THE EFFECT OF INTERMITTENT HYPOXIC TRAINING UNDER OXIDATIVE STRESS PARAMETERS IN WISTAR RATS FED ON STANDARD AND HIGH FAT DIET

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ABSTRACT

Considering that higher fat diets lead to oxidative stress, causing injuries in cells and, intermittent hypoxic training (IHT) increases the endogenous antioxidant system in several situations, the aim of this study was to evaluate the effect of IHT on oxidative stress parameters and antioxidants defenses in liver of Wistar rats fed on standard and high fat diet. Animals were divided into groups fed on standard or high fat diets. The groups were submitted into intermittent hypoxia (IH), 15 minutes IH (14-11% O_2) and 5 min re-oxigenation or normoxia (N) (21% O_2) sessions, per 2 hours day during 30 days.

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The Wistar rats fed on standard diet, and submitted to IHT, showed a reduction of 37,7% in levels of thiobarbituric acid reactive substances (TBARS) and increase of 34,66% and 39,8% in the content of superoxide dismutase (SOD) and catalase (CAT), respectively, as compared with its control (normoxia). In the group with high fat diet there was not statistic difference between subgroups IH and N. Our data, showing that THI have antioxidant effect in the liver of Wistar rats, argues in favor of using alternative intermittent hypoxia protocols in future overview in certain pathologies.

Keywords*:* intermittent hypoxia, antioxidants, free radicals, rats, diets.

O EFEITO DO TREINAMENTO HIPÓXICO INTERMITENTE SOBRE PARÂMETROS DE ESTRESSE OXIDATIVO EM RATOS WISTAR ALIMENTADOS COM DIETA PADRÃO E RICA EM GORDURA.

RESUMO

Considerando-se que dietas ricas em gordura levam ao estresse oxidativo, causando lesões nas células e que o treinamento hipóxico intermitente (THI) aumenta as defesas antioxidantes endógenas em diversas situações, o objetivo deste estudo foi avaliar o efeito do THI em parâmetros de estresse oxidativo e defesas antioxidantes em fígado de ratos Wistar alimentados com dieta rica em gordura e/ou dieta padrão. Ratos Wistar foram divididos em grupos alimentados com dieta padrão ou rica em gordura. Os grupos foram submetidos a hipóxia intermitente (HI), 15 minutos HI (14-11% O_2) intercalados com cinco minutos de re-oxigenação ou sessões de normóxia (N) (21% O_2), por um período de duas horas diárias durante 30 dias. Os ratos Wistar alimentados com dieta padrão, e submetidas a HI, apresentaram uma redução de 37,7% na concentração de substâncias reativas ao ácido tiobarbitúrico (TBARS) e aumento de 34,66% e 39,8% no conteúdo de superóxido dismutase (SOD) e catalase (CAT), respectivamente, em comparação com o seu controlo (normoxia). No grupo com dieta rica em gordura, não houve diferença estatística entre os subgrupos HI e N. Nossos dados, que demonstram que o THI possui efeito antioxidante no fígado de ratos Wistar, argumentam em favor do uso alternativo de protocolos de hipoxia intermitente no tratamento de determinadas patologias.

Palavras-chave: hipoxia intermitente, antioxidantes, radicais livres, ratos, dietas.

INTRODUCTION

The global prevalence of overweight and obesity is increasing rapidly worldwide since the consumption of fats and carbohydrates in different populations is higher than that recommended (ACHESON, 2004), resulting in a great incidence of problems, such as dyslipidemias (FRIED et al., 2008), cardiovascular diseases (LAVIE et al., 2008), type II diabetes mellitus (PAGOTTO et al., 2008) and fatty liver (YALNIZ et al., 2007). Free radicals have been implicated in over one-hundred human diseases (HALLIWELL et al., 1992) although researchers emphasized that oxidative damage could be just as much consequence of tissue injury as a cause of it (HALLIWELL and GUTTERIDGE, 1984). The oxidative stress related with tissue injury, could then contribute significantly to worsening the tissue injury, or it might be irrelevant (HALLIWELL, 1994). Regardless of the controversy of the subject, it is known that a profound influence is exerted by the diets on the antioxidant defenses and can have pro-oxidant or antioxidant effects depending on its composition (LUKASHI et al., 2000). Thus, the intake of high fat diets, especially with saturated fat (LUDKE and LÓPEZ, 1999), is considered to be an important factor in the development of oxidative stress (OS) in tissues (DRÖGE, 2002; DALLE-DONNE et al., 2003; CARDOSO et al., 2010), and increase in blood biomarkers of oxidative stress (SIES et al., 2005; WALCZEWSKA et al., 2010).

The oxidative stress occurs when the balance between reactive oxygen species (ROS) production and their elimination is disturbed leading to their enhanced steady-state level (BLOOMER and WELLMAN, 2010; LUSHCHAK, 2011). The raise of ROS production can lead to oxidation of lipids, proteins and DNA, which impair the physiological functions in cells (MEERSON et al., 1982; SASTRE et al., 2003; ZHANG et al., 2005).

The damage could be attenuated by increasing the antioxidant concentrations in tissues (BECKMAN and AMES, 1998) or the use of alternative methods with antioxidant properties. Alternative methods may be considered desirable for clinical use. In this sence, the intermittent hypoxic training (IHT) that appeared in the 30's in the Soviet Union and relates to the method that uses repeated hypoxia episodes (16-9% O_2) interspersed with normoxia episodes (21% O_2) (SEREBROVSKAYA, 2002) could be an alternative method, once studies showed that IHT increase of antioxidants defenses, decrease or kept in normal levels the products of lipidic peroxidation, enhance the tissue activity of antioxidants enzymes (SEMENOV and YAROSH, 1991; MEERSON et al., 1992b; SEREBROVSKAYA et al., 2001; JUNG ET AL., 2008).

Unavailable reports about the intermittent hypoxic training effects under oxidative profile in rats fed on high fat diets led us to evaluating the effect of intermittent hypoxia on pro-oxidant and antioxidant defenses in hepatic tissue of rats fed on standard and high fat diets.

MATERIAL AND METHODS

Animals

Male Wistar rats (236 ± 34 g) were obtained from the Animal Breeding Center of the Federal University of Santa Maria, RS, Brazil. All animals were transferred to the

Department of Physiology and Pharmacology and housed for 7 days to allow acclimatization to the new environment. Rats were maintained under controlled temperature (21–23°C) at 12/12h light-dark photoperiod with lights on at 07:00 h. All experimental protocols were conducted in accordance with guidelines of the Brazilian College of Animal Experimentation and were approved by the Ethics Committee for Animal Research of the Federal University of Santa Maria.

Diets and groups

Rats (n = 40) were randomly divided into two groups and were supplied with standard diet (S) (3018 kcal/kg) or high fat diet (HF) (4181 kcal/kg) consisting of standard chow added with 20% lard and 10% sugar, which were prepared and offered each two days. Diets composition are shown in Tab 1. Both S and HF groups were subdivided into four subgroups (n=10) and submitted to intermittent hypoxia (IH) or normoxia (N) sessions. Subgroups were S/N (control), S/IH, HF/N and HF/IH. Water and food were provided ad libitum. Body weight and food intake were evaluated every two days.

Table 1 - Diets Composition

Diets composition %	Standard diet (3018 kcal)	High fat diet (4181 kcal)
Proteins	14,43	10,10
Saccharides	61,32	52,72
Lipids	4,04	21,83
Raw fiber, vitamins and minerals	20,21	15,35

IH conditioning

The intermittent hypoxia consisted in placing rats in a custom-made 4-room acrylic chamber, with five animals in each room. The air composition could be quickly and precisely adjusted using compressed gas (GO_2 Altitude Hypocator equipment). The IH program was continued for 30 days and consisted of 15 minutes of hypoxic exposure, followed by 5 minutes re-oxygenation for a total duration of two consecutive hours per day. The O_2 concentrations were adjusted to 14% (week 1), 13% (week 2), 12% (week 3) and 11% (week 4). The normoxia group was placed in the same chamber but with normal sea level O_2 concentrations (21%).

Oxidative stress analysis

After this period, rats were anesthetized with halothane and the hepatic tissue was collected for oxidative stress and antioxidants defenses analysis. The euthanasia was performed through heart removal.

Hepatic tissues were homogenized in medium consisting of 120 mM KCl and 30 mM buffer sodium phosphate (pH 7.4) containing 1 mM PMSF. The homogenates were centrifuged at 1000 × g for 10 min, between 0 and 4 °C to eliminate nuclei and cell debris, and the supernatant fraction obtained was frozen at −70 °C for further analyses. Supernatants were used for analysis of lipid peroxidation (LPO), superoxide dismutase (SOD) and catalase (CAT).

Lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) (BUEGE and AUST, 1978). Aliquots of the supernatant were added to a pyrex tube containing TCA (10%) and TBA (0.67%) and incubated at 100 °C for 15 min. The mixture was allowed to cool on ice for 5 min. The mixture was centrifuged at 1000 × g for 15 min in order to extract the resulting chromogen (Schiff's base). The absorbance of the organic phase was determined at 535 nm in spectrophotometer. Results were expressed as nmol mg protein^{-1} using ε_{535} = 156 mM^{-1} cm^{-1}. The protein content of homogenate was measured using the method described in Lowry and Rosebrough (1951) using bovine serum albumin as standard.

Total superoxide dismutase activity was determined as the inhibition rate of autocatalytic adenochrome generation at 480 nm (ε_{480} nm = 4.0 mM^{-1} cm^{-1}) in a reaction medium containing 1 mM epinephrine and 50 mM glycine/NaOH (pH 10,2). Enzyme activity was expressed as SOD units mg protein^{-1}. One SOD unit was defined as the amount of enzyme needed for 50% inhibition of adenochrome formation (MISRA and FRIDOVICH, 1972).

Catalase activity was evaluated by measuring the decrease in the absorption at 240 nm (ε_{240} nm = 40 M^{-1} cm^{-1}) in reaction medium consisting of 50 mM buffer phosphate (pH 7.4) and 2 mM H_2O_2, thereby determining the pseudo-first-order constant reaction (k') of the decrease in H_2O_2 absorption (BOVERIS and CHANCE, 1973). Results were expressed as nmol mg protein^{-1}.

Statistical analyzes

The Shapiro-Wilk test was carried out for normality. After finding that values did not follow a normal curve, we performed the Mann-Whitney and Kruskal Wallis non-parametric tests. The Dunn test was used to evaluate the significances. The significance level of used for all tests was 5%. Statistica 7, SAS 9.1 and Bioestat 5. were the softwares used.

RESULTS

IHT effects on tissue lipid peroxidation

Figure 1a shows the lipoperoxidation (LPO) values in liver of rats, as measured by TBARS. We observed significant difference among S subgroups (S/N and S/IH),

whose LPO levels were reduced (37,7%) in animals submitted to IH. There was no statistic difference between subgroups (HF/N=HF/IH) in the group with high fat diet.

IHT effects on enzymatic antioxidants

Results of antioxidant enzymes SOD and CAT in the liver of rats are shown in Fig. 1b and 1c respectively. SOD and CAT activity in standard diet group showed statistic difference between S subgroups. The SOD and CAT activity showed a increase of 34,66% and 39,8%, respectively, in animals submitted to IH. In the group with high fat diet there was no statistic difference between subgroups (HF/N=HF/IH).

DISCUSSION

Our study suggest that IHT has antioxidant effect since these alternative methods reduced significantly the TBARS levels and increased SOD and CAT enzymatic activity in the liver of rats fed on the standard diet.

It is known that ROS are physiologically essential in controlled amounts due to their contribution to vascular regulation, cell growth and proliferation, gene transcription, mitochondrial biogenesis and cell signaling (CHANDEL and SCHUMACKER, 2000). However, when its quantity is higher than antioxidant defenses, pathologies can emerge (REAVEN et al., 2004; AMIRKHIZI et al., 2007).

It is well known that the high ingestion of fat diets increase ROS production and results in excessive macromolecule oxidation (SIES et al., 2005). Consumption of high fat content diets may alter oxygen metabolism since fatty deposits are vulnerable to suffering oxidation reactions. If the production of these ROS exceeds the antioxidant cell capacity, the oxidative stress resulting in lipid peroxidation could contribute to the development of several illnesses (KHAN et al., 2006). Lipid peroxidation is a cascade of biochemical events resulting from the action of free radicals on the unsaturated lipids of cell membranes, leading to destruction of their structure, alteration in the mechanisms of metabolites exchange that may trigger sequence in cell injury and even cell death (VACA et al., 1988; BENZIE, 1996). One technique for assessing lipid peroxidation (LPO) is through TBARS levels and the literature describes that increases in TBARS levels indicate enhanced LPO that leads to tissue injury (KASAPOGLU and OZBEN, 2001; AMIRKHIZI et al., 2007). The data presented in this study showed that IHT significantly decreases TBARS levels in animals fed on standard diets, suggesting that this alternative method might exert a protective activity in membranes, reducing the LPO.

It has been shown that the body has an effective mechanism to prevent the free radical induced tissue cell damage by a set of endogenous antioxidant enzymes, such as SOD and CAT (BLOKHINA et al., 2002). SOD and CAT are enzymes that provide a primary defense line against ROS generated during hypoxic exposure (ESTEVA et al., 2010). SOD catalyzes $^{\cdot}O_2$ elimination by dismutation and hydrogen peroxide formation and CAT catalyzes the conversion of hydrogen peroxide into water (NOVELLI et al., 2005).

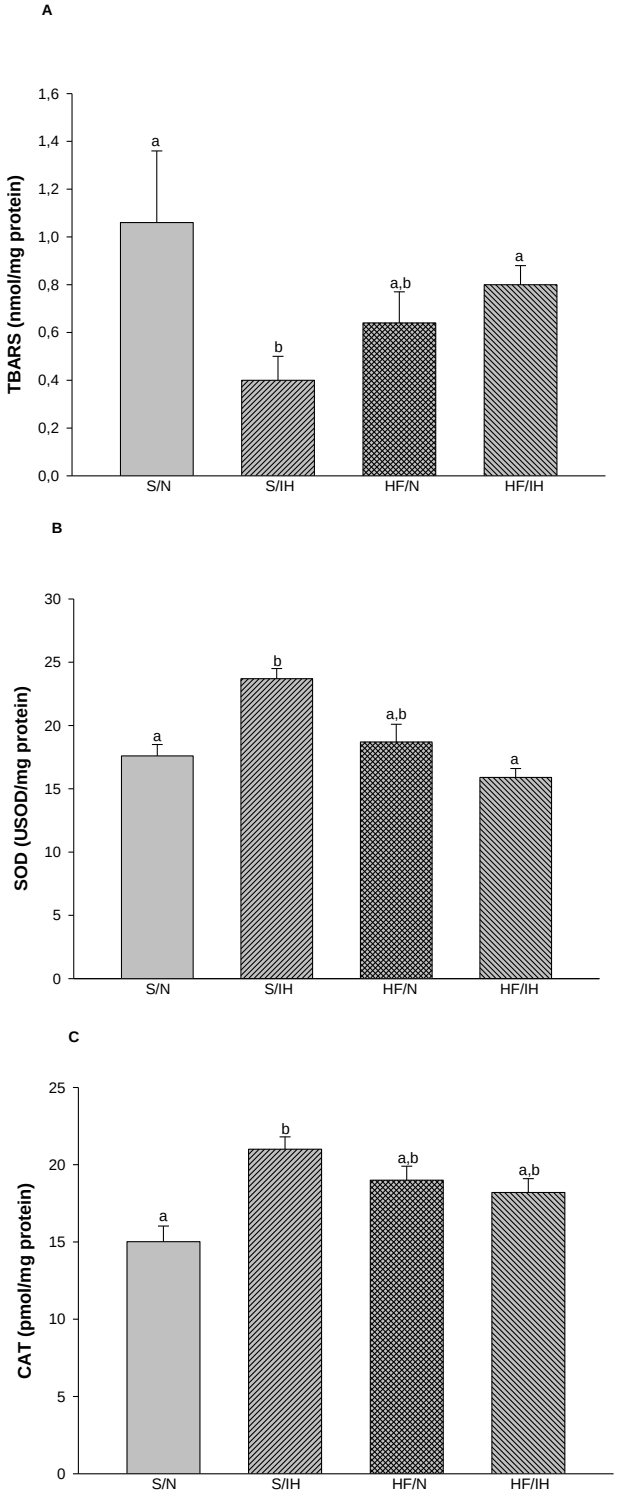

Figure 1 - Effects of different diets and O_2 condition in TBARS (A), SOD (B) and CAT (C) levels in rats liver. Values are given as mean ± S.E.M. for group of nine animals each. Results with equal letters do not differ statistically ($p<0,05$).

Our results with TBARS levels, SOD and CAT activity are in agreement with other previous findings. Semenov and Yarosh (1991) demonstrated that IHT in rats kept under simulation of 4000 m, 7 hours a day for 2 weeks prevented lipid peroxidation. Later, Meerson et al. (1992b) found reduction of lipid peroxidation and in the activity of enzymes SOD and CAT with continuous hypoxia exposition (2100 m altitude) during 30 days. Otherwise, when rats were exposed 6 hour a day for 30 days, with harder hypoxia (5000 m), the activity of antioxidant enzymes increased and the LPO content remained normal. Our results, and those of aforementioned authors, show that the IHT acts on the oxidative profile.

The significant increase of SOD and CAT in rats fed on standard diet obtained in our study suggests that the IHT may potentiate the action of these antioxidant enzymes, and these data associated with reduced levels of TBARS indicates a beneficial IHT effect. It can be suggested that these results are related to the fact that hypoxia is known by directly affects various cellular processes through effects on the gene expression. A direct effect of hypoxia, as well as the intermittent hypoxia on gene expression is exercised by the hypoxia-inducible factor (HIF) (BUNN and POYTON, 1996; SEMENZA, 2000). HIF-1 is regulated by proteasomes, which degrade the subunit of HIF-1 in the presence of oxygen, or oxygen levels directly regulate the α expression component in a dose-dependent manner (BUNN and POYTON, 1996). Under hypoxic conditions, the HIF-1α decomposition is inhibited, leading to nuclear accumulation of protein, which is associate with HIF-1β and it binds to the hypoxia responsive elements (HRE) (SEMENZA, 2003; FUKUDA et al., 2007). Thus, HIF-1 states the regulation of a variety of genes contributing to the adaptative metabolic changes that could protect the body against unfavorable situations. One of the target genes is the mitochondrial manganese superoxide dismutase content which catalyzes $^{\cdot}O_2$ elimination by dismutation and hydrogen peroxide formation which in turn is destroyed by CAT; then the HIF indirectly increases the CAT activity (HERVOUET et al., 2008).

The fact of SOD and CAT enzymes do not increase with the IHT and there is no reduction of TBARS levels in the high fat content diet group could be related to diet composition, rich in saturated fat, which is considered an important factor in the development of oxidative stress in tissues (DRÖGE, 2002; DALLE-DONNE et al., 2003; CARDOSO et al., 2010). To the best of our knowledge, this is the first study to analyze the effects of IHT in rats fed on different content diet, thereby we did not find in literature studies that helped us to explain our results. In this way we can suggest that the diet composition plus shorter time exposure and the hypoxic load (O_2 concentration) could be insufficient to hypoxia had significant effects.

CONCLUSION

Our results, which show LPO reduction and activation of SOD and CAT enzymes in animals fed on standard diet suggesting antioxidant effect of the intermittent hypoxic training. These effects may be due to the action of intermittent hypoxia on the HIF, which activates genes involved in the synthesis of MnSOD and consequently increases the CAT. The results argues in favor of using intermittent normobaric hypoxia protocols in many well established uses, such as altitude pre-acclimation and athletic performance improvement and for other potential future overview in certain

pathologies. However, more studies on different protocols of load and time of hypoxia exposure and more suitable and rigorously-validated methodologies in animals fed on high fat content diets are necessary to evaluate if the intermittent hypoxic training could have protective effect under reactive oxygen species.

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