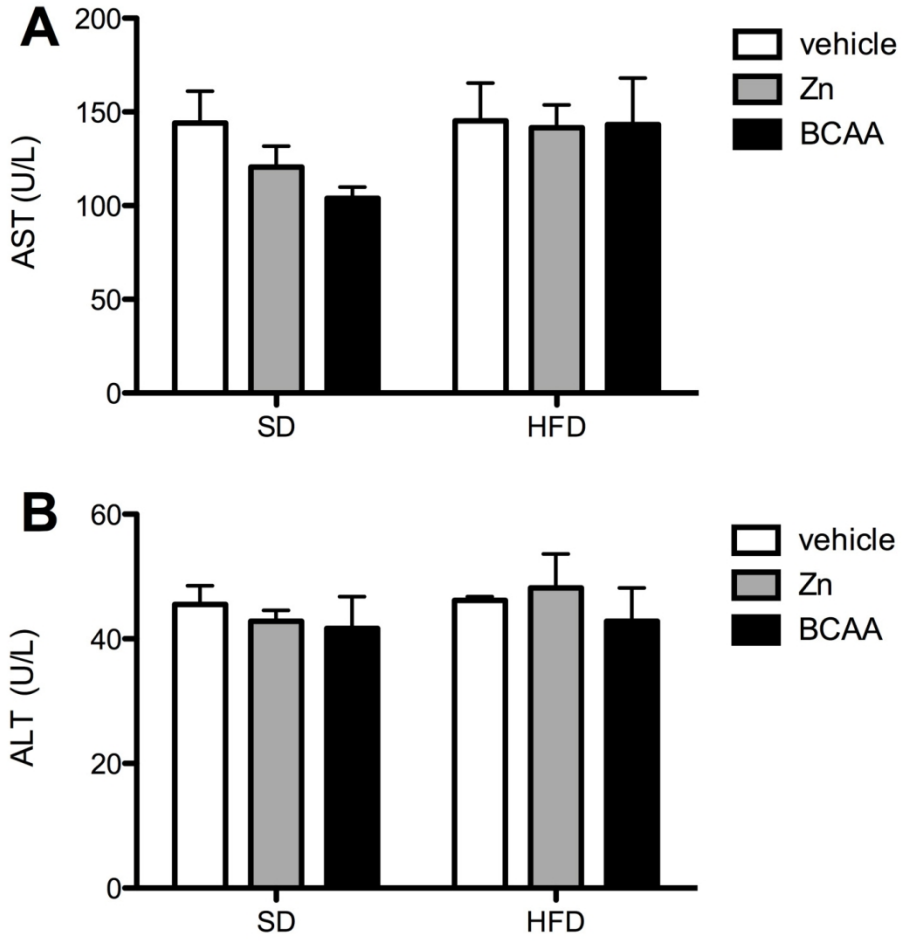

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