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Occupational exposure to isoflurane impairs liver redox status in mice

Marina Zanin[1], Antonio Sergio Varela Junior[2], Julia Eisenhardt de Mello[3], Rosélia Maria Spanevello[3], Martielo Ivan Gehrcke[2], Maria Eduarda Bicca Dode[2], Mariana Gobbo Braz[4,5], Carine Dahl Corcini[1]

[1]Programa de Pós-Graduação em Ciências Fisiológicas, Instituto de Ciências Biológicas, Universidade Federal do Rio Grande (UFRG) – Rio Grande (RS), Brazil

[2]Faculdade de Medicina Veterinária, Universidade Federal de Pelotas (UFPel) – Pelotas (RS), Brazil

[3]Programa de Pós-Graduação em Bioquímica e Bioprospecção, Universidade Federal de Pelotas (UFPel) – Pelotas (RS), Brazil

[4]Unidade de Pesquisa Experimental (UNIPEX), Faculdade de Medicina, Universidade Estadual Paulista (UNESP) – Botucatu (SP), Brazil

[5]Laboratório de Genotoxicidade (GENOTOX), Faculdade de Medicina, Universidade Estadual Paulista (UNESP) – Botucatu (SP), Brazil

Corresponding author: Marina Zanin - Universidade Federal do Rio Grande - Avenida Itália Km 8 – CEP: 96203-900 - Rio Grande (RS), Brazil – E-mail: mariinazanin@gmail.com

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ABSTRACT

Introduction: Isoflurane, widely used in medical and scientific settings, plays a significant role in the generation of waste anesthetic gas. Occupational exposure to this anesthetic can result in serious health impairments due to chronic exposure, particularly concerning oxidative impact. The liver, being the primary organ responsible for the metabolism of this anesthetic, is a key target for these adverse effects. **Objective:** To evaluate the effects of chronic occupational exposure to isoflurane on the hepatic redox status of mice and investigate whether discontinuation of exposure can restore oxidative balance. **Methods:** Twenty mice were exposed to 50 ppm of isoflurane for 5 hours a day over a period of 30 days. Following exposure, animals were euthanized either on the first day after exposure cessation (immediate euthanasia group - G2 – n:10) or 20 days after the last exposure (late euthanasia group - G3 – n:10) and compared to the control group (G1 – n:10). Their liver tissue samples were subjected to biochemical analyses, assessing the concentration of reactive oxygen species and thiobarbituric acid reactive substances, as well as the activity of catalase and superoxide dismutase enzymes. **Results:** Occupational exposure to isoflurane can affect the activity of antioxidant enzymes in liver tissue. Exposed animals showed a reduction in catalase activity, associated with a late and non-physiological increase in superoxide dismutase activity. **Conclusion:** Isoflurane at purportedly safe concentrations disrupts the hepatic redox status of exposed individuals, and discontinuation of exposure does not guarantee a return to oxidative balance.

Keywords: occupational exposure; isoflurane; oxidative stress; liver.

INTRODUCTION

For decades, anesthesia has been crucial for conducting invasive medical and experimental procedures. Halogenated compounds are currently the most widely used anesthetics, capable of inducing and maintaining general anesthesia by blocking the central nervous system, thereby providing analgesia and loss of consciousness[1]. Isoflurane, chemically known as 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, has emerged as the anesthetic of choice due to its low metabolic rate and solubility, which facilitate rapid induction and recovery[2]. However, isoflurane, along with other volatile anesthetic agents, contributes to the production of waste anesthetic gases (WAG); trace amounts of which are present in the ambient air of operating and experimental rooms, posing potential occupational hazards to exposed personnel[3]. The Occupational Safety and Health Administration[4] estimates that over 250,000 healthcare professionals, including doctors, dentists, and veterinarians, may face potential exposure to WAG and their associated risks. Notably, these figures exclude employees of vivarium and animal experimentation laboratories, where isoflurane is also commonly used[5].

The potential health risks associated with exposure to inhalational anesthetics have been a topic of ongoing debate for several years, with a lack of definitive data regarding the adverse effects on exposed organisms. Karabiyik et al.[6] identified genotoxic lesions in patients who administered isoflurane anesthesia, while Wei et al.[7] proposed a connection between isoflurane exposure and the induction of neuronal apoptosis. Epidemiological studies have linked chronic exposure to low doses of these anesthetics with an increased incidence of leukemia, reproductive disorders, and liver diseases[8,9]. Regarding the redox status, previous research has suggested an association between isoflurane and oxidative stress[3,10]. This has been correlated with cytogenetic and inflammatory damage when examining occupational exposure and its potential risks[11]. Oxidative stress refers to an imbalance between the production and elimination of reac-

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tive oxygen species (ROS), which, while essential for physiological processes such as cell signaling, can lead to cellular dysfunction when produced in excess[12]. Despite numerous studies over recent decades investigating the effects of isoflurane and other halogenated agents on oxidative stress, the findings have been inconsistent[13]. Nevertheless, exposure to isoflurane has consistently been associated with oxidative damage in multiple studies. Its hepatic metabolism is a major pathway to produce free radicals, facilitated by the enzymatic activity of the cytochrome P450 family[14]. Furthermore, isoflurane appears to diminish the capacity of the tissue's antioxidant defense mechanisms[15].

Given the extensive use of isoflurane in both operating rooms and animal experimentation settings, it is crucial to investigate its potential impact on oxidative status, particularly in individuals with chronic and occupational exposure. Considering the lack of studies on the liver's oxidative profile following chronic exposure to isoflurane in a controlled experimental environment.

This study aimed to evaluate the occurrence of oxidative stress in exposed mice by measuring ROS levels, thiobarbituric acid reactive substances (TBARS), and the activities of catalase (CAT) and superoxide dismutase (SOD) in liver tissues.

METHODS

All procedures were carried out in accordance with the Ethics and Animal Experimentation Committee of the Federal University of Pelotas (UFPel) - Opinion 18/2020/CEEA/Reitoria - registration number 23110.036567/2019-91 and complied with the ARRIVE guidelines. Thirty 6-week-old male Swiss albino mice were obtained from the Central Animal House of the UFPel. The sample size was decided based on a power analysis for one-way ANOVA followed by Tukey and Dunnett tests, with an alpha value of 0.05, standard deviation of 1.85, and a sample size of 10; the power of the study is 0.90 for three treatments and

a sample size of 30 animals (10 per treatment). The animals were acclimatized for 1 week before beginning the experiments. In the vivarium, mice were kept in three polypropylene boxes with wood shavings, with 10 individuals in each box. The mice were subjected to a light/dark cycle of 12 h light/12 h dark, temperature of 22°C ±1°C, and humidity of 55% ±5%, with ad libitum water and a commercial pelleted diet specific for mice, provided by the Central Animal House. At the end of the experiment, the mice were euthanized by cervical dislocation, as exposure to anesthetics during euthanasia could potentially influence the experimental outcomes (our unpublished data). Subsequently, the tissues were carefully dissected.

The animals were randomly assigned to three groups, each consisting of 10 individuals, through simple randomization. To mitigate confounding factors, each group's boxes were color-coded. The control group (G1) included individuals not exposed to waste anesthetic gases (WAG), such as isoflurane. The second group (G2) experienced chronic exposure to the anesthetic and was euthanized immediately after the exposure period, on the 31st day. The third group (G3) represented the late group, where individuals were also chronically exposed to WAG isoflurane but remained without contact for 20 days before euthanasia. The animals were placed daily in three 21.6 L acrylic boxes, equipped with holes for gas entry and exit, for 5 hours per day over 30 days[16].

Throughout the exposure period, all boxes were filled with wood shavings, and a 100% oxygen supply at a rate of 1 L/min for each box was maintained. Box one (G1) served as the control, with 10 animals exposed solely to oxygen. The other two boxes, corresponding to the exposure groups, employed a continuous drip system. This system delivered a microdrop (5 µl) of isoflurane to a gauze every 10 minutes[16], allowing vaporization and maintaining an ambient isoflurane WAG concentration of approximately 50 parts per million (ppm)[17]. These values were predetermined through a pilot experiment conducted at the Botucatu Medical School, São Paulo State University (Brazil), using the InfraRan 4-Gas Anesthetic Specific Vapor Analyzer

(Wilks Enterprise/Spectro Scientific, Norwalk, CT), a real-time infrared spectrophotometer. At the conclusion of the exposure period, the boxes were opened, the oxygen supply ceased, and the mice were returned to their maintenance boxes in the vivarium until the next day's exposure.

The daily exposure regimen lasted for 30 days. After this period, exposures were halted. Animals in groups G1 and G2 were euthanized immediately on the first subsequent day, while animals in G3 were euthanized on the 20th day after exposure cessation (late euthanasia). After euthanasia, performed by cervical dislocation, laparotomy was performed, and liver tissues were collected.

Tissue preparation

After organ resection, tissues were homogenized in sodium phosphate buffer (pH 7.4) containing KCl (1:10 w/v), and the homogenate was centrifuged at 2500 ×g for 10 min at 4°C. The pellet was discarded, and the supernatant was used for biochemical analysis[18].

Thiobarbituric acid reactive substances (TBARS) quantification

To determine TBARS levels, the homogenates were mixed with 10% trichloroacetic acid and 0.67% thiobarbituric acid, and subsequently incubated in a dry block at 100°C for 30 min. TBARS levels were determined by measuring the absorbance at 535 nm, and the results were reported as nmol of TBARS per mg of protein[18].

Reactive oxygen species (ROS) quantification

ROS formation was determined according to Ali et al..[19] Oxidation of dichloro-dihydro-fluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) was measured, and the fluorescence intensity was measured at an excitation wavelength of 488 nm and emission

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wavelength of 525 nm 60 min after the addition of DCFH-DA to the medium. ROS levels were expressed as μmol/g of tissue[18].

Superoxide dismutase (SOD) activity

To measure total SOD activity, the method described by Misra and Fridovich[20] was used. This assay is based on the inhibition of superoxide-dependent auto-oxidation of epinephrine to adrenochrome using a spectrophotometer set at 480 nm. The intermediate in this reaction is superoxide, which is then eliminated by SOD. One unit of SOD was defined as the amount of enzyme required for 50% inhibition of epinephrine autoxidation. Specific SOD activity is reported as units per mg of protein (U/mg of protein)[18].

Catalase (CAT) activity

The CAT activity was determined using the method of Aebi.[21] The decomposition of 30 mM hydrogen peroxide (H_2O_2) in 50 mM potassium phosphate buffer (pH 7.0) was continuously monitored using a spectrophotometer at 240 nm for 180 s at a temperature of 37°C. One unit of the enzyme was defined as 1 nmol of H_2O_2 consumed per minute, and the specific activity was reported as units per mg of protein[18].

Protein determination

Protein was determined by Lowry et al.[22] with bovine serum albumin as a standard.

It should be noted that the researchers responsible for the evaluation of results and data were unaware of the allocation of groups, respecting blindness during the experiment.

Statistical analysis

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The data obtained were analyzed for normality using the Shapiro–Wilk test, for homoscedasticity using Hartley's test, and for the independence of residuals by graphic analysis. Because the data met all assumptions for parametric analysis, they were subjected to analysis of variance using the F test ($p \leq 0.05$). In case of statistical significance, the groups were compared with each other using the Tukey test ($p \leq 0.05$), and the treatments were compared with the controls individually using the Dunnett test ($p \leq 0.05$). The software used was SAS 8.2.

RESULTS

The level of ROS, TBARS, CAT, and SOD activity, as markers of oxidative stress in the liver of young mice occupationally exposed to isoflurane, is presented in table 1. ROS and TBARS levels in the liver tissues did not change. The activity of antioxidant enzymes was evaluated in the liver of mice exposed to isoflurane. CAT activity significantly decreased in the groups exposed to the anesthetic, remaining reduced even after the exposure was interrupted (Figure 1). SOD activity, in turn, as can be observed, did not show a significant difference in the immediate euthanasia group (G2) when compared to the control group; it showed, however, an increase in its activity in the group subjected to late euthanasia (Figure 2). The statistical measures of F value, degrees of freedom, and p-value are presented in table 2.

DISCUSSION

The present study is the first to demonstrate, through a scientific experimental method under controlled conditions, that chronic exposure to an environmental concentration of isoflurane deemed safe by the latest ACGIH recommendation[17] adversely affects the hepatic redox status of mice. The possible relationship between occupational exposure to WAG and oxidative stress has been investigated for the last decade, yet it remains a relatively misunderstood field[3]. In this work, the groups exposed to isoflurane WAG exhibited a decrease in catalase (CAT)

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antioxidant activity, which persisted at reduced levels even 20 days after the last exposure, demonstrating that cessation of exposure may not be sufficient to restore enzymatic antioxidant capacity.

According to Kharasch et al.[14] the isoflurane metabolism, by the enzymatic action of cytochrome P450 in the liver, leads to the production of free radicals and constitutes a significant pathway for the establishment of a pro-oxidative environment. These reactive species can interact with molecules and cause oxidative damage, or even inhibit antioxidant enzymes, reducing their activity[15,23], as noted in the present study. However, its role in oxidative stress may involve other components, even in the absence of an increase in the concentration of free radicals. According to Durak et al.[10] the observed inhibition of the CAT enzyme may be due to isoflurane and/or one of its metabolites. Fluoride, a constituent of isoflurane, may inhibit antioxidant enzymes by forming complexes with their cofactor metals such as Cu, Zn, and Fe[10].

Nonetheless, its action on various pro-oxidant and antioxidant balance pathways can be suggested by its relationship with other factors. In addition to its association with trace elements that are intricately linked to the antioxidant machinery[15] and, Venkatapuram et al.[24] investigated the impact of isoflurane on various tissues, analyzing redox status even without an increase in ROS, and found a reduction in the level of p53 protein. This transcription factor is involved in oxidative stress and seemingly linked to catalase activity. According to several studies[24-26], the modulation of GSK-3 (glycogen synthase kinase) by isoflurane indirectly culminates in the degradation of p53. The catalase enzyme, by presenting a protein binding site for p53, is enhanced in the presence of this transcription factor[27]. However, in the absence of p53 due to isoflurane, there is a diminished stimulus on catalase, reducing its activity.

Furthermore, O'Connor et al.[28], while examining p53's role in oxidative stress, identified p53 binding sites in the promoter regions of catalase, which, according to the authors, un-

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derscores its role in influencing the gene expression of this enzyme. From this, it can be suggested that the decreased CAT activity following chronic exposure to isoflurane is closely associated with a reduction in p53 levels induced by this WAG. Additionally, the likely interference in catalase gene expression may explain the sustained reduction in activity even 20 days after cessation of anesthetic exposure. According to Stewart and Pientenpol[29] the inactivation of p53 leads to genomic instability, which can have delayed effects.

The findings of this study are consistent with previous research. Durak et al.[10] reported a decrease in CAT activity and total antioxidant potential in the kidney tissue of pigs subjected to inhalational anesthesia with isoflurane, resulting in oxidative stress and isoflurane-induced kidney toxicity. Similarly, Rocha[30] and Türkan et al.[15] demonstrated a reduction in total antioxidant capacity in individuals exposed to this anesthetic, with CAT playing a significant role in tissue antioxidant capacity. Simultaneously, it is suggested that there was an increase in the activity of the antioxidant enzyme glutathione peroxidase (GPx), which acts in a similar way to CAT. The elevation in GPx activity in the liver may result from reduced CAT activity, and this finding could potentially explain the normal levels of reactive oxygen species (ROS) in the liver and thereby maintain homeostasis in TBARS in this study[31].

Interestingly, SOD activity, as measured, exhibited an increase in the G3 group (Figure 2), which was analyzed after the cessation of exposure to WAG. Bibliographic data vary regarding the precise effect of isoflurane exposure on the activity of this enzyme, oscillating, however, between reduced or unchanged activity after anesthetic exposure[10,15,31]. In the present study, the late increase in SOD activity may be an indirect consequence of exposure to the anesthetic. According to Türkan et al.[32], individuals chronically exposed to WAG isoflurane presented high levels of plasma zinc. This element, which serves as a cofactor for the SOD enzyme, may contribute to its stability and stimulation[33]. These findings are consistent with

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those of Arslan et al.[34], who showed an elevation in SOD levels following exposure to halogenated anesthetics, concomitantly with an increase in GPx.

However, unlike the antioxidant activity of CAT and GPx, which act in a complementary manner, SOD specifically acts on a particular free radical, the superoxide anion. Although previously discussed studies show a suppression of antioxidant activity upon exposure to anesthetic residues, several studies have considered a supposed protective role of isoflurane[35]. According to the aforementioned authors, individuals subjected to tissue oxidative stress and exposed to isoflurane showed an improvement in the antioxidant response, especially through an increase in SOD activity[36]. Furthermore, a study developed by Mu et al.[37] demonstrated that organisms in an inflammatory state can benefit from exposure to subanesthetic concentrations of isoflurane; this agent is capable of increasing serum SOD activity, resulting in improvement in oxidative status. From another perspective, yet, the effect of this anesthetic is capable of increasing the production of mitochondrial superoxide anion. This free radical, being the main substrate of SOD, can explain the increase in enzymatic activity[35].

Despite this, it would be premature to state that occupational exposure to isoflurane can beneficially stimulate the antioxidant system, since, in addition to reduced CAT activity, the stimulus to high SOD activity comes from oxidative or inflammatory damage not observed in healthy tissues[38]. Thus, in line with previous studies[3,10,15] the present study demonstrates that chronic exposure to WAG, particularly isoflurane, adversely affects the hepatic oxidative environment. Our data reveal oxidative imbalance and impact on the antioxidant machinery in the hepatic tissue of mice, when examining the effect of ambient concentrations of isoflurane, WAG considered safe for occupational exposure[17]. Additional studies that consider the measurement of p53 and trace elements, as well as addressing the aspects of catalase gene expression in the face of isoflurane exposure, are recommended. However, the decrease in CAT activity,

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whether associated with increased SOD activity in the present study, further supports the effect of isoflurane on the balance of antioxidant defenses in the exposed individuals.

The authors recognize that the lack of a second control group for the experimental group G3 is a limitation of this study. However, the present experiment was performed in this way, advocating the 3 R's principle of reduction and considering the absence of differences in antioxidant enzymatic activity among groups with little age difference[39]. Additionally, another limitation is the lack of continuous monitoring of WAG concentration in the exposure environment. However, conducting pilot tests with a portable anesthetic gas analyzer to assess exposure levels proved useful. Although continuous monitoring may not be fully dependable, studies indicate that most occupational risk research is conducted without measuring WAG[40]. Thus, this experimental design can illustrate the real occupational risk scenario observed in work environments. Exposure to isoflurane waste anesthetic gases (WAGs) at doses considered supposedly safe by some occupational health guidelines may impair the oxidative balance in the liver, which could have significant implications for the health of exposed individuals, given the liver's crucial role in the metabolism of toxic substances.

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Figure 1: Effect of isoflurane on catalase activity. Catalase (CAT) activity in liver tissues, expressed as U/mg of protein, after occupational exposure to isoflurane. G1, control group; G2, immediate euthanasia group; G3, late euthanasia group. *significant, comparing groups G2 and G3 with control G1, individually, by Dunnett's test ($p \leq 0.05$)

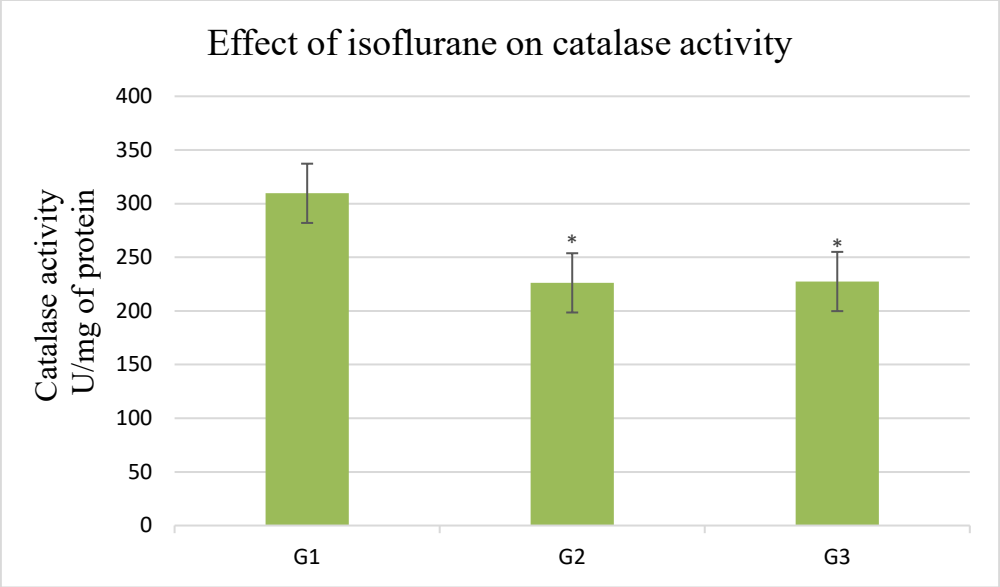

Figure 2: Effects of isoflurane on superoxide dismutase activity. Superoxide Dismutase (SOD) activity in liver tissues, expressed as U/mg of protein, after occupational exposure to isoflurane. G1, control group; G2, immediate euthanasia group; G3, late euthanasia group. *significant, comparing groups G2 and G3 with control G1, individually, by Dunnett's test ($p \leq 0.05$)

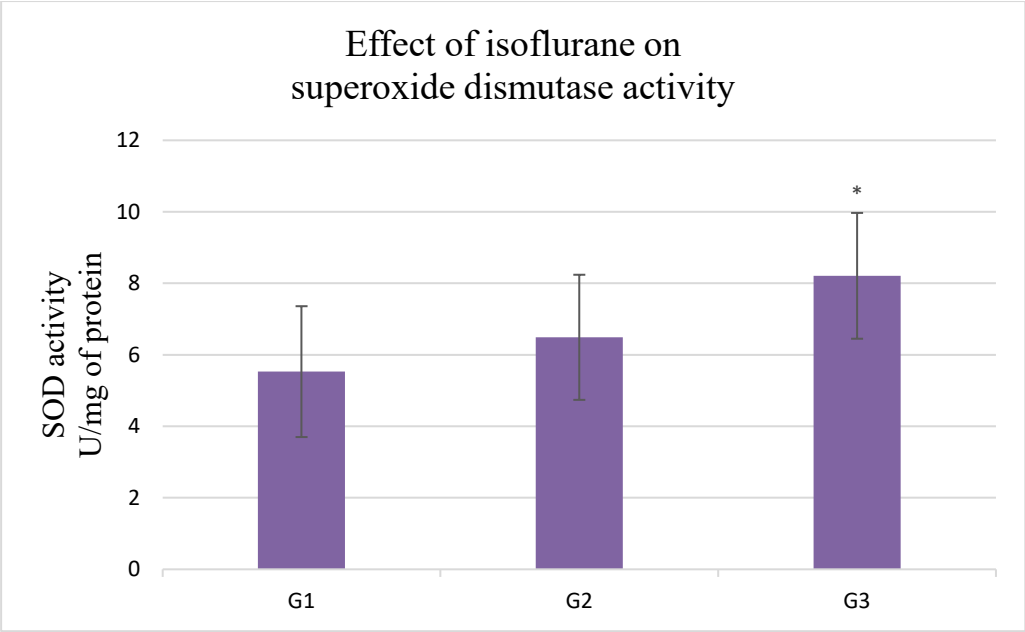

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Table 1: Reactive oxygen species (ROS) (µmol DCF/mg of protein), superoxide dismutase (SOD) activity (SOD/mg of protein), catalase (CAT) activity (CAT/mg of protein), and TBARS (TBARS/mg of protein) in mouse liver samples.

Group	ROS	SOD	CAT	TBARS
G1	9.18 a	5.53 b	309.06 a	1.41 a
G2	10.73 a ^{ns}	6.49 b ^{ns}	226.14 b *	1.61 a ^{ns}
G3	10.70 a ^{ns}	8.21 a *	227.42 b *	1.91 a ^{ns}
Coefficient of variation	0.28	0.26	0.39	0.62

Means followed by the same letter in the column do not differ when comparing the three groups by the Tukey test ($p \leq 0.05$).
* significant, ^{ns} non-significant - comparing groups G2 and G3 with control G1, individually, by Dunnett's test ($p \leq 0.05$).

Table 2: Analysis of variance Summary

Variable	**Degrees of Freedom (df)**		**F Value (F.V.)**	**p-Value**
Reactive Oxygen Species – ROS	Treatment: 2,	Residue: 24	0.71	0.5009^{ns}
Enzyme Activity of Superoxide Dismutase – SOD	Treatment: 2,	Residue: 24	5.08	0.0145*
Enzyme Activity of Catalase – CAT	Treatment: 2,	Residue: 24	4.83	0.0013*
Thiobarbituric Acid Reactive Species – TBARS	Treatment: 2,	Residue: 24	0.51	0.6069^{ns}

^{ns} not significant, * statistically significant.

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