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Trans-resveratrol reduces cardiac oxidative stress in rats exposed to cigarette smoke

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ABSTRACT. Differences between reactive oxygen species and antioxidant defense system unbalance the redox status. The exposure to cigarette smoke can increase this imbalance. Trans-resveratrol is a polyphenol with great antioxidant action that reduces the oxidative stress. This study investigated the effect of the trans-resveratrol supplementation on the cardiac oxidative stress in rats exposed to cigarette smoke. Male Wistar rats were randomized into four groups: Control Group (CG), Exposure to Smoke Group (ESG), Antioxidant Group (AG) and Exposure to Smoke plus Antioxidant Group (ESAG). Animals were exposed to cigarette smoke and supplemented with trans-resveratrol (6.0 mg kg^{-1}) for two months. The lipid peroxidation (TBARS) and the enzymatic activity of catalase (CAT) were measured in the cardiac muscle. The ESG presented the highest lipid peroxidation level compared with CG ($p < 0.001$), AG ($p < 0.001$) and ESAG ($p < 0.006$). The CAT activity was higher in the AG ($p < 0.001$) and ESAG ($p < 0.001$) compared with CG. The ESG presented lower CAT activity compared with the ESAG ($p < 0.001$). The supplementation of Trans-resveratrol attenuated the cardiac oxidative stress and increased the activity of catalase. Our findings evidenced the cardioprotective effect of trans-resveratrol in rats exposed to cigarette smoke.

Keywords: smoking, myocardium, reactive oxygen species, catalase.

O trans-resveratrol reduz o estresse oxidativo cardíaco de ratos expostos à fumaça de cigarro

RESUMO. Diferenças entre espécies reativas de oxigênio e sistema de defesa antioxidante desequilibram o estado redox. Exposição à fumaça de cigarro pode aumentar esse desequilíbrio. Trans-resveratrol é um polifenol com ação antioxidante que reduz o estresse oxidativo. O objetivo do presente estudo foi investigar os efeitos da suplementação com trans-resveratrol no estresse oxidativo cardíaco de ratos expostos à fumaça de cigarro. Randomização de 32 ratos Wistar machos em quatro grupos: Controle (CG), Exposição à Fumaça (ESG), Antioxidante (AG) e Exposição à Fumaça+Antioxidante (ESAG). Animais foram expostos à fumaça de cigarro e suplementados trans-resveratrol (6,0 mg kg^{-1}) durante dois meses. Lipoperoxidação (TBARS) e atividade enzimática da catalase (CAT) foram mensuradas no músculo cardíaco. ESG apresentou maiores níveis de lipoperoxidação quando comparado ao CG ($p < 0,001$), AG ($p < 0,001$) e ao ESAG ($p < 0,006$). Atividade da CAT foi maior no AG ($p < 0,001$) e no ESAG ($p < 0,001$) quando comparados ao CG. ESG apresentou a menor atividade da CAT quando comparado ao ESAG ($p < 0,001$). A suplementação com trans-resveratrol atenuou o estresse oxidativo cardíaco e aumentou a atividade enzimática de defesa catalase. Esses resultados sugerem evidências de efeitos cardioprotetores do trans-resveratrol em ratos expostos à fumaça de cigarro.

Palavras-chave: hábito de fumar, miocárdio, espécies de oxigênio reativas, catalase.

Introduction

Smoking is one of the leading risk factors for non-communicable diseases, such as cardiovascular disease, diabetes, chronic respiratory diseases, and cancer (WHO, 2012). About 40% of children and 35% of adults are at high risk for developing ischemic heart disease, asthma, respiratory infections and disability-adjusted life-years because of the exposure to secondhand smoke (OBERG et al., 2011). Larger quantities of free radicals (reactive oxygen species [ROS]) present in the cigarette smoke increases the production of endogenous oxidants and may lead to an inflammatory immune response through the nuclear factor κβ pathway

activation (MORGAN; LIU, 2011). Despite participating in normal physiological events, large amounts of ROS can impair the body (ALBERG, 2001).

The imbalance between oxidant and antioxidant agents induces a state of oxidative stress defined as an interrupt signaling and cellular redox control. The Hydroxyl radical (OH^-), Superoxide anion radical (O_2^-) and Hydrogen peroxide (H_2O_2) comprise the main ROS (HALLIWELL; GUTTERIDGE, 1986), whereas their main opposing molecules are the enzymatic antioxidants (e.g., Superoxide dismutase, Catalase, Glutathione peroxidase) and non-enzymatic antioxidants (e.g., Vitamin A, C, E, Uric acid, Coenzyme Q, Lipoic acid) (POLJSAK et al., 2013). The ROS and nitrogen may lead to a macromolecular damage and potential imbalance of redox pairs via deregulation of the cellular signaling pathways (JONES, 2006). All cell components and main biomolecules (lipids, proteins, nucleic acids) are susceptible to oxidative damage and can suffer change in the structure and function (THÉROND et al., 2000). This contributes to the development and/or progression of the diseases associated with smoking (WHO, 2012), exposure to cigarette smoke (OBERG et al., 2011), and cardiovascular dysfunctions such as atherosclerosis, ischemia-reperfusion injury, chronic ischemic heart disease, cardiomyopathy, congestive heart failure, and arrhythmias (TAVERNE et al., 2013).

Using antioxidant micromolecules in appropriate doses can lower the oxidizing and harmful effects of cigarette smoke under such conditions of ROS increased by uncontrolled endogenous antioxidants (POLJSAK et al., 2013). Trans-resveratrol is the most bioavailable isoform that crosses the cell membrane and promotes balance of ROS (CAO et al., 1997). This antioxidant is found in large quantities in grapes, berries, peanuts, and red wine. Previous studies have shown its benefits for preventing and/or treating cardiovascular diseases, ischemic lesions, cancer, and inflammation (MAIER-SALAMON et al., 2013). However, its protective effects on the heart muscle of animals exposed to cigarette smoke remain unclear. The aim of this study was to investigate the effect of trans-resveratrol supplementation on the parameters of the cardiac oxidative stress from the exposure to cigarette smoke in rats.

Material and methods

Animals

Thirty-two male Wistar rats weighing 200-250 g were included in the study. The experimental protocol used was approved by the Ethics Committee on Animal Use of the Universidade Federal de Santa Maria under the project number 079/2011 – CEUA/UFSM, and adhered to the Brazilian Guiding Principles for the Care and Use of Animals. Animals were fed according to a standard rat chow diet, having free access to water and food. The environmental conditions were controlled (12-h photoperiod and 20 ± 2ºC) throughout the experimental period.

Study groups

After one week of adaptation to individual cages, animals were divided into four groups: Control Group (CG), Exposure to Smoke Group (ESG), Antioxidant Group (AG) and Exposure to Smoke plus Antioxidant Group (ESAG). Animals of the CG were housed in a separate room during the experimental protocol and experienced the same stressful conditions of the other groups without the exposure to cigarette smoke and trans-resveratrol supplementation.

Exposure to cigarette smoke

Adapted from the protocol previously described (WANG et al., 1999), the exposure machine was a transparent chamber with dimensions of 40 x 45 x 30 cm, connected to a smoking device that absorbed the cigarette smoke and released it into the transparent chamber. According to the protocol described by our research group (VELOSO et al., 2013), animals of the ESG and ESAG were adapted to the smoke from five cigarettes burned for 30 min. (twice a day, in the morning and afternoon) for one week. From the second week by the end of two months, the number of cigarettes burned for 30 min. was progressively increased until 10 cigarettes (twice in the morning and twice in the afternoon). The cigarettes used were composed of 0.7 mg of nicotine, 9.0 mg of carbon monoxide and 8.0 mg tar.

Trans-resveratrol supplementation

The AG and SEAG received 6.0 mg kg^{-1} (REAGAN-SHAW et al., 2008) of trans-resveratrol by the gavage technique for two months. The trans-resveratrol (Pharma Nostra™, Brazil – 99.9 purity) was diluted with canola oil to facilitate the administration.

Venous blood sample collection

After the experimental protocol, a blood collection was performed in all rats by puncturing the retro-orbital plexus after prior mild anesthesia with isoflurane. Part of the blood was centrifuged in tubes (3000 RPM, 5 min.) to obtain serum for the analysis of liver enzymes (alanine aminotransferase

and gamma glutamyl transferase) and albumin. Other part of the blood was placed in tubes with ethylene diaminetetraacetic acid and used to perform the complete blood count and leukogram. After the last blood sample collection, the animals were euthanized and the hearts were removed for analysis.

Homogenate and quantification of heart tissue protein

A piece of each heart was placed in tubes with 115% potassium chloride (0 - 4ºC) and homogenized for 30 s. This homogenized was centrifuged (1000 RPM, 10 min., 0 - 4ºC), and aliquots of the supernatant were used to perform the ThioBarbituric Acid Reactive Substances (TBARS) and evaluation of the enzymatic activity of Catalase (CAT). The quantification of the protein was performed following the Lowry method (LOWRY et al., 1951), where the maximum absorbance for the solution of Folin–Ciocalteau, because of its interaction to the albumin protein of the bovine serum, occurs at 625 ηm.

Lipid peroxidation of heart tissue

The estimation of the lipid peroxidation in heart tissue was performed using the TBARS assay as previously described (BUEGE; AUST, 1978), in which the authors quantified the colorimetric reaction of the lipid peroxidation of the product malondialdehyde (MDA) with thiobarbituric acid (TBA). The reaction produces a colored compound that absorbs maximally at 532 ηm. One gram of heart tissue in 5 mL of potassium phosphate (0.1 M, pH 7.4) was homogenized using a Polytron mixer (Kinematica AG, Luzern, Switzerland). After heating at 90°C to react with TBA, the tubes were cooled and centrifuged (3000 RPM, 10 min.). The organic layer (supernatant) was collected and the absorbance was read at 532 ηm using a spectrophotometer. Data were encoded in ηmoles mg^{-1} of protein.

Enzymatic activity of Catalase

The catalase activity was measured as a function of the decrease in the absorption of H_2O_2 at 25ºC as previously described (AEBI, 1984). The method is based on the removal of H_2O_2 by the CAT and the loss of absorbance at 240 ηm. The results were expressed in ηmoles mg^{-1} of protein.

Statistical analysis

The descriptive analyses comprised mean and standard deviation (± DP); the normality assumption was evaluated by the Kolmogorov-Smirnov test. The groups were compared using the one-way and two-way analyses of variance (before and after) for repeated measures (time, group and interaction), followed by the Tukey's *post-hoc* test. A value of $p < 0.05$ was considered statistically significant.

Results

Physical and metabolic characteristics of the groups

No difference was observed among groups during the initial and final evaluations of the body weight ($p < 0.216$), although all groups increased about 50% in weight ($p < 0.021$) throughout the study (Table 1). The biochemical tests such as hematocrit, red blood cells, hemoglobin, total leukocyte and leukocyte fractions (not shown), creatinine, urea, gamma glutamyl transferase, alanine aminotransferase, and albumin did not differ among groups (Table 2).

Table 1. Values of body weight in all groups before and after the experimental protocol.

	CG n=8	ESG n=8	AG n=8	ESAG n=8	ANOVA two-way p value Time	Group	Interaction
Before	203 ± 16	201 ± 27	215 ± 23	225 ± 29	<0.001	0.216	0.116
After	331 ± 21★	308 ± 29★	329 ± 19★	324 ± 14★			

Data expressed as mean ± standard deviation; CG - Control Group; ESG - Exposure to Smoke Group; AG - Antioxidant Group; ESAG - Exposure to Smoke plus Antioxidant Group; ★ - Significant difference before vs. after.

Table 2. Values of the biochemical tests after the experimental protocol.

Variable	CG n = 8	ESG n = 8	AG n = 8	ESAG n = 8	ANOVA one-way p value
Hematocrit (%)	41.2 ± 4.8	40.7 ± 2.7	39.0 ± 3.8	38.7 ± 2.7	0.510
Erythrocytes ($10^5 mm^{-3}$)	7.2 ± 0.9	7.3 ± 0.7	7.0 ± 0.4	7.3 ± 0.5	0.793
Hemoglobin ($g\ dL^{-1}$)	12.9 ± 1.0	12.7 ± 0.9	12.4 ± 1.0	12.3 ± 0.9	0.676
Leukocytes Total ($10^3 mm^{-3}$)	6920 ± 3779	7037 ± 3610	5800 ± 2260	5085 ± 1340	0.359
Creatinine ($mg\ dL^{-1}$)	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.092
Urea ($mg\ dL^{-1}$)	33.5 ± 5.2	33.3 ± 5.7	29.5 ± 4.3	27.8 ± 2.3	0.158
GGT ($u\ L^{-1}$)	4.7 ± 2.0	5.1 ± 2.0	3.1 ± 1.4	5.1 ± 3.0	0.156
ALT ($u\ L^{-1}$)	78.3 ± 14.7	63.8 ± 11.2	62.4 ± 17.2	55.4 ± 15.5	0.115
Albumin ($g\ dL^{-1}$)	2.1 ± 0.4	2.5 ± 0.8	3.1 ± 1.0	3.3 ± 0.9	0.081

Data expressed as mean ± standard deviation. CG - Control Group; ESG - Exposure to Smoke Group; AG - Antioxidant Group; ESAG - Exposure to Smoke plus Antioxidant Group; GGT - Gamma Glutamyl Transferase; ALT - Alanine Aminotransferase.

TBARS

The data analyses for oxidative damage are shown in Figure 1A. The CG (3.2 ± 0.37 ηmoles mg^{-1} protein), AG (3.63 ± 0.55 ηmoles mg^{-1} protein) and ESAG (3.72 ± 0.24 ηmoles mg^{-1} protein) revealed no difference in the myocardial lipid peroxidation. However, in the ESG (4.3 ± 0.23 ηmoles mg^{-1} protein), the lipid peroxidation was respectively 34.4% ($p < 0.001$) and 18.4% ($p < 0.001$), values greater than in the CG and AG. Animals exposed to cigarette smoke plus the trans-resveratrol supplementation showed a decrease of 13.5% ($p < 0.006$) in this oxidation when compared with the ESG.

Catalase

The CAT activity on the CG for the cardiac muscle (0.25 ± 0.11 ηmoles/mg protein) was similar to the ESG (0.45 ± 0.9 ηmoles mg^{-1} protein; $p < 0.094$). By comparing the CG and the groups with trans-resveratrol supplementation, the CAT activity was approximately twice higher in the AG (0.57 ± 0.17 ηmoles mg^{-1} protein; $p < 0.001$) and six times higher in the ESAG (1.65 ± 0.2 ηmoles mg^{-1} protein; $p < 0.001$). The ESG showed lower CAT activity when compared with the ESAG ($p < 0.001$). The ESAG had higher means of CAT when compared with all the other groups, with an increase of 660% when compared with the CG, 367% for the SEG and 290% for the AG (data shown in Figure 1B).

Discussion

Our results provide evidences that the trans-resveratrol supplementation decreases the amount of lipid peroxidation caused by the exposure to cigarette smoke, and increases the enzymatic activity of the CAT in heart tissue of rats. The animals exposed to cigarette smoke obtained the highest values of lipid peroxidation, suggesting higher cardiovascular risk under this condition. The mainstream exposure to cigarette smoke is equivalent to the active smoking, which is composed of about 8% of tar and 92% of gaseous components that contain or induce the formation of ROS (SMITH; FISCHER, 2001). The exposure to cigarette smoke affects a wide variety of oxidizing compounds, such as ROS, which promotes the oxidation of lipids of the cell membrane and increases the systemic oxidative stress (KUNITOMO et al., 2009). Lipid peroxidation leads to a decrease in the membrane fluidity and alignment of receivers, which interferes in the transport and provokes rupture and cell lysis (MACHLIN; BANDICH, 1987). One of the first implications of aggression to the blood vessels is the oxidative modification of low-density lipoprotein (LDLc), which can promote the development of atherosclerosis from the endothelial dysfunction with subsequent formation of foam cells and complex plaques composed of necrotic cellular debris, lipid and fibrous tissue (TINKEL et al., 2012).

A report about the cardioprotective action of a plant, source of amino acids, minerals and vitamins, in rat, as experimental model in the exposure to cigarette smoke, showed an increase of lipid peroxidation and a decrease of enzymatic activity of the CAT in relation to the other groups without the exposure to cigarette smoke (RAMESH et al., 2008).

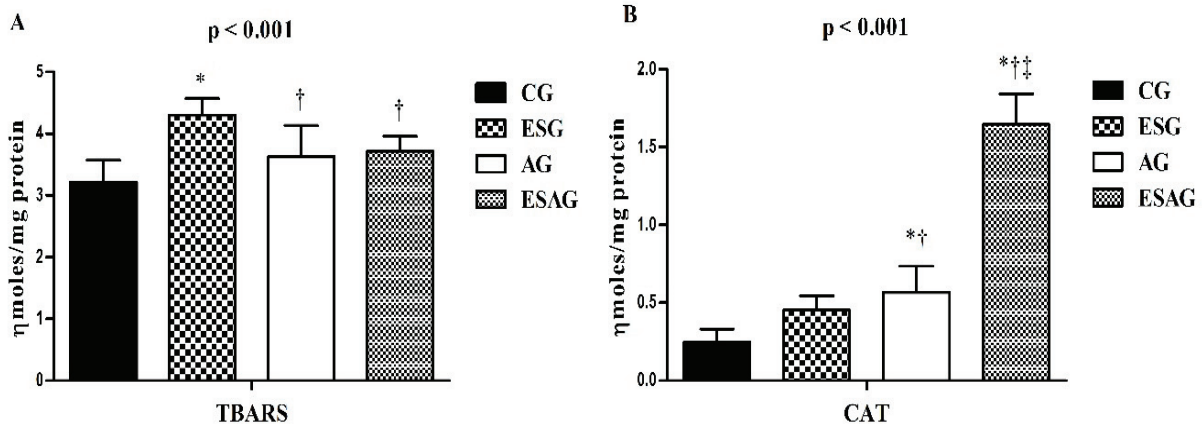

Figure 1. Effects of the exposure to the cigarette smoke and/or trans-resveratrol supplementation on markers of lipid peroxidation by the TBARS (A) and the antioxidant capacity measured by the CAT (B); CG - Control Group; ESG - Exposure to Smoke Group; AG - Antioxidant Group; ESAG - Exposure to Smoke plus Antioxidant Group; * - Significant difference in relation to the CG; † - Significant difference in relation to the ESG; ‡ - Significant difference in relation to the AG.

Similar to trans-resveratrol, this action suggests that the cigarette smoke is responsible for the oxidative damage and for reducing the endogenous antioxidants that cause the imbalance of ROS. These changes cause irreversible damages to lipids, essential proteins and DNA bases or the interruption of important cellular signaling (THÉROND et al., 2000). The antioxidants that are endogenous or come from diet, such as carotenoids and flavonoids, can prevent or regenerate these subsequent damages by the presence of excessive ROS in the essential biological systems (POLJSAK et al., 2013).

Catalase is an enzyme from the endogenous antioxidant defense that reduces the ROS, turning H_2O_2 into H_2O and O_2^-, in which prevents the formation of other more aggressive oxidants, such as OH^- (TAVERNE et al., 2013). Furthermore, the accumulation of H_2O_2 and OH^- may unbalance the expression and activity of this enzyme (MANDRAFFINO et al., 2010). The reduction of the CAT activity in the ESG may generate a reduction in the protein content of sulfhydryl groups because of the exposure to nicotine and other gases. It causes oxidation in the thiol proteins by the inhibition of the expression of reducing enzymes (AL-MALKI; MOSELHY, 2013). However, the nose-only exposure to cigarette smoke caused an increase in the activity of the antioxidant enzyme Superoxide dismutase (SOD) in heart, kidneys and liver. This suggests that the oxidative damage can be accompanied by adaptive responses, which counterbalance the ROS activity (NEMMAR et al., 2013). Similar to trans-resveratrol, in a study with α-tocopherol the authors found results with high CAT activity in the cardiac tissue of rats exposed to cigarette smoke and with supplementation (KOUL et al., 2001). Nevertheless, rats treated with subcutaneous injections of nicotine and epicatechin intragastric supplementation obtained an increase for the CAT activity in the kidney tissue, but without additional effect when associated with α-tocopherol (AL-MALKI; MOSELHY, 2013).

Resveratrol is associated with the increase of nitric oxide (NO) production and expression of NO synthase, suppression of interleukin-6, and reduction of the peroxidation of LDLc (XU; SI, 2012). NO is the main molecule responsible for endothelium-dependent vasodilatation, but it can react with O_2^- and form peroxynitrite ($ONOO^-$). This oxidant reduces the NO bioavailability to the endothelium and may cause inflammatory response (BONETTO et al., 2009). Another oxidizing species such as H_2O_2 may oxidize the cofactor tetrahydrobioptein for the synthesis of NO (DA LUZ; COIMBRA, 2004). Red wine flavonoids are also associated with decreased endothelin-1 production and activation and/or expression of the nuclear factor κβ (DA LUZ; COIMBRA, 2004). Flavonoids can increase the production and bioavailability of NO and protect against vascular diseases resulting from the exposure to cigarette smoke, because the improvement of the endothelial function decreases the risk of cardiac events (DA LUZ; COIMBRA, 2004).

The increasing blood pressure, the decrease in the cardiac output and the functional and morphological changes are also acute cardiac effects of nicotine (MINICUCCI et al., 2009). An experimental study demonstrated that the secondhand smoke is responsible for alteration in the protein products from the advanced oxidation in the plasma, and subsequent development of endothelial dysfunction, intimal hyperplasia and increases in thickness of the myocardial wall (BOOR et al., 2009). The endothelial cells may suffer morphological changes such as disruption of junctional complexes, abnormal formation of cytoplasmic vacuoles, impairment of microtubules, and increasing expression of adhesion molecules on the cell surface (MINICUCCI et al., 2009). Therefore, the cigarette smoke is directly associated with damage and imbalance in endothelial system, which contributes to the development of vascular diseases.

Our data demonstrated that the supplementation with trans-resveratrol increased the CAT activity, which represents a protective effect against the exposure to cigarette smoke. This finding can be explained by the great stimulation of enzymatic pathways such as CAT, required for the ROS, scavenger in non-physiological conditions and of potential oxidation (YU, 1994). The association of these data may represent the restoration of the endogenous antioxidant activity with the trans-resveratrol supplementation.

The absence of vascular and cardiac histopathology analyses that would macroscopically demonstrate tissue damage and other oxidative parameters of stress are the limitations of our study. These variables would make possible a better understanding of the pathophysiological mechanisms caused by the exposure to cigarette smoke as well as of the protective effects of the trans-resveratrol in the cardiovascular system. The measurement of the oxidative stress in other tissues such as lung, kidneys and liver could broaden the findings related to the local and systemic effects of the inhalation of smoke, and especially regarding the trans-resveratrol metabolism in this condition.

Conclusion

In conclusion, our study identified the harmful effects of the exposure to cigarette smoke as increased the lipid damage in rat myocardium. Our findings also indicate cardioprotective effects of trans-resveratrol through the potentiation of enzymatic defenses in response to excessive formation of ROS. This shows that the trans-resveratrol may be a tool to assist the population exposed to the secondary damages of the inhalation of cigarette smoke (smoking and secondhand smoke), who will be benefited by the use of this antioxidant. However, clinical studies are necessary to evaluate the appropriate dosage of trans-resveratrol in order to have its cardioprotective effect.

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