



Original/Investigación animal

## Effect of long term intake of white tea on acute oxidative stress in rats

Cristóbal Espinosa, Daniel González-Silvera, Francisca Pérez-Llamas, José Ángel López-Jiménez y Salvador Zamora

Physiology Department, Faculty of Biology, University of Murcia, 30100 Murcia (Spain).

### Abstract

**Introduction:** the well known antioxidant properties of white tea include the prevention of cancer, neurodegenerative diseases and oxidative stress. Adriamycin can generate an amount of oxidative stress *in vivo*.

**Objective:** evaluate long term intake of white tea on plasma antioxidant capacity and on the fatty acid profile of liver and heart microsomes in animals subjected to acute oxidative stress.

**Methods:** rats were given distilled water (controls), 15 mg/d (dose 1) or 45 mg/d (dose 2) of solid white tea extract/per kilogram of body weight for 12 months. After this time, all the animals received an injection of adriamycin (ADR) (10 mg/kg body weight), except half of the control group, which were given an injection of saline solution. Samples of plasma and liver and heart were taken. The antioxidant activity, the carbonyl groups and hydroperoxide concentration were analyzed in plasma, and the fatty acid profiles of liver and heart microsomes were obtained.

**Results & discussion:** only the hydroperoxides showed significant changes, while slight tendencies were observed in antioxidant activity and the carbonyl groups. Although the long term intake of white tea and the administration of adriamycin did not change the fatty acid profile, slight tendencies existed for the SFAs, MUFAs and PUFAs.

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Key words: *Adriamycin. Fatty acids. Oxidative stress. Polyphenols. Plasma. White tea.*

### EFFECTO DE LA INGESTIÓN A LARGO PLAZO DE TÉ BLANCO SOBRE EL ESTRÉS OXIDATIVO AGUDO EN RATAS

### Resumen

**Introducción:** las propiedades antioxidantes del té son ampliamente conocidas, entre las que se incluyen la prevención del cáncer, diversas enfermedades neurodegenerativas y otras patologías relacionadas con el estrés oxidativo. Por otro lado, la adriamicina es un agente antitumoral que tiene la capacidad de generar estrés oxidativo *in vivo*.

**Objetivo:** valorar el efecto de la ingesta de té blanco a largo plazo sobre la capacidad antioxidante plasmática y el perfil de ácidos grasos de microsomas de hígado y corazón en animales sometidos a estrés oxidativo agudo.

**Métodos:** se dispuso de ratas a las que se les administró diferentes dosis de té blanco: 0,15 y 45 mg de extracto sólido de té/kg de peso corporal durante 12 meses. Tras este periodo de tratamiento con té blanco, todos los animales recibieron una inyección intraperitoneal de adriamicina (ADR), 10 mg/kg de peso corporal, excepto la mitad del grupo control, que recibieron una inyección de solución salina. Fueron obtenidas muestras de sangre, corazón e hígado. Se analizó la capacidad antioxidante total y se realizaron análisis de oxidación proteica y lipídica en plasma. Además, se obtuvo la fase microsomal de hígado y corazón.

**Resultado y discusión:** se observó una fuerte oxidación lipídica en plasma y una recuperación en los animales tratados con las diferentes dosis de té. La actividad antioxidante y la oxidación proteica, aunque relevantes, solo muestran una ligera tendencia a recuperarse con el tratamiento con té. En cuanto al perfil de ácidos grasos, solamente se observan ligeras tendencias en el porcentaje de ácidos grasos saturados, monoinsaturados y poliinsaturados.

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Palabras clave: *Adriamicina. Ácidos grasos. Estrés oxidativo. Polifenoles. Plasma. Té blanco.*

**Correspondence:** Cristóbal Espinosa Ruiz.  
Department of Physiology, Faculty of Biology.  
University of Murcia. Campus de Espinardo.  
30100 – Murcia, Spain.  
E-mail: cer48658@um.es, cespinosas31416@gmail.com

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## Abbreviations

ABTS: 2,2'-azino-bis-3-(ethylbenzothiazoline-6-sulphonic acid).

ADR: Adriamycin.

EGCG: Epigallocatechin gallate .

ECG: Epicatechin gallate .

EGC: Epigallocatechin .

HUFAs: Highly unsaturated fatty acids .

MUFAs: Monounsaturated fatty acids.

PUFAs: Polyunsaturated fatty acids.

PXI: Lipid Peroxidation Index .

ROS: Reactive Oxygen species.

SFAs: Saturated fatty acids

UI: Unsaturation Index.

## Introduction

Reactive oxygen species (ROS) are naturally produced during in cellular pathways of aerobic metabolism, including oxidative phosphorylation, electron transport chains in mitochondria and microsomes, the activity of oxido-reductase enzymes, or even immunological reactions such as active phagocytosis<sup>1</sup>. These free radicals are neutralized by an elaborate antioxidant defense system consisting of enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and numerous non-enzymatic antioxidants, including vitamins A, E and C, glutathione, ubiquinone, flavonoids and others.

Polyphenols are the most abundant antioxidants in the diet. Indeed, fruits, vegetables, beverages (tea, wine, juices), plants, and some herbs are loaded with powerful antioxidant polyphenols. Phenolic compounds have received increasing interest from consumers and manufacturers because numerous epidemiological studies have suggested associations between the consumption of polyphenol-rich foods or beverages and the prevention of certain chronic diseases such as cancers and cardiovascular diseases<sup>2,3</sup>.

Tea (*Camellia sinensis*) has beneficial properties in the prevention of diseases such as cancer, heart disease and neurodegenerative diseases<sup>4</sup>. Such effects have been attributed to the flavonoid content of tea, which may be beneficial in pathological situations associated with the high production of free radicals<sup>5</sup>.

The amount of fatty acids and the level of antioxidants found in biological membranes differ between species and the tissues of the same species. The variation in peroxidable long chain fatty acids and antioxidants found in membranes makes them vulnerable to lipid peroxidation. Unsaturated fatty acids are more susceptible to ROS induced damage, the sensitivity to lipid peroxidation increasing as a function of the number of double bonds<sup>6</sup>. The measurement of lipid peroxidation is one of the most commonly used assays for radical induced damage<sup>7,8</sup>.

Adriamycin (ADR) is a quinone belonging to the group of anthracyclines. ADR is used in chemotherapy for treating different kind of cancers, including lymphomas, leukaemia or solid tumours due to its ability to prevent cell replication<sup>9</sup>. In addition, ADR produces ROS *in vivo* once it is metabolized, causing toxicity in patients and representing a serious problem in the treatment of cancers. However, clinical studies in humans have shown that treatment with certain antioxidants does not fully reverse the cardiotoxic effect of ADR, leading to and there is a search for new agents to reverse this secondary effect<sup>10</sup>.

Our previous research showed that long term treatment with white tea inhibits the oxidative effect of ADR in different organs (liver, brain, heart)<sup>11</sup>, and looked at how the mechanisms involved in white tea prevent the oxidative damage of ADR in liver<sup>12</sup>. The aim of this study is to analyze the effects of ADR and the long term intake of white tea extract on plasma and on the fatty acid profile of liver and heart in rats.

## Materials and methods

### Experimental design

The study protocol was in accordance with the Helsinki Declaration and was approved by the Bioethical Committee of Murcia University, Spain. Twenty-two weaned Sprague–Dawley rats were purchased from the University Animal Center REGA-ES300305440012 (Murcia, Spain). The animals received a commercial diet for rodents (Harlan 2014 Global Rodent Maintenance, Barcelona, Spain) and were kept in cages in groups of three or four, with males separated from females.

The animals, n=22 (an equal mixture of males and females) were maintained in a room under controlled conditions: temperature  $23 \pm 2$  °C, relative humidity  $55 \pm 5\%$ , ventilation frequency 18 times/h and a 12:12 h light–dark photoperiod. For 12 months, the rats received different doses of tea in their drinking water. The control (n=12) received distilled water; dose 1 group (D1) (n=5) received 15 mg of solid tea extract/kg body weight (4 mg polyphenols/kg body weight), and dose 2 group (D2) (n=5) received a dose of 45 mg of solid tea extract/kg body weight (12 mg polyphenols/kg body weight) dissolved in distilled water. The drink was available continuously, and rats drank *ad libitum*. We estimated that these doses contained amounts of tea equivalent to those consumed by humans drinking between zero and three cups of tea per day, reflecting Western habits<sup>13</sup>, and up to nine cups of tea per day, reflecting oriental habits<sup>14</sup>. After 12 months, all the animals received an intraperitoneal injection of ADR (10 mg/kg body weight), except for half of the animals in the control group that received an injection of saline solution. In this way, four experimental groups were established: control, control + ADR (C + ADR), white tea dose 1 + ADR (D1 + ADR) and white tea dose 2

+ ADR (D2 + ADR). Forty-eight hours after the administration of ADR, and following overnight fasting, the rats were weighed and sacrificed under anaesthesia (sodium thiopental). The samples of plasma were obtained from recently anesthetized rats at the beginning of the operation and were kept frozen at  $-80^{\circ}\text{C}$  until analysis. The heart and liver were removed and immediately rinsed in cold saline solution. The microsomes, obtained, as described by Philipp and Shapiro (1979), were kept frozen at  $-80^{\circ}\text{C}$  prior to analysis<sup>15</sup>.

#### *Preparation of tea infusion*

The tea extract was prepared in Barcelona (Spain) following the protocol described by Almajano<sup>16</sup>. The tea infusions were freeze dried, and the extracts were kept at  $-20^{\circ}\text{C}$  until use. The total phenol content was analysed by the Folin–Ciocalteu method, and the profile of the main catechins was determined by capillary electrophoresis. The white tea used contained  $2180 \pm 161$  mg of total polyphenols/100 g tea leaves, expressed as mg gallic acid equivalent per litre of infusion. The individual catechin content of the white tea was analysed by RP-HPLC<sup>17</sup>. The main catechin observed was epigallocatechin-3-gallate (EGCG) ( $1525 \pm 113.4$  mg/100 g tea leaves). Several other polyphenols were also present (mg/100 g tea leaves), including epigallocatechin ( $159 \pm 15.2$ ) and epicatechin ( $54.3 \pm 2.5$ ), as well as methylxanthines including theophylline and caffeine<sup>17</sup>.

#### *Hydrophilic antioxidant activity (HAA)*

HAA was measured in the plasma using the method described by Arnao, Cano and Acosta (1999), which is based on the ability of the antioxidants in the sample to reduce the radical cation of 2,2'-azino-bis-3-(ethylbenzothiazoline-6-sulphonic acid) (ABTS), as determined by the decolouration of ABTS<sup>+</sup>, and measuring the quenching of the absorbance at 730 nm. This activity is calculated by comparing the values of the sample with a standard curve of ascorbic acid and expressed as ascorbic acid equivalents (mmol) per milligram of protein<sup>18</sup>. The samples were analyzed in triplicate.

#### *Hydroperoxides*

The technique described by Jiang, Hunt and Wolff (1992) was used to determine hydroperoxides. This is based on the reaction in which  $\text{Fe}^{2+}$  is oxidised to  $\text{Fe}^{3+}$  in acidic conditions. The reaction is characterised by the donation of a negative anion mediated by the action of Fox's reagent. It uses a dye, xylenol orange, sensitive to the oxidation of iron. In the case of hydroperoxides, iron acts as a transition metal. The orange colour intensity of the xylenol increases with the presence of hydroperoxides. The AMN (ammonium

ferrous sulphate) is the source of iron for lipid peroxidation, while AAPH (2-2 azobis amidinopropane) is a strong inducer of lipid peroxidation<sup>19</sup>.

#### *Carbonyl groups*

The concentration of carbonyl groups in plasma, which represents the degree of protein, oxidation was determined by 2,4-dinitrophenylhydrazine (DNPH) reagent, according to the method of Reznick and Packer (1994), modified by Bailey (2001). Microsomal samples were divided into two aliquots of at least 1 mg/ml of protein in each. To one of them, 2 ml of 2 N HCl were added before incubating at room temperature for 1 h, stirring intermittently. This served as the Control tube. To the other tube, 2 ml of 10 mM DNPH dissolved in 2 N HCl was added. This was incubated and stirred as above, serving as the experimental sample. After incubation, the reaction in both cases was stopped by adding TCA (tri-chloro acetic acid) at 20% and then the mixture was centrifuged at 5,000g for 13 min. The pellets were washed with 10% TCA and re-spun. The precipitate was washed three times with 2 ml of a solution of ethanol-ethyl acetate (1:1). The final protein precipitate was resuspended in 6 M guanidine–HCl and centrifuged, reading the supernatant by a sweep of 344–390 nm. The carbonyl content was expressed in nmol/mg protein<sup>20,21</sup>.

#### *Lipid extraction and fatty acid analysis*

The total amount of lipids in samples was determined gravimetrically after extraction, essentially as described by Folch et al. (1957). The total lipid extracts were subjected to acid-catalysed transmethylation using the method described by Christie (2003). The fatty acid methyl-esters (FAME) obtained were separated and quantified by gas–liquid chromatography using a Hewlett–Packard 5890 gas chromatograph equipped with a capillary column (SPTH-2560, SUPELCO, 100 m $\times$ 0.25 mm I.D., 0.20  $\mu\text{m}$  thickness). Peaks were identified by comparing their retention times with appropriate FAME standards (purchased from Sigma Chemical Company, St. Louis, MO, USA). Concentrations of individual fatty acids were expressed as percentages of total fatty acids. Various parameters of interest, such as the sum of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), highly unsaturated fatty acids (HUFA, fatty acids of 20 or more carbon atoms and 3 or more double bonds) and the peroxidation index (PI, as a susceptibility index) were calculated<sup>22,23</sup>.

#### *Statistical analyses*

The results were expressed as means  $\pm$  SD. The normality of the variables was confirmed by the Shapiro–

Wilk test and homogeneity of variance by the Levene test. Statistical differences among the four groups of treatments were assessed by one-way ANOVA analyses, followed by the Bonferroni or the Games Howell test, depending on the homogeneity of the variables. The fatty acid composition of whole animals is represented as the mean  $\pm$  SD. Comparisons between values from the groups were made using a one-way ANOVA, and a Tukey-b post hoc test for multiple comparisons. The significance level was 95% in all cases ( $P < 0.05$ ). All data were analysed by the computer application SPSS for Windows® (version 15.0, SPSS Inc., Chicago, USA).

## Results

During the 12 months that the dietary treatment lasted, food consumption was similar in all the groups, and no differences were detected in the body weights of the animals as a result of consuming tea (Data not shown).

The analysis of HAA (Figure 1) and carbonyl groups (Figure 2) did not show any significant differences between the groups. However, it was shown that antioxidant capacity show a trend to decrease in C+ADR group and recover its levels in tea groups and the carbonyl group pointed to a increase in oxidation of amino acids in control + ADR group but not in D1+ADR and D2+ADR group.

The analysis of hydroperoxides (Figure 3) pointed to a significant increase in the control + ADR group. The dose 1 + ADR group showed no difference from the control + ADR group and dose 2 + ADR group. The dose 2+ADR group showed a restoration of basal levels.

The fatty acids analysis of liver and heart microsomes (Table I and Table II, respectively) identified no differences between the groups. However, the PXI showed a trend to increase in C+ADR group and to decrease in D1+ADR and D2+ADR groups compared with the control.

## Discussion

In a previous study, we analyzed different organs (liver, brain and heart) observing a response to the long-term intake of white tea, which prevented ADR from damaging the tissues<sup>24</sup>.

In plasma the results were not as clear as previously reported. The pro-oxidant effect of toxin caused led to the C+ADR group, showing a tendency to decrease the hydrophilic antioxidant capacity (Figure 1). Weiss et al. (1992), considered adriamycin to exert a pro-oxidant effect when it is metabolized, producing oxidative damage near the site where the free radicals are formed (mitochondria, microsomes, etc.), but not reaching the plasma; that is, it causes great damage in the intracellular environment than in the extracellular<sup>25</sup>. Indeed, this would explain the attenuated effect of this substance observed in plasma.

The effect of adriamycin on plasma lipids and proteins, was more evident than on the AAT in the plasma. There was a significant increase in the concentration of hydroperoxides and a tendency for the concentration of carbonyl groups to increase in plasma after administration of the drug. ADR caused oxidation in plasma, an effect that was even more noticeable in the hydroperoxide analysis, perhaps because the FOX is the most sensitive, convenient and simple method for determining the lipid oxidation in plasma<sup>19</sup>.

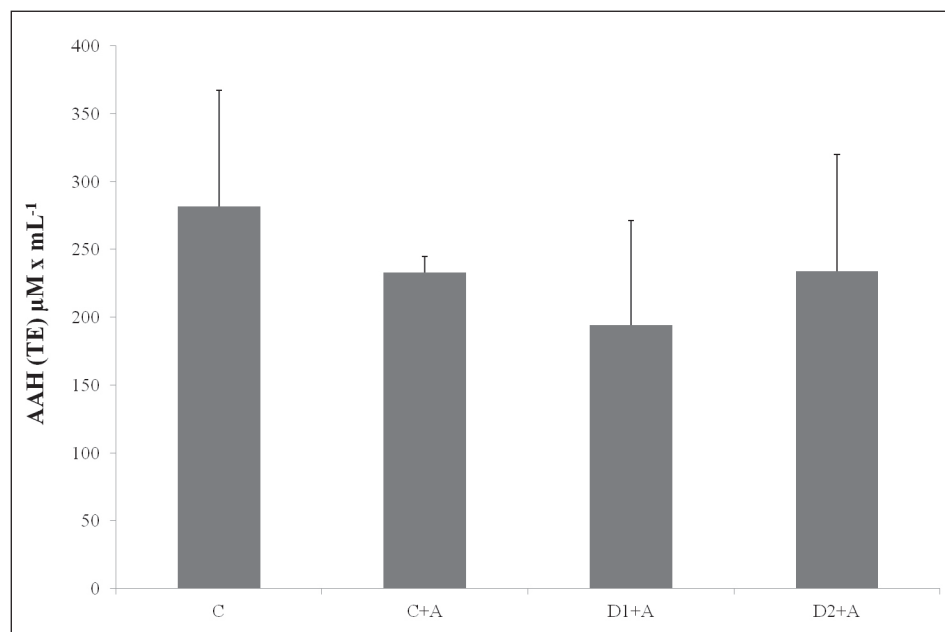


Fig. 1.—Hydrophilic antioxidant activity (HAA) expressed in equivalents of ascorbic acid (M) per mg of protein. Values are the mean  $\pm$  SD of 5 animals per group. Values with different letters indicate statistically significant differences among treatments ( $p < 0.05$ ).

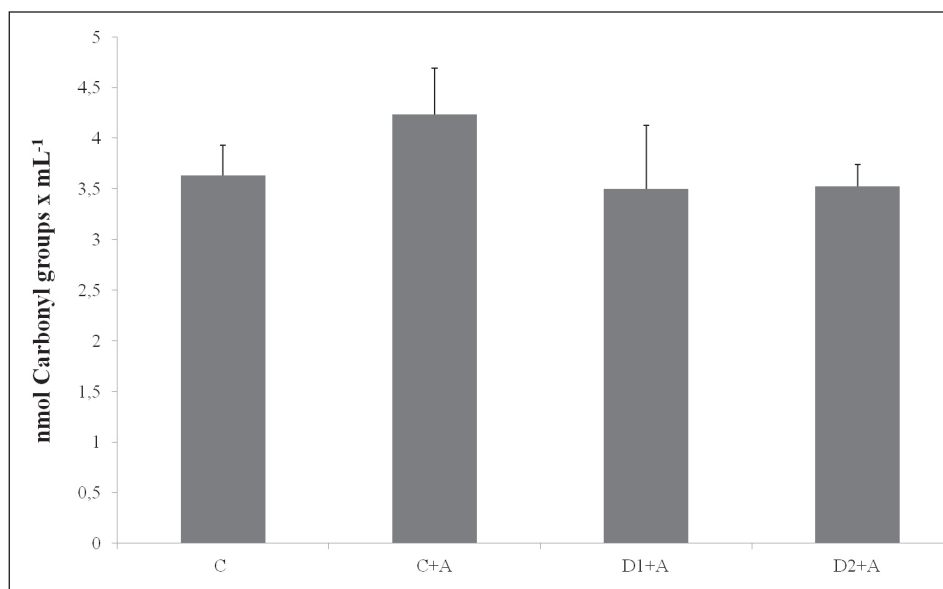


Fig. 2.—Concentration of carbonyl groups expressed in nanomols per mg of protein. Values are the mean  $\pm$  SD of 5 animals per group. Values with different letters indicate statistically significant differences among treatments ( $p < 0.05$ ).

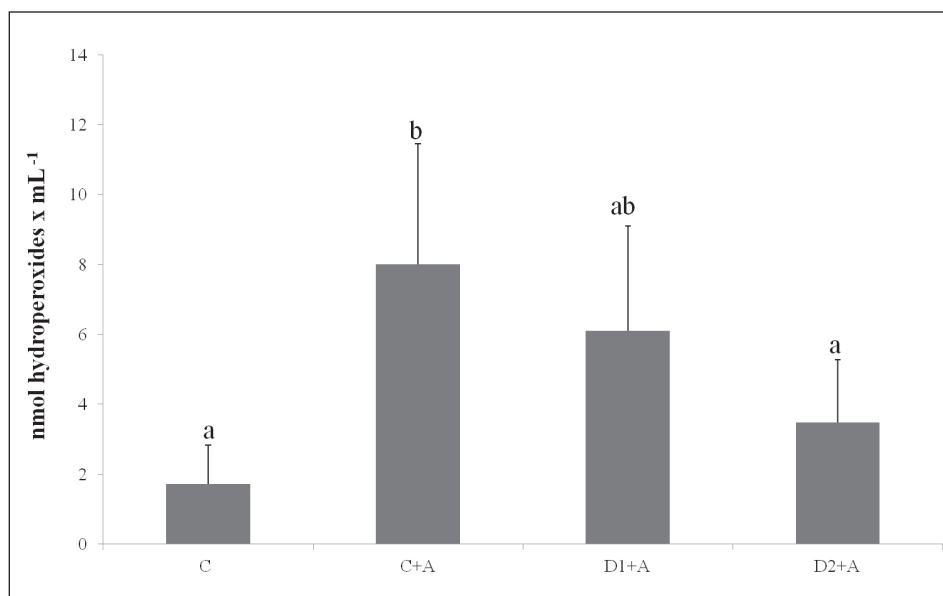


Fig. 3.—Concentration of hydroperoxides expressed in nanomols per mg of protein. Values are the mean  $\pm$  SD of 5 animals per group. Values with different letters indicate statistically significant differences among treatments ( $p < 0.05$ ).

It has been reported that tea has antitumoral and antimutagenic properties since it blocks the binding of substances that promote tumors to receptors<sup>26</sup>, such as hormones and growth factors<sup>27,28</sup> and have been attributed palliative effects in neurodegeneration caused by ischemia<sup>29</sup>. Most of these studies were conducted with green tea, but there are few studies involving white tea. Some studies have shown the protective effect of both types of tea in mice, in which the total antioxidant capacity increased in different organs and tissues such as heart, lung<sup>30</sup> and colon<sup>31</sup>.

In plasma samples, the protective effect of tea has been assessed by the AAT technique described by Arnao et al. (1999). None of the analyses showed a significant increase in plasma antioxidant capacity in rats treated with tea compared with control rats (Figure 1).

Similar results have been described in mice treated with white tea, which did not significantly increase plasma antioxidant activity<sup>30</sup>. Furthermore, it has been reported that, upon the administration of antioxidants such as selenium in order to counteract the oxidative damage caused by adriamycin, significant changes were observed in liver, but not in plasma<sup>32</sup>. The decrease in oxidative damage in plasma due to the administration of white tea was not reflected in the concentration of carbonyl groups, which only showed a slight trend to increase in C + ADR group (Figure 2). However, this tendency becomes statistically significant in the analysis of hydroperoxides, which returned to baseline levels at the lowest dose of white tea (Figure 3). These data are consistent with other studies conducted with green tea in the plasma of rats, which showed a reco-

very of the peroxide caused by oxidative damage due to ethanol<sup>33</sup> and an increase in antioxidant activity and decrease in lipid peroxidation<sup>34</sup>, and with our previous research which described the protective effect of white tea intake<sup>11,12</sup>.

Very few studies in the literature have looked at the possible pro-oxidant effect of adriamycin and tea on the profile of fatty acids (FA) in liver microsomes, and none, to our knowledge, have studied the effect on heart microsomes.

This study did not show significant changes in the fats profile of liver microsomes after the administration of adriamycin (Table I). Only a slight trend was observed towards a lower proportion of total FSA. The results would agree with those described by Català et al. (2007), who found a decrease, although statistically significant in their case, in the amounts of

SFA and the arachidonic and docosahexaenoic acids in mitochondria of hepatocytes after treatment with adriamycin<sup>35</sup>. The effects on mitochondria could be explained because this is where the drug exerts its pro-oxidant action<sup>36</sup>.

Our results also failed to show significant changes in the proportion of total SFA and in the ratio of n-3 / n-6, and only a slight upward trend in total MUFAs was observed. Similar results after treatment with the same drug were also described by other authors<sup>37</sup>. Indeed, in rat hepatocytes treated with adriamycin and / or selenium, the latter as an antioxidant, Bordoni et al. (2008) observed no change in the ratios of SFA, MUFAs and PUFAs. There was only a slight increase in the concentration of arachidonic acid because adriamycin had no effect but the selenium increased the activity of the  $\delta$ -6-desaturase<sup>32</sup>.

**Table I**  
*Fatty acid composition (% of total fatty acid) of lipids in rat liver.*

	<i>Control</i> (n=4)	<i>Control + ADR</i> (n=6)	<i>Dose 1 + ADR</i> (n=5)	<i>Dose 2 + ADR</i> (n=5)
SFA	39,9 ± 0,3	37,7 ± 8,9	41,5 ± 1,4	41,7 ± 1,3
MUFA	7,7 ± 0,2	9,1 ± 3,8	9,7 ± 0,8	8,3 ± 1,3
n-6 PUFA	42,6 ± 0,3	42,7 ± 7,4	39,2 ± 2,5	40,5 ± 1,9
n-3 PUFA	9,6 ± 0,7	10,4 ± 1,8	9,4 ± 1,4	9,4 ± 0,9
Total PUFA	52,3 ± 0,3	53,2 ± 6,4	48,7 ± 1,4	49,9 ± 1,1
n-3/n-6	0,22 ± 0,01	0,25 ± 0,06	0,24 ± 0,05	0,23 ± 0,003
UI	202,6 ± 3,8	206,5 ± 12,9	190,3 ± 7,4	194,2 ± 4,6
PXI	195,8 ± 6,1	198,8 ± 10,3	181,6 ± 13,2	187,1 ± 8,4

Some minor fatty acids (<0.1 g/100 g of fatty acids) are not shown. Data are expressed as mean ± standard deviation. UI: Unsaturation Index. PXI: Lipids Peroxidation Index.

**Table II**  
*Fatty acid composition (% of total fatty acid) of lipids in rat heart*

	<i>Control</i> (n=4)	<i>Control + ADR</i> (n=6)	<i>Dose 1 + ADR</i> (n=5)	<i>Dose 2 + ADR</i> (n=5)
SFA	35,0 ± 13,1	48,9 ± 5,7	43,5 ± 6,9	41,7 ± 11,3
MUFA	29,7 ± 18,9	12,6 ± 1,9	20,0 ± 3,8	28,1 ± 10,3
n6 PUFA	23,7 ± 10,3	30,5 ± 5,6	25,1 ± 7,2	17,3 ± 6,4
n3 PUFA	11,4 ± 8,6	7,8 ± 0,9	11,3 ± 3,5	12,8 ± 7,6
Total PUFA	35,1 ± 10,9	38,3 ± 6,1	36,4 ± 8,2	30,1 ± 1,2
n-3/n-6	0,6 ± 0,77	0,26 ± 0,06	0,47 ± 0,19	0,97 ± 0,81
UI	160,2 ± 45,7	149,9 ± 20,9	149,7 ± 26,8	146,1 ± 27,1
PXI	134,0 ± 62,7	135,5 ± 22,1	128,8 ± 27,6	126,1 ± 30,6

Some minor fatty acids (<0.1 g/100 g of fatty acids) are not shown. Data are expressed as mean ± standard deviation. UI: Unsaturation Index. PXI: Lipids Peroxidation Index.

Our study did not identify significant changes in the fat profile of heart microsomes after the administration of adriamycin (Table II) and only showed tendencies in SFA, MUFAs and PUFAs. Also, there was a trend for the UI to decrease and the PXI to increase, which could be explained by the oxidative stress oxidizing the double bonds of fatty acids, which are susceptible to damage by ADR. As the heart is one of the most damaged organs by ADR<sup>38</sup>, the observed effect of white tea can be considered an important finding of our research.

In conclusion, the protective effect of white tea administered long term was only evident in the analysis of hydroperoxides in plasma, probably because the method used was appropriate for use in plasma. However, it is evident that ADR caused oxidative damage in plasma and treatment with the white tea extract protected against the oxidative stress. The fatty acid profiles did not show any significant change, although the slight trends observed could have been caused by treatment with ADR and the protective effect of white tea against oxidative stress.

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## Conflict of Interest

The authors declare that they have no conflicts of interest.

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