Research Article

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Long-term intake of white tea prevents oxidative damage caused by adriamycin in kidney of rats

Cristóbal Espinosa,[a*] José A López-Jiménez,[a] Francisca Pérez-Llamas,[a] Francisco A Guardiola,[b] Maria A Esteban,[b] Marino B Arnao[c] and Salvador Zamora[a]

Abstract

BACKGROUND: White tea infusion (*Camelia sinensis*) has antioxidants properties. The infusion contains polyphenols that have been proposed to induce antioxidant response element (ARE) response via nuclear factor E2-related factor 2 (NRF2). Adriamycin (ADR) has antitumour properties and oxidative effects. Oxidative stress is related to a variety of kidney diseases. Prevention of the oxidative stress through long-term intake of white tea and the study of the molecular mechanisms involved in protection could be of great interest. Rats were given distilled water, 0.015 or 0.045 g of solid white tea extract kg^{-1} body weight for 12 months. Animals received an injection of ADR. In kidney, oxidative stress parameters were measured, the expressions of nuclear factor E2-related factor 2 gene (*Nrf2*), and detoxifying and antioxidants genes were analysed, and the activities of catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) were measured.

RESULTS: ADR administration increased oxidative parameters and decreased the antioxidant activity; significantly increased the expression of analysed genes and the activity of CAT and SOD and decreased GR activity. The highest white tea dose protected redox status and inhibited ARE response.

CONCLUSION: Long-term intake of white tea protected kidney against the oxidative stress. ADR activated the ARE response but in animals treated with the highest dose of white tea, this response was inhibited, probably for antioxidant protection.

Keywords: adriamycin; ARE response; kidney; *Nrf2*; polyphenols; oxidative stress; white tea

INTRODUCTION

The beneficial properties of tea have been known for centuries and have been studied for decades. These properties include the prevention of various diseases, such as cancer, heart disease and neurodegenerative processes,[1] and are attributed to the flavonoids present in tea, whose benefits are associated with pathologies characterised by a high production of oxidative stress.[2] However, the physiological mechanisms which relate the antioxidant properties of polyphenols with their effects on health are far from being understood.[2]

The differences between types of tea arise from the origin of basic material and their subsequent processing. For example, to produce green tea, the young leaves of *Camelia sinensis* are rolled and steamed to minimise oxidation, while white tea is steamed and dried immediately after picking the very young leaves or buds to prevent oxidation.[3]

The main catechins in white tea are catechin (C), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), epigallocatechin (EGC) and epicatechin (EC), the exact quantities of each depending on different types of tea. EGCG is the major compound in white and green tea, although in white tea caffeine, gallic acid, theobromine, EGC and ECG are present in greater proportions, which may be related to its higher antioxidant activity.[4]

The incidence of chronic kidney disease is increasing in developed and developing countries, although it is generally recognised that many patients with chronic kidney disease are more likely to die of cardiovascular events before renal dysfunction.[5] Of all the common symptoms in chronic kidney disease (endothelial dysfunction, inflammation, oxidative stress, anaemia, proteinuria, changes in the metabolism of vitamin D, etc.) oxidative stress has attracted the greatest interest among researchers.

A cohort study comprising more than 13 000 elderly patients revealed that the increased incidence of cardiovascular events may in part be related to the fact that patients with kidney

* *Correspondence to: Cristóbal Espinosa Ruiz, Department of Physiology, Faculty of Biology, University of Murcia, Campus de Espinardo, 30100 Murcia, Spain. E-mails: cespinosa31416@gmail.com; cer48658@um.es*

a *Physiology Department, Faculty of Biology, University of Murcia, 30100 Murcia, Spain*

b *Fish Innate Immune System Group, Department of Cell Biology and Histology, Faculty of Biology, University of Murcia, 30100 Murcia, Spain*

c *Department of Plant Biology (Plant Physiology), Faculty of Biology, University of Murcia, 30100 Murcia, Spain*

disease are less likely to receive preventive treatments against such diseases.[6] However, the mechanisms linking susceptibility to cardiovascular events in patients with chronic kidney disease are not entirely clear, but it might be interesting to prevent this type of pathologies.

Nuclear factor E2-related factor 2 (NRF2) is a transcription factor of the cap 'n' collar basic region leucine zipper (cnc bZip) family, controlling the expression of various cytoprotective antioxidant enzymes.[7–10] This transcription factor is found in many tissues, and is activated in response to a wide range of oxidative and electrophilic stimulation, including reactive oxygen species (ROS) and some chemical agents. Given that NRF2 controls a broad range of cellular antioxidant defence mechanisms, this pathway may also contribute to the multi-factorial phenotype associated with the aging process. Recent studies demonstrate that NRF2 modulates the expression of antioxidant genes through interaction with antioxidant stress element, antioxidant response element (ARE).[9] Under normal physiological conditions and low oxidative stress, NRF2 is confined to the cytoplasm associated with the suppressor protein kelch-like ECH-associated protein 1 (KEAP1), and is degraded by the ubiquitin proteasome pathway. Oxidative and electrophilic stress factors stimulate dissociation of the NRF2–KEAP1 complex, thereby promoting the release and translocation of NRF2 into the nucleus where it upregulates the expression of NRF2/ARE-linked antioxidant and detoxifying genes.[7,10] These genes include haem oxygenase (*Ho1*), superoxide dismutase (*Sod*), peroxyredoxine 1, NAD(P)H, quinone oxidoreductase 1 (*Nqo1*), glutamate cisteine ligase, and glutathione-*S*-transferase (*Gst*).[11–13]

Adriamycin (ADR) (doxorubicin) is a quinone belonging to the anthracyclines, a class of antibiotic first isolated from *Streptomyces peucetius* in the 1960s. Adrimycin prevents DNA replication by intercalating between base pairs of the DNA.[14] In humans, it is used as a chemotherapeutic agent against a variety of tumours, including lymphomas, leukaemias and solid tumours. *In vivo*, ADR is capable of generating a wide variety and number of free radicals with cytotoxic effect.[15,16] Human clinical studies have shown that treatment with some antioxidants does not completely reverse the cardiotoxic effects of ADR and investigations are continuing to find agents that prevent or alleviate such damages.[17]

This paper is preceded by two publications, in which long-term white tea treatment protected against acute stress caused by ADR in different tissues,[18] and the second article ratified these findings and attempted to clarify the mechanisms through which tea protects against ADR in the liver.[19]

The aim of this work was to identify potential molecular mechanisms triggered in the kidney as a consequence of long-term consumption of white tea and how this can moderate the response of the tissue to an oxidant insult. The kidney is a special tissue due to the variety of its functions such as filtering blood, regulating blood pressure and (or) gluconeogenesis. There are various pathologies in these organs related to oxidative stress and, thus, it would be interesting to prevent as well as to find the manner to attenuate the side effects of ADR. We will analyse *Nrf2* expression, the expression of genes to which it regulates (*Nqo1*, *GST-α1*, *Ho1*, *Cat*, *Sod 1*, *Sod 2*, *Sod 3* and *Gr*), and the enzyme activities relevant in the cellular redox state (SOD, CAT and GR). We also analyse the total antioxidant activity and oxidative protein and lipid parameters in kidney. We hope to understand the genetic and enzymatic mechanisms of response in kidney against oxidative stress and if the long-term treatment with white tea could prevent that oxidative damage.

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. In addition, it is conforms to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1985).

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; Served by University Animal Center REGA-ES300305440012, Murcia, Spain) and were kept in cages in groups of three or four, with males separated from females.

The animals (an equal mixture of males and females) were maintained in a room under controlled conditions: temperature 23 ± 2 °C, relative humidity 55 ± 5%, ventilation frequency 18 times h^{-1} and a 12:12 h light–dark photoperiod. For 12 months, the rats received different doses of white tea in their drinking water. The control ($n = 12$) received distilled water; dose 1 group (D1) ($n = 5$) received 0.015 g of solid white tea extract kg^{-1} body weight (0.004 g polyphenols kg^{-1} body weight), and dose 2 group (D2) ($n = 5$) received a dose of 0.045 g of solid white tea extract kg^{-1} body weight (0.012 g polyphenols kg^{-1} body weight). 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,[20] and nine cups of tea per day, reflecting oriental habits.[21] After 12 months, all the animals received an intraperitoneal injection of ADR (0.010 g kg^{-1} body weight), except half of the animals of 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 kidneys were removed and immediately rinsed in a cold saline solution. For the enzymatic assays and the measurement of hydrophilic antioxidant capacity, carbonyl group and hydroperoxides, part of the samples were homogenised in a phosphate buffer.

Preparation of the tea infusion

The white tea extract was prepared in Barcelona (Spain) following the protocol described by Almajano *et al.*[22] The white tea infusions were freeze dried, and the extracts were kept at −20 °C until use. The tea was prepared daily and at the described doses. The total phenol content was analysed by the Folin–Ciocalteu method, and the profile of the main catechins was determined by capillary electrophoresis (Table 1). The white tea used contained 21.80 ± 1.61 g of total polyphenols kg^{-1} 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.[23] The main catechin observed was epigallocatechin-3-gallate (EGCG) (1525 ± 113.4 g 100 g^{-1} tea leaves). Several other polyphenols were also present (g kg^{-1} tea leaves) including epigallocatechins (1.590 ± 0.152) and epicatechin (0.543 ± 0.025), as well as methylxanthines including theobromine and caffeine.[23] White tea extract with ethanol was analysed by HPLC. Data are shown in Table 2.[24] No problems were founded in white tea solubility, and the white extract solution was

Table 1. Content of the main catechins and caffeine (mg 100 g^{-1} tea leaves) determined by capillary electrophoresis

Compound	Amount
Caffeine	6.760 ± 0.312
Gallic acid	0.548 ± 0.064
EGC	1.510 ± 0.152
EGCG	15.250 ± 1.134
EG	0.573 ± 0.027
EGC	3.010 ± 0.293
TP	2.180 ± 0.1616
TEAC value	4.546 ± 0.054
PAC	2.1

TP, total phenolics in g gallic acid equivalent per litre of infusion.
TEAC value, Trolox equivalent capacity (mol of trolox per litre of infusion) determined by the ABTS method.
PAC, phenol antioxidant coefficient, calculated as the ratio TEAC/total phenolics.
Content of total phenolics (by the Folin–Ciocalteu method), TEAC value and ratio of these parameters (mean ± SD, $n = 6$)[22]

Table 2. Concentration of polyphenols found in MeOH white tea extracts by HPLC[24]

Analysis compound	Retention time (min)	Concentration (g kg^{-1} dry tea leaves)
EGC	1.913	2.609
EC	2.91	0.949
EGCG	4.715	21.138
ECG	7.757	3.822

protected from light-oxidation all the time. Probes were used to minimise the oxidation.

Hydrophilic antioxidant activity

Hydrophilic antioxidant activity (HAA) was measured in kidney using the method described by Arnao *et al.*, which is based on the ability of the antioxidants in the sample to reduce the radical cation of 2,20-azino-bis-3-(ethylbenzothiazoline-6-sulfonic acid) (ABTS), determined by the decolouration of $ABTS^{A+}$, and measuring the quenching of the absorbance at 730 nm.[25] 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.

Carbonyl groups

The concentration of carbonyl groups in kidney proteins was determined using 2,4-dinitrophenylhydrazine (DNPH) according to the method described by Reznick and Packer[26] and modified by Bailey.[27] Kidney samples were divided into two aliquots of at least 1 mg mL^{-1} of protein in each. To one of them, 2 mL of 2 mol L^{-1} HCl were added before incubating at room temperature for 1 h, stirring intermittently. This served as the Control tube. To the other tube was added 2 mL of 10 mmol L^{-1} DNPH dissolved in 2 mol L^{-1} HCl. This was incubated and stirred as above, and served as the experimental sample. After incubation, the reaction in both cases was stopped by adding trichloroacetic acid (TCA) at 20% and then the mixture was centrifuged at 5000 × *g* 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 mol L^{-1} guanidine-HCl and centrifuged, and the supernatant was read by a sweep of 344–390 nm. The carbonyl content was expressed in nmol mg^{-1} protein.

Hydroperoxides

The technique described by Jiang *et al.* was used to determine hydroperoxides.[28] This is based on the reaction in which Fe^{2+} is oxidised to Fe^{3+} in acidic conditions, and 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 intensity of the orange colour of the xylenol increases with the increasing presence of hydroperoxides. The AMN (ammonium ferrous sulfate) acts as the source of iron for lipid peroxidation, while 2,2-azobis(amidinopropane) (AAPH) is a strong inducer of lipid peroxidation.

Quantitative real-time PCR

The mRNA levels of *Nrf2*, *Ho1*, *Nqo1*, *Gst-α1*, *Cat*, *Gr*, *Sod-1*, *Sod-2* and *Sod-3* were determined by quantitative real-time PCR. Total RNA was extracted from 0.5 g of rat kidney tissue using TRIzol Reagent (Invitrogen, Madrid, Spain). It was then quantified, and the purity was assessed by spectrophotometry; the 260:280 ratios were 1.8–2.0. The RNA was then treated with DNase I (Promega, Milan, Italy) to eliminate genomic DNA contamination. Complementary DNA (cDNA) was synthesised from 1 µg of total RNA using the SuperScript III reverse transcriptase (Invitrogen) with an oligo-dT_{18} primer. The expression of nine selected redox-relevant genes was analysed by real-time PCR, which was performed with an ABI PRISM 7500 instrument (Applied Biosystems, Madrid, Spain) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures [containing 10 µL of 2 × SYBR Green supermix, 5 µL of primers (0.4 µmol L^{-1} each) and 5 µL of cDNA template] were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C and, finally, 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. For each mRNA, gene expression was corrected by the RNA β-actin subunit content in each sample. The primers used are shown in Table 3. Sequences were obtained from researches published by Chan *et al.*,[29] Wang *et al.*[30] and Chen *et al.*[31] In all cases, each PCR was performed with triplicate samples. The reference gene was stable in the kidney.

Enzyme assays

The activity of CAT was measured by monitoring the consumption of H_2O_2 at 240 nm.[32] This method is based on the principle that the absorbance will decrease due to the decomposition of hydrogen peroxide by catalase at 240 nm. The amount of H_2O_2 converted into H_2O and O_2 in 1 min under standard conditions is accepted as the enzyme reaction rate. GR was measured by the method modified by Carlberg and Mannervik.[33] The reaction was initiated by adding 0.1 mmol L^{-1} NADPH to the mixture of enzyme in 50 mmol L^{-1} potassium phosphate buffer pH 7.0 containing 2 mmol L^{-1} EDTA and 0.5 mmol L^{-1} GSSG. The change in absorbance was monitored at 340 nm for 3 min by a UV–visible Thermo Scientific model Evolution 300 dual-beam spectrophotometer (Thermo Scientific, Madrid, Spain). One unit of GR activity is defined as the amount of enzyme that catalyses the reduction of 1 µmol of NADPH per minute ($\varepsilon_{340\,nm}$ for NADPH 6.22 mM^{-1}

Table 3. PCR primer sequences[29–31]

Target gene	Sense primer (5′ to 3′)	Antisense primer (5′ to 3′)	Annealing temperature (°C)	No. of cycles	Size (bp)
NRF2	CCATGCCTTCTTCCACGAA	AGGGCCCATGGATTTCAGTT	–	40	–
NQO1	GCGTCTGGAGACTGTCTGGG	CGGCTGGAATGGACTTGC	60	40	170
HO1	GCGAAACAAGCAGAACCCA	GCTCAGGATGAGTACCTCCCA	58	40	185
GST-α1	CGGTACTTGCCTGCCTTTG	ATTTGTTTTGCATCCACGGG	59	40	248
SOD1	CACTCTAAGAAACATGGCG	CTGAGAGTGAGATCACACG	60	40	40
SOD2	TTCAGCCTGCACTGAAG	GTCACGCTTGATAGCCTC	60	40	669
SOD3	CTTCACCTGGTTGAGAAGATAG	GATCTGTGGCTGATCGG	60	40	735
CAT	ATGGCTTTTGACCCAAGCAA	CGGCCCTGAAGCTTTTTGT	60	40	68
GPx	GCGGGCCCTGGCATTG	GGACCAGCGCCCATCTG	60	40	132
β-Actin	CCCATCTATGAGGGTTACGC	TTTAATGTCACGCACGATTTC	60	40	150

NRF2, nuclear factor E2-related factor 2; NQO1, NAD(P)H:quinone oxidoreductase-1; HO1, haem oxygenase 1; GST-α1, glutathione *S*-transferase α1; SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

cm^{-1}). SOD activity was determined based on the inhibition of the reduction of cytochrome c in the presence of SOD at 550 nm.[34] The SOD-like activity of the complexes was studied at 298 K by an indirect method using cytochrome c as superoxide oxidant (indicator). The superoxide radical anion was generated *in situ* by the xanthine oxidase reaction, and detected spectrophotometrically by monitoring the formation of the reduced form of cytochrome c at 550 nm in a Thermo Scientific Evolution 300 dual-beam spectrophotometer.

Statistical analyses

The results are expressed as means ± 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 Games Howell test, depending on the homogeneity of the variables. The significance level was 95% in all cases ($P < 0.05$). All the data were analysed by the computer application SPSS for Windows® (version 15.0; SPSS Inc., Chicago, IL, USA).

RESULTS

During the 12 months of the dietary treatment, 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 white tea.

Figure 1 shows the antioxidant activity and the concentration of hydroperoxides and carbonyl groups (A, B and C). The antioxidant activity suffered a significant decrease in the C + ADR group, with a response in the D1 + ADR and D2 + ADR groups, in which the antioxidant activity levels of the control was recovered (Fig. 1A). The carbonyl groups and hydroperoxides showed a similar behaviour pattern. Oxidation increased in the C + ADR group while D1 + ADR and D2 + ADR groups gradually recovered baseline levels (Fig. 1B and C).

Figure 2 depicts the *Nrf2*, *Nqo1*, *Ho1* and *Gst-α1* genes. The present study shows that the ADR caused activation of the ARE response in kidneys. As shown in Fig. 2A, the expression of *Nrf2* levels was high in the C + ADR group. In D1 + ADR and D2 + ADR groups the expression of *Nrf2* was lower than in C + ADR and in group C.

The study shows that in the expression of antioxidant and detoxifying genes (*Nqo1*, *Ho1*, *Gst-α1*, *Gr*, *Cat* and *Sod1*) increased in the C + ADR group (Fig. 2B and Fig. 3). However, *Sod3* and *Sod2* expression did not differ significantly compared with the control (Fig. 3). The expression of *Nqo1* and *Ho1* showed a decrease in the D1 + ADR and D2 + ADR groups, returning to baseline in the D2 + ADR group. The expression of *Gst-α1*, *Gr* and *Cat* in the D1 + ADR and D2 + ADR groups remained below that of the C + ADR group and also significantly below the control group, being decreased their expression (Fig. 2 and Fig. 3). The *Gr* expression increased in the C + ADR group but there were no significant differences among the other experimental groups (Fig. 3).

The SOD and CAT activities increased in C + ADR group with respect to the control group and diminished their activities in D1 + ADR and D2 + ADR groups, returning to normal activity in the D2 + ADR group (Fig. 4). The GR activity diminished in the C + ADR group with respect to the control group and showed an increase in their activity in D1 + ADR and D2 + ADR groups, returning to normal activity in the D2 + ADR group (Fig. 4).

DISCUSSION

The findings shown in Fig. 1 confirm the view that the long-term ingestion of white tea protects against oxidative stress caused by ADR. To understand the mechanisms involved in this antioxidant effect another series of tests was conducted, as described below.

The expression of transcription factor *Nrf2* was quantified. Under normal unchanged redox status conditions NRF2 is attached to the KEAP1 protein complex which is degraded via proteasome ubiquitination.[35–37] In cases of oxidative stress, the NRF2–KEAP1 complex dissociates, and the NRF2 searches the nucleus.[38] Once in the nucleus, it recognises the ARE sequences scattered over the DNA and binds to them, promoting the expression of the antioxidant gene response and phase II enzymes.

The study showed that ADR caused an increased expression of *Nrf2* in the kidney (Fig. 2A). This increase in NRF2 factor causes the activation of other genes, as observed in the analysis of *Gst-α1*, *Nqo1*, *Ho1*, *Cat*, *Sod1* and *Gr*. All these were significantly increased compared to the control (Fig. 2B and Fig. 3) which can be considered a flexible response on the part of factor NRF2 mediated by the oxidative stress caused by ADR. These findings are consistent with others that showed that transcription factor NRF2 activates this set of genes in the face of a significant oxidative

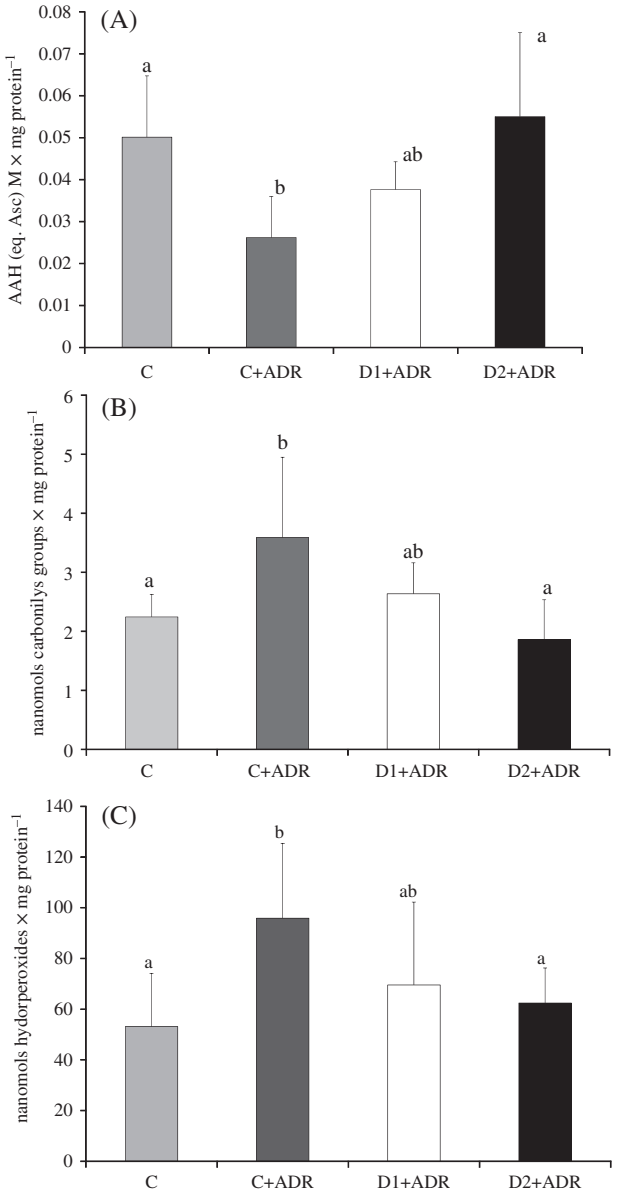

Figure 1. (A) Hydrophilic antioxidant activity (HAA) expressed in equivalents of ascorbic acid (mmol L^{-1}) per mg of protein. (B) Concentration of carbonyl groups expressed in nanomols per mg of protein. (C) Concentration of hydroperoxides expressed in nanomols per mg of protein. Values are the mean ± SD of five animals per group. Values with different letters indicated statistically significant differences among treatments ($P < 0.05$).

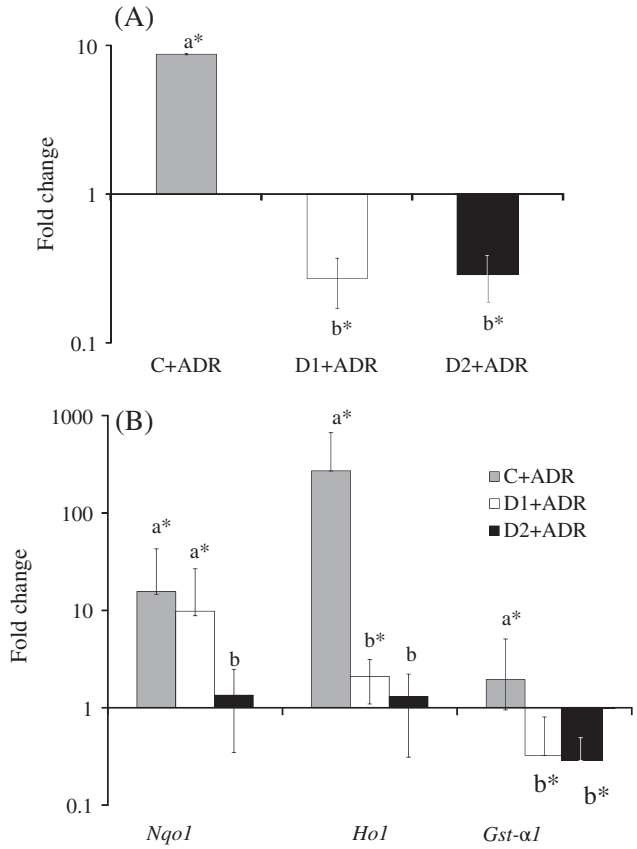

Figure 2. (A) Expression of the *Nrf2* gene determined by real-time PCR in the kidney of rats after intraperitoneal injection of ADR. (B) Expression of the *Nqo1*, *Gst* and *Ho1* genes determined by real-time PCR in the kidney of rats after intraperitoneal injection of ADR. The bars represent the mean ± SD ($n = 5$) fold change relative to the control. Values with different letters indicate statistically significant differences between treatments; *Indicates significant differences compared with the control ($P < 0.05$).

stimulus: for example, Sahin *et al.*,[39] found that in response to the oxidative stress caused by increased temperature, NRF2/ARE is activated, as in many others studies.[40] In the present study, the increase in the gene expression of *GST-α1*, *Nqo1*, *Ho1*, *Cat*, *Gr* and *Sod1* genes was followed by a further increase in the activity of the enzymes tested (CAT and SOD) (Fig. 4A and B). However, that increase was not enough to decrease the oxidative stress, as indicated in Fig. 1.

The decrease in GR activity in the face of oxidative stress is an interesting observation because no such decrease was recorded in a previous study in liver.[19] The liver increased its activity in the C + ADR group, in response to acute oxidative stress. However, their activity diminished in the kidney, in the same experimental group (Fig. 3 and Fig. 5), consistent with other observations in kidney by Yeung[41] who indicated that the GR does not react in the same way against acute oxidative stress in the liver as in the kidney, so their regulation seems to differ. This may be due to the relationship established between the GR and other enzymes such as CAT, SOD and/or GPx, which need reduced glutathione (which is renewed by the GR) to function. The supply of reduced glutathione may decrease the lifetime of the enzyme, its activity in the face of an acute oxidative stress, precisely when there is a need to manufacture more enzyme. However, in the liver, possibly due to its detoxifying function, this enzyme is enhanced and shows high activity in high-demand situations (antioxidant or oxidative stress situations) (Fig. 5).

The long-term consumption of white tea extract protected against the oxidative damage caused by ADR, as seen from the results obtained by measuring the antioxidant activity, the concentration of carbonyl groups and hydroperoxides (Fig. 1), whose levels returned in D2 + ADR to those observed in control animals. These results are also consistent with previous observations that the supply of an antioxidant was able to neutralise the oxidative damage caused by ADR, in liver, heart and brain.[18,19] However, the results in the literature are contradictory in this matter, a reduction of the toxic effects of the ADR by an antioxidant was also observed, for example, when vitamin E was administered, with no decrease in the effectiveness of the chemotherapeutic agent.[42–47] In contrast, Alberts *et al.*[48] showed that vitamin

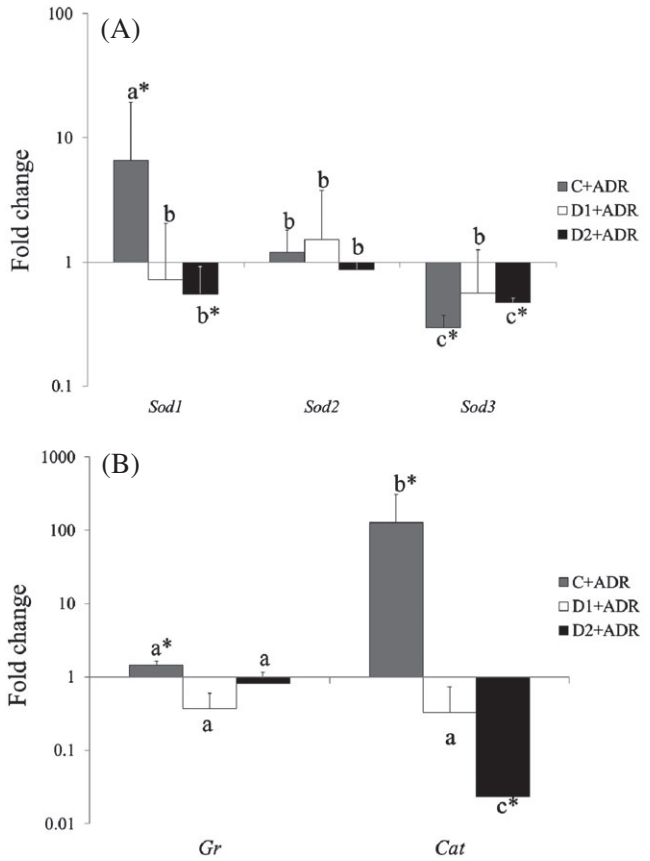

Figure 3. (A) Expression of the *Sod* (1, 2 and 3) genes determined by real-time PCR in the kidney of rats after intraperitoneal injection of ADR. (B) Expression of the *Cat* and *Gr* genes determined by real-time PCR in the kidney of rats after intraperitoneal injection of ADR. The bars represent the means ± SD ($n = 5$) fold change relative to the control. Values with different letters indicate statistically significant differences among treatments; *Indicates significant differences compared with the control ($P < 0.05$).

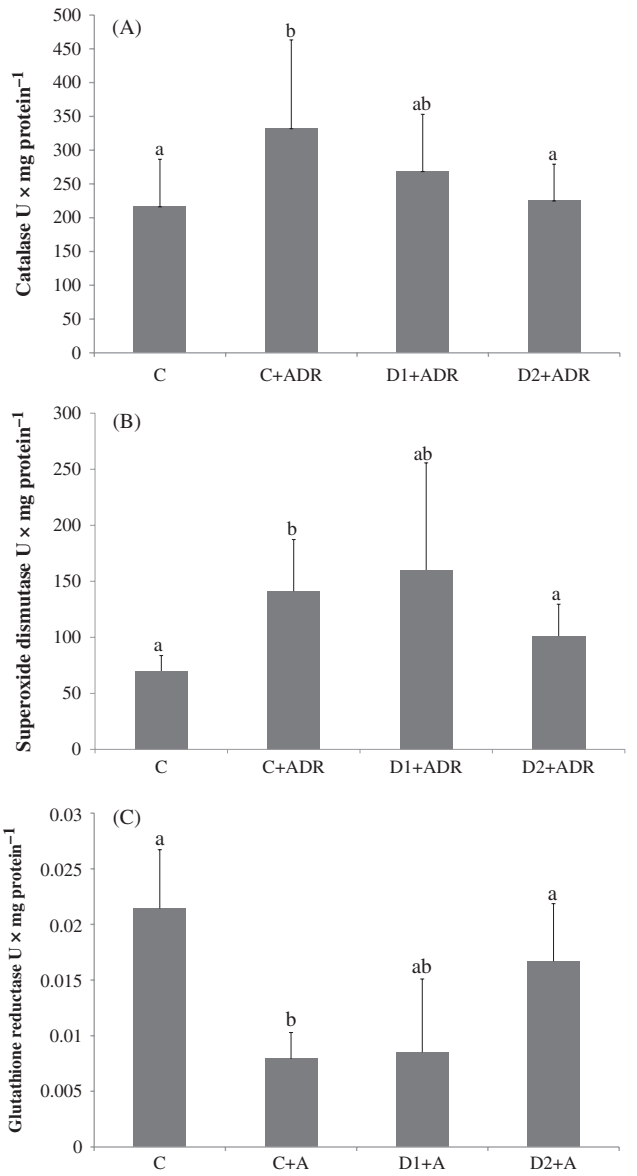

Figure 4. (A) Kidney catalase (CAT) activity expressed in units of CAT per milligram of protein. (B) Kidney superoxide dismutase (SOD) activity expressed in units of SOD per milligram of protein. (C) Kidney glutathione reductase (GR) activity expressed in units of GR per milligram of protein. Values are the mean ± SD of five animals per group. Values with different letters indicate statistically significant differences among treatments ($P < 0.05$).

E increased the toxic effect of ADR, or by other researches that vitamin E had no effect against the administration of ADR.[49–51] Kurbacher *et al.*[52] observed synergistic toxic effects following the simultaneous administration of ascorbic acid in two lines of breast tumour cells (MCF-7 and MDA-MB-231). These authors suggested that mechanisms by which the synergistic toxic effect occurred was related with the antioxidant properties of ascorbic acid, as also suggested by Miura *et al.*[53] This suggests that not only for its antioxidant capacity could the white tea has been protected against the oxidative stress.

The *Nrf2* expression contrasted with those observed in our previous publication where white tea administered long term greatly increased expression of *Nrf2*, promoting the expression of antioxidant and detoxifying response enzymes.[19] However, in kidney, the response of the D1 + ADR and D2 + ADR groups resulted in a significant decrease in the expression of *Nrf2* to the point that it was significantly lower than in the control group (Fig. 2A). This negative regulation can be used to explain a similar expression of the *Nqo1*, *Ho1*, *Sod1* genes, and the significantly decrease compared with the control group of the *Gst-α1*, *Cat* and *Gr* genes (Fig. 2B and Fig. 3).

The analysis of gene expression points to show a similar logical pattern that relates the activity of each enzyme with its expression (except in the case of *Sod2*, *Sod3* and *Gr*). These genes (*Cat* and *Sod1*) increased their expression and activity in the C + ADR group and maintained or decreased their expression and activity in the D2 + ADR groups (Fig. 3 and Fig. 4). These results agree with those observed in a previous work,[19] where we suggested the possibility that white tea polyphenols, after functioning for a relatively long period, due to their antioxidant properties and their relatively high amounts were sufficient to neutralise the negative effects of ADR. Therefore, through a mechanism of negative feedback regulation, gene activation was controlled, thereby decreasing the synthesis of the antioxidant. This hypothesis would be supported by the fact that the polyphenols were absorbed, due to the good bioavailability of polyphenols of white tea[54] and that they are stored or located in the cell in the membrane-cytosol,[55] a very susceptible target to the first attack by the oxidative ADR. There have been

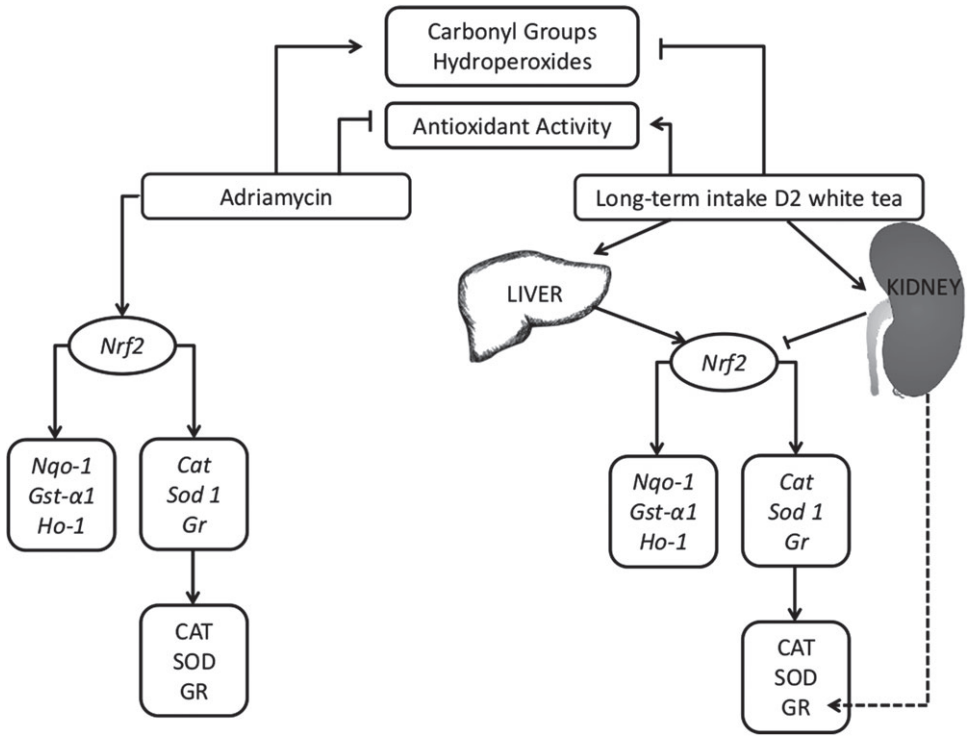

Figure 5. Diagram showing the effects of adriamycin and the long-term intake of doses 2 of white tea in the expression of the transcription factor Nrf2 and detoxifying genes (*Nqo-1*, *Gst-α1* and *Ho-1*) and antioxidants (*Cat*, *Sod-1* and *Gr)* and the effect in enzymes activity (CAT, SOD and GR) in liver[18,19] and kidney.

various studies with liposomes composed of phosphatidylcholine and phosphatidylserine that show that the (−)-epicatechin (EC) adsorbs oligomers on the liposome surface and inhibits lipid oxidation by hydrophobic oxidants.[55] The strength of the interactions established between the oligomers of EC and the surface of the membrane depends greatly on the chemical nature of the polar groups of the phospholipids, in the order of galactolipids > phosphatidylcholine > phosphatidylserine.[56] Through such interactions, flavonoids may be trapped at the membranes surface and exert local protective effects. If these polyphenols are located on the membrane surface are relatively functional, they will neutralise the oxidation caused by the ADR, and subsequent activation of the antioxidant enzymes would be required. Additional research is needed in this regard.

In a similar study made in the liver, the expression of *Nqo1*, *Ho1* and *GST-α1* was high in the D1 + ADR and D2 + ADR groups than in the C and C + ADR groups (Fig. 5).[19] However, in the present study the expression of these enzymes is lower than in the C + ADR group, and similar to the expression in the control group in the case of the *Ho1* and *Nqo1* genes, and even significantly lower in the *GST-α1* gene (Fig. 2B). More specific studies are needed to clarify the different expression levels observed in these genes.

As regards superoxide dismutase, three isoenzymes were analysed: the homodimeric cytosolic SOD Cu/Zinc (SOD1), homotetrameric mitochondrial Mn SOD (SOD2) and the SOD bound to proteoglycan Cu/Zinc or extracellular (SOD3).[57] These SODs catalyse the conversion of $O_2^{\bullet -}$ to a less harmful product, H_2O_2, which is converted to H_2O by catalase (CAT) and glutathione peroxidase (GPx). The three enzymes are involved in a cascade process.

The analysis of the three SOD isoenzymes showed that expression was unchanged in *Sod 2*, so we concluded that neither ADR nor long-term treatment with white tea affected the expression of SOD in the mitochondria. The *Sod 3* expression remained repressed compared to the control in the C + ADR group, D1 + ADR and D2 + ADR, suggesting that long-term treatment with white tea had no effect on the expression of *Sod 3*. These results could indicate a different activation route on the part of this enzyme, or that *Sod 3* does not have an ARE sequence targeted by NRF2. This behaviour might also be explained by the fact that this enzyme activity occurs outside the cell. These results are consistent with those described in similar a work in liver.[19] Further studies are needed to explain or justify the proposed hypotheses, necessitating further research in this area.

As mentioned above, GR might have be another kind of regulation. Both the activity and expression differ between organs and among antioxidant enzymes,[19] but the differences in expression could be explained by the relation between the enzymes, as previously mentioned. The activity of GR in the groups treated with the two doses of white tea returned to the levels observed in the control group with the higher dose of white tea. Finally, treatment with white tea returned GR to its basal levels.

CONCLUSION

Long-term administration of white tea protects kidney against oxidative damage caused ADR both at protein and lipid levels, maintaining the antioxidant activity of the control animals. The associated NRF2/ARE response is activated in order to detoxify and neutralise the oxidative damage produced by ADR before it occurs: however, the animals that were not treated with white tea could not counteract this damage (Fig. 5). The results are clear as regards the properties of white tea to neutralise oxidative damage and cytotoxicity caused by ADR. The antioxidant

activity of white tea and its ability to be stored and/or placed at the lipid-interface cytosol appear to play an important role in the defense against oxidative stress and activation of the NRF2 response, which enables or inhibits the synthesis of antioxidants and phase II enzymes. These results reveal that the quality and quantity of polyphenols and catechins in white tea administered on the experimental conditions of this study were sufficient to neutralise free radicals and the resulting oxidative stress. It was not necessary for the antioxidant and phase II enzymes to be activated, which was confirmed and reinforced by the response observed with increasing doses of white tea. There were differences in this response caused by ADR between the liver and kidney (Fig. 5). Additional studies are needed to understand such differences between organs and to clarify the mechanisms involved in the regulation of genes related to the antioxidant defence and modulated by different components administered in the diet.

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