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Naringin Abrogates Cisplatin-Induced Cognitive Deficits and Cholinergic Dysfunction Through the Down-Regulation of AChE Expression and iNOS Signaling Pathways in Hippocampus of Aged Rats

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Abstract Chemotherapy-related cognitive deficits are a major neurological problem, but the underlying mechanisms are unclear. However, very few studies have looked at the possible ways of preventing this stress-induced deficit. Thus, we investigated the relationship between cisplatin (Cis) exposure to acetylcholinesterase, ATPase, oxidative stress biomarkers, and impaired behavior performance and the possible protecting mechanism of naringin (Nar), a plant-derived flavonoid, in aged rats. The experimental procedures were divided in two sets of experiments. In the first, the animals were divided into four groups: vehicle, Nar 25 mg/kg, Nar 50 mg/kg, and Nar 100 mg/kg. In the second, the animals were divided into four groups: Cis (5 mg kg^{-1} $week^{-1}$ for five consecutive weeks), Cis plus Nar (25 mg/kg), Cis plus Nar (50 mg/kg), and Cis plus Nar (100 mg/kg). Results showed that Cis exposure leads to the increase in acetylcholinesterase associated with a significant increase in mRNA levels of acetylcholinesterase and the inducible nitric oxide synthase (iNOS) in the hippocampus. Moreover, a decrease in membrane-bound ATPase enzyme activities and enzymatic and nonenzymatic antioxidant activities in the hippocampus and an increase in the levels of malondialdehyde (MDA), protein carbonyls (PCO), nitrite formation (NO), and reactive oxygen species (ROS) levels were found. Further, Cis-induced neuronal alterations were evidenced by impairment behavioral performance. Treatment with Nar significantly and dose-dependently prevented all the behavioral, biochemical, and molecular alterations in aged rats treated with cisplatin. Thus, findings from the current study demonstrate the possible involvement of oxidative-stress-mediated inflammatory signaling in Cis-induced cognitive dysfunction and also suggests the effectiveness of naringin in preventing cognitive deficits in chemotherapy-induced peripheral neuropathy.

Keywords Cisplatin · Aged rat · Hippocampus · Cognitive deficit · Cholinergic function · Oxidative stress

Introduction

The cognitive deficits observed after treatment with chemotherapeutic drugs (e.g., cisplatin) are a significant clinical problem in the world. Up to 30–40 % of cancer patients that receive these agents experience pain, and about 20 % of patients are unable to complete a full course of cisplatin therapy (Windebank and Grisold 2008; Markman 2006). Due to the neurotoxicity potential of the cisplatin, peripheral neuropathy was frequently seen in patients and causes dose-limiting problems in the treatment process (Brouwers et al. 2009; Siegal and Haim 1990). Although cisplatin is very effective in cancer therapy, it is extremely toxic, and it has severe side effects such as neurotoxicity, nephrotoxicity, ototoxicity, and vomiting (Li et al. 2006; Paksoy et al. 2011; Ta et al. 2006). The adverse effects of cisplatin on nervous system were demonstrated in animals and humans with electrophysiological and histopathological examination of the peripheral nerve in the literature (Carozzi et al. 2009; Krarup-Hansen et al. 2007;

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Turan et al. 2014). Similarly, neurotoxicity was observed in human treated with cisplatin in an autopsy study (Krarup-Hansen et al. 1999). Several pathophysiological mechanisms are proposed to explain the cisplatin-induced neurotoxicity such as oxidative damage, inflammation, mitochondrial dysfunction, DNA damage, and apoptotic cell death in the nervous system (Gill and Windebank 1998; Englander 2013; Kim et al. 2014). These adverse effects from cisplatin exposure have led to the search for preventative treatments (Campbell et al. 1996, 1999). Many agents have been proposed to manage chemotherapy-induced neuropathy (acetylcysteine, amifostine, calcium and magnesium, diethyldithiocarbamate, glutathione, or vitamin E), but to date, the data are insufficient to conclude that any of the purported agents prevent or limit the neurotoxicity of platinum drugs among human patients (Albers et al. 2011). The absence of effective treatments for chemotherapy-evoked neuropathy makes the identification of alternative analgesics a crucial medical need.

There is a growing interest in the potential of phytochemicals to improve memory, learning, and general cognitive ability (Spencer 2008a, b). A large amount of excellent reviews highlighting the effects of phytochemicals as modulators of brain function (Kandhare et al. 2012; Baluchnejadmojarad and Roghani 2006). Recent evidence has indicated that a group of plant-derived compounds known as flavonoids may exert particularly powerful actions on mammalian cognition and may reverse age-related declines in memory and learning (Youdim et al. 2000; Hartman et al. 2006; Shif et al. 2006).

Naringin (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside) is one of the flavonoids, which is a major bittering principal in citrus juices. It is extensively distributed in citrus and occurs in grapefruits, mandarins, etc. (Kumar et al. 2010a,b,c). Many biological activities of naringin have been recognized, such as antioxidant and antiradical agents (Choe et al. 2001), antilipid peroxidation activities (Rajadurai and Prince 2009), anti-inflammatory and antimutagenic effects (Kanno et al. 2006), inhibition of tumor development, and suppression of tumor cell proliferation (Benavente-Garcia and Castillo 2008). Naringin is hydrolyzed by intestinal microflora to yield naringenin (4′,5,7-trihydroxyflavanone). The latter is readily absorbed and also has good penetration across the blood–brain barrier (Zbarsky et al. 2005). A recent study has reported naringin's neuroprotective potential against deltamethrin-induced cognitive impairment and oxidative stress in mice (Mani and Sadiq 2014). Further, our previous work on naringenin, a metabolite of naringin, has demonstrated the hepatoprotective potential of this compound against cholesterol-induced hepatic inflammation by modulating matrix metalloproteinases-2,9 via inhibition of nuclear factor κB pathway in rats (Chtourou et al. 2015). Therefore, it is worthwhile to assess the neuroprotective property of naringin against Cis-induced brain dysfunction in aged rats in view of its multiple favorable pharmacological activities. However, the effects of naringin on cisplatin-induced oxidative stress, memory deficits, and cholinergic dysfunction especially in aged rats remain unknown.

With this background, the present study was designed to explore the possible role of naringin against Cis-induced hippocampus dysfunction mediated memory deficits, inflammatory signaling, and oxidative stress in aged rats.

Materials and Methods

Chemicals and Reagents

Naringin and all other chemicals required for biochemical assays were obtained from Sigma Chemicals Co. (St. Louis, France).

Animals

Middle-aged (18-month-old, 57–61 % lifespan) Wistar rats were purchased from the Central Pharmacy (SIPHAT, Tunisia). Animals were kept in an air conditioned room (22±2 °C) with free access to water and commercial diet supplied by the Company of Animal Nutrition, Sfax, Tunisia. All manipulations were carried out between 8:00 A.M. and 4:00 P.M. All animal procedures were conducted in strict conformation with the local Institute Ethical Committee Guidelines for the Care and Use of Laboratory Animals of our faculty.

Cisplatin Exposure and Treatment with Naringin

Experimental procedures were divided in two sets of experiments. In the first set, animals were randomly divided into four groups (ten rats in each group): control (CT), naringin (25 mg/kg body weight) (Nar 25), naringin (50 mg/kg body weight) (Nar 50), or naringin (100 mg/kg body weight) (Nar 100).

In the second experimental set, animals were divided into four groups (ten rats in each group): cisplatin (Cis) group (5 mg kg^{-1} $week^{-1}$ for five consecutive weeks), cisplatin with naringin (25 mg/kg body weight) (Cis+Nar 25), cisplatin with naringin (50 mg/kg body weight) (Cis+Nar 50), and cisplatin with naringin (100 mg/kg body weight) (Cis+Nar 100). Naringin was diluted with buffered saline and propylene glycol 25/75 (*v*/*v*), administered by oral gavage, and did not exceed 1.0 ml/kg body weight.

Behavioral Tests

Behavioral tests were performed in order to evaluate how cisplatin treatment influenced both locomotor and learning

and memory performances in the different experimental groups. They were performed at 48 h before the killing of animals.

The following behavioral testes were used.

Open Field Test

This test was used in order to evaluate possible toxic effects of cisplatin that could affect locomotor performance of the animals, which could influence their performance in specific behavioral tests to address Cis-induced learning and memory deficits.

In order to control for possible sensorimotor effects induced by Cis and Nar, behavior during exploration of an open field was evaluated 24 h before the end of the experiment (killing of animals). The open field was a 40-×45-cm arena surrounded by 50-cm high walls, made of plywood with a frontal glass wall. The floor of the arena was divided into 16 (4×4) equal squares by black lines. Animals were placed in the central case to explore the field freely for 5 min. Line crossings and rearings were counted (Walsh and Cummins 1976). At the end of each test, the apparatus was thoroughly cleansed with cotton wool dipped in 70 % ethanol. The observed parameters were as follows: crossing frequency (number of squares crossed), number of rearing, and the time spent in the central case.

Elevated Plus Maze

The elevated plus maze test developed by Pellow et al. (1985) was employed to unveil the effect of cisplatin on anxiety. Briefly, the maze apparatus consisting of two open (55× 10 cm) and two closed arms (55×10×12 cm) having an open roof with the entire plus maze elevated 50 cm from the floor was used to observe anxiolytic behaviour in rats. Each rat was placed individually at the center of the elevated plus maze with its head facing an open arm. During the 5-min test, the preference of the animal for the first entry, the number of entries into the open/closed arms, and the time spent in each arm of the maze were recorded.

Biochemical Analysis of the Hippocampus

After the behavioral tests (48 h later), animals were euthanized by cervical decapitation to avoid stress conditions. Brain structures were quickly removed from skull, rinsed in ice-cold Tris–HCl buffer (10 mM, pH 7.4), placed on filter paper moistened with the same buffer on top of a Petri dish filled with ice, and the following hippocampus regions were dissected using consistent anatomical landmarks as criteria for dissection and was homogenized in the appropriate buffer as indicated in the procedures of each parameter measurements.

The homogenates were then centrifuged at 12,000×*g* for 15 min at 4 °C to remove nuclei and debris. The supernatants were separated, aliquoted, and stored at −80 °C until analysis.

Determination of Lipid Peroxidation and Oxidative Stress Markers

Lipid peroxidation in the hippocampus tissue was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS), which were expressed in terms of malondialdehyde content according to Draper and Hadley's (1990) method. Briefly, 100 μl of trichloroacetic acid (5 %) was added to 100 μl of hippocampus supernatants and centrifuged at 4000×*g* for 10 min. One hundred microliters of the supernatants was transferred to a Pyrex tube and incubated with 200 μl of thiobarbituric acid reagent (TBA, 0.67 %) on a boiling water bath for 15 min. The TBARS were determined in microplate reader at 532 nm. The MDA values were calculated using 1,1,3,3-tetraethoxypropane as the standard and expressed as nanomoles of MDA/milligram protein.

Protein carbonyl (PCO) contents were detected by the reaction with 2,4-dinitrophenylhydrazine (DNPH) method as reported by Levine et al. (1990). Briefly, the DNPH reaction proteins were precipitated with an equal volume of 20 % (*w*/*v*) trichloroacetic acid and washed three times with 2 ml of an ethanol/ethyl acetate mixture (1:1). Finally, the precipitates were dissolved in 6 M guanidine HCl solution. The absorbance was measured at 370 nm, using the molar extinction coefficient of DNPH, e=22,000 M^{-1} cm^{-1}, and the results were expressed as nanomoles/milligram protein.

Hydroperoxide assay (H_2O_2) was determined by the method of Gay et al. (1999). Briefly, 50 μl of the sample was added to 950 μl of FOX 1 reagent (25 mM sulfuric acid, 250 μM ferrous ammonium sulfate, 100 μM xylenol orange, and 0.1 M sorbitol) and incubated for 30 min at room temperature. This assay is based on the ability of H_2O_2 to oxidize the ferrous Fe^{2+} ions to the ferric Fe^{3+} ions, which react with xylenol orange to a colored complex. The absorbance of the samples was read at 570 nm, and the concentration of H_2O_2 was determined using standard peroxide solutions in the same microtiter plate.

Determination of Nonenzymatic and Enzymatic Antioxidants

Reduced glutathione in hippocampus was determined by the method of Ellman (1959) based on the development of a yellow color when 5,5-dithiobis-2-nitrobenzoic acid (DTNB) was added to compounds containing sulfhydryl groups. The absorbance was measured at 412 nm after 10 min using a microplate reader. Total GSH content was expressed as nanomoles/milligram protein.

Acid ascorbic (vitamin C) content was determined spectrophotometrically by dinitrophenyl-hydrazine method described

by Jacques-Silva et al. (2001). Briefly, the ascorbic acid in the homogenate was oxidized by Cu^{2+} to form dihydro-ascorbic acid, which reacts with acidic 4-dinitrophenyl hydrazine to form a red hydrazone. Final color development was achieved with 65 % sulfuric acid, which was measured at 540 nm. The calibration curve was prepared using ascorbic acid as standard. Results were expressed as microgram/microgram protein.

Catalase (CAT) activity was assayed by the decomposition of hydrogen peroxide according to the method of Aebi (1984). A decrease in absorbance due to H_2O_2 degradation was monitored at 240 nm for 1 min, and the enzyme activity was expressed as l mol H_2O_2 consumed/min/mg protein.

Total superoxide dismutase activity (SOD) was evaluated by measuring the inhibition of pyrogallol activity as described by Marklund and Marklund (1974). One unit corresponded to the enzyme activity required to inhibit the half of pyrogallol oxidation. SOD activity was expressed as units/milligram protein.

Glutathione peroxidase activity (GPx) was measured according to Flohe and Gunzler (1984). The enzyme activity was expressed as nanomoles of GSH oxidized/minute/milligram protein.

Assay of Hippocampus Nitric Oxide

Nitric oxide production was determined based on the Griess reaction (Green et al. 1982). Briefly, 50 μl of deproteinized sample was incubated with 50 μl of the Griess reagent at room temperature for 10 min. Absorbance was measured at 550 nm using a microplate reader. Nitrite concentration was determined from a standard nitrite curve generated using $NaNO_2$.

The results were expressed as micromoles per milligram of protein.

Determination of Oxygen Reactive Species Levels in the Hippocampus

In order to determine the presence of oxidative imbalance caused by cisplatin, oxygen reactive species levels in hippocampus from aged rats were measured. According to the method of Driver et al. (2000). Supernatant was incubated with 10 μl of 5 mM 2′,7′ dichlorofluorescein diacetate (DCHF-DA). The oxygen reactive species levels were determined by a spectrofluorimetric method, using DCHF-DA assay. The oxidation of DCHF-DA to fluorescent dichlorofluorescein is measured for the detection of intracellular oxygen reactive species. The fluorescence intensity was measured using a fluorescence plate reader with an excitation wavelength of 485 nm and emission detection at 530 nm.

Determination of Acetylcholinesterase Activity

Acetylcholinesterase (AChE) activity was measured using a colorimetric assay based on the method of Ellman et al. (1961). Brievly, The reaction mixture contained 100 mM K^+ phosphate buffer, with pH 7.5 and 1 mM 5,5′-dithio-bisnitrobenzoic acid (DTNB). The method is based on the formation of the yellow anion, 5,5′-dithio-bis-acid nitrobenzoic, measured by absorbance at 412 nm during 2 min of incubation at 25 °C. The reaction was initiated by adding 0.8 mM acetylthiocholine iodide (AcSCh), the protein content was adjusted (0.8 μg/μl), and the results are expressed as micromoles per minute per milligram of protein.

Determination of Na^+, K^+, Ca^{2+}, and Mg^{2+} ATPase Activities in the Hippocampus

Na^+/K^+ ATPase, Ca^{2+} ATPase, and Mg^{2+}ATPase activities were assayed as previously described by Meng et al. (2008) and Carfagna et al. (1996). The inorganic phosphate–molybdate complex was quantitated by a spectrophotometer at 690 nm. The Na^+/K^+-ATPase activity was calculated as the difference between the total ATPase activity and the ATPase activity inhibited by ouabain, the specific-inhibitor of Na^+/K^+ ATPase. In the presence of Ca^{2+}, Ca^{2+}-ATPase activity appeared and was measured in the presence of Ca^{2+}, Na^+, K^+, and Mg^{2+} ions and ouabain. The ATPase activity was expressed as nanomoles Pi per milligrams of protein per minute.

Protein Quantification

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin as the standard.

Total RNA Isolation and Semiquantitative Reverse Transcription Polymerase Chain Reaction Amplification

Expression of inducible nitric oxide synthase (iNOS), acetylcholinesterase (AChE), and GAPDH genes in hippocampus tissues of experimental rats were measured using a reverse transcriptase polymerase chain reaction (RT-PCR) technique. Total RNA was extracted using the iScriptTM RT-qPCR sample preparation reagent and according to the manufacturer's instructions (170–8898, Bio-Rad). RNA concentrations and purity were determined by measuring the absorbance A_{260}/A_{280} ratios. The cDNA was produced from 5 μg of total messenger RNA (mRNA) by reverse transcription with superscript reverse transcriptase (Invitrogen, France) using oligo(dT)18 as a primer in a total volume of 20 μl. After incubation for 50 min at 42 °C, the reaction was terminated by denaturating enzyme for 10 min at 70 °C. PCR was performed according to the recommended protocol using the

PCR Master Mix (Invitrogen) and primers sequences used for the gene amplification: GAPDH (FP 5′-CCTCTCTCTTGC TCTCAGTAT-3′, RP 5′- CCTCTCTCTTGCTCTCAGTAT-3′), iNOS (FP 5′-GCATCACCCCTGTGTTCCACCC-30, RP 5′-TGGGGCAGTCTCCATTGCCA-3′), and ACHE (FP 5′-CCTGTGCGGGCAAAATTG-3′, RP 5('CTGGATCCCT CGCTGAA-3′) (Chtourou et al. 2012).

The amplification profile consisted of an initial denaturation at 94 °C for 5 min followed by denaturation at 94 °C, annealing from 55 to 60 °C, and extension at 72 °C for 1 min.

Expression of the housekeeping gene GAPDH served as the control. The number of amplification cycles was determined using individual primer sets to maintain exponential product amplification (25–30 cycles). Amplicons were separated by electrophoresis in 2 % agarose gel, visualized by staining with ethidium bromide (0.5 μg/ml), and the intensities of bands on the gels were calculated by ImageJ (National Institute of Health, MD, USA). All signals were normalized to mRNA levels of the house keeping gene, GAPDH, and expressed as a ratio.

Statistical Analyses

Results were expressed as mean±standard errors (mean±SEM). All analyses were carried out with GraphPad Prism 6.0 for Windows (GraphPad Software, San Diego, CA, USA). Significant differences between treatment effects were determined by one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons with statistical significance of $p<0.05$.

Results

Behavioral Tests

Naringin Prevents Behavioral Impairment Induced by Cisplatin Exposure

Figure 1 shows the effect of oral administration of naringin alone (Nar) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (Cis; 5 mg kg^{-1} $week^{-1}$), and their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on the open field data. Statistical analysis (one-way ANOVA) of testing showed that naringin alone at different doses (Nar 25, Nar 50, or Nar 100) did not modify open field step-down latencies ($p>0.05$) compared with control group (CT) (Fig. 1). Further, open-field data (one-way ANOVA) revealed that cisplatin (Cis) alone decreases the time spent in the central case of aged rats [$F_{(7, 72)}=8.302$, $p<0.001$], the number of crossing [$F_{(7,72)}=10.14$, $p<0.001$], and rearing [$F_{(7,72)}=6.815$, $p<0.001$] responses in a subsequent open-field test session compared with control group (CT), suggesting that Cis caused gross motor disabilities at testing (Fig. 1). Administration of naringin with cisplatin (Cis+Nar 50 and Cis+Nar 100 mg/kg) prevents the behavioral impairment observed in the Cis group, although Cis cotreatment with Nar at 25 mg/kg (Cis+Nar 25) did not prevent these modifications.

Effect of Cisplatin Exposure and Naringin Treatment on Anxiolytic-Like Behavior in the Elevated T-Maze Test

Table 1 shows the effect of oral administration of naringin alone (Nar) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (Cis; 5 mg kg^{-1} $week^{-1}$), and their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on the anxiolytic-like behavior in the elevated plus-maze task. Statistical analysis of testing (one-way ANOVA) showed that rats exposed to cisplatin alone (Cis) spent more time in closed arms [$F_{(7, 72)}=7.49$, $p<0.001$] and number of enteries in the closed arms [$F_{(7, 72)}=13.04$, $p<0.001$] compared with control group (CT), indicating anxiogenic effect of Cis. Moreover, administration of naringin with cisplatin (Cis+Nar 25, Cis+Nar 50, and Cis +Nar 100 mg/kg) prevents this increase in the time in closed arms and the number of entries in closed arms compared to Cis group. Statistical analysis showed that exposure to Cis (5 mg/kg) significantly increased the time spent on the open arms [$F_{(7, 72)}=11.64$, $p<0.001$] and of open-arm entries [$F_{(7, 72)}=8.84$, $p<0.001$]. Administration of naringin alone at different doses (Nar 25, Nar 50, or Nar 100) did not modify these parmeters measured in the elevated plus-maze task ($p>0.05$) compared with control group (CT).

Naringin Prevents the Increase in Acetylcholinesterase Activity Induced by Cisplatin in the Hippocampus

Figure 2a shows the effect of oral administration of naringin alone (Nar) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (Cis; 5 mg kg^{-1} $week^{-1}$), and their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on acetylcholinesterase (AChE) activity in the hippocampus of aged rats. One-way ANOVA showed that AChE activity [$F_{(7,64)}=4.418$, $p<0.0028$] was significantly increased in Cis group compared with the control group. Further, administration of naringin with cisplatin (Cis+Nar 50 and Cis+Nar 100 mg/kg) prevent this increase in the hippocampus observed in Cis group, although treatment with Nar at 25 mg/kg (Cis+Nar25) did not prevent the increase in AChE activity induced by Cis in the hippocampus. The administration of naringin alone (Nar 25, Nar 50, or Nar 100 mg/kg body weight) did not affect AChE activity in the hippocampus.

A one-way ANOVA analysis revealed significant differences in gene expression of mRNA AChE in the hippocampus [$F_{(7, 25)}=7.22$, $p<0.001$]. As shown in Fig. 2b, treatment with Cis produced a statistically significant increase in the relative

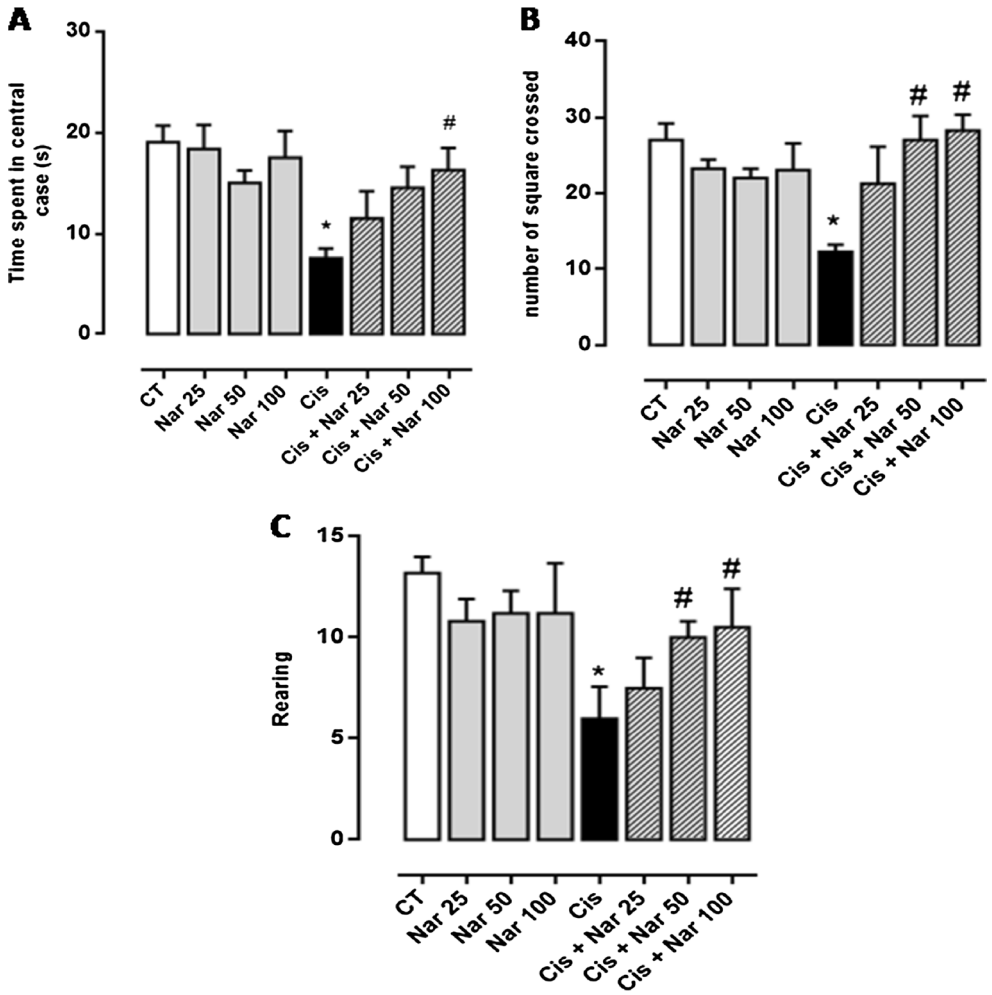

Fig. 1 Effect of oral administration of naringin alone (*Nar*) at different doses of Nar 25, Nar 50 and Nar 100 (mg/kg of body weight), cisplatin alone (*Cis*; 5 mg kg^{-1} $week^{-1}$) or their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on the open field data: the time spent in central case (**a**), the number of crossing (**b**), and the rearing responses (**c**) in aged rats. *Bars* represent means ±SEM of 10 rats in each group. One-way ANOVA-student followed by Tukey's post hoc test. *$p<0.05$: cisplatin group (*Cis*) (*dark bar*) compared with control group (*CT*) (*white bar*). $^{\#}p<0.05$: (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) groups (*stripped bars*) compared with cisplatin group (*Cis*) (*dark bar*)

Table 1 Effect of oral administration of naringin alone (Nar) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (Cis; 5 mg kg^{-1} $week^{-1}$) and their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on time spent in closed arms, number of entries in closed arms, time spent in open arms, and number of entries in open arms measured in the elevated plus maze over the 5-min test

Groups	Open arm		Closed arm	
	Time spent (s)	No. of entries	Time spent (s)	No. of entries
Control	53.25±4.14	4.17±0.65	141.05±3.87	7.12±1.30
Nar 25	51.05±2.11	5.76±1.32	139.1±5.33	9.43±1.52
Nar 50	53.98±2.76	4.65±0.92	137.98±2.54	8.07±0.65
Nar 100	51.87±1.84	4.21±0.52	139.76±3.98	7.66±0.32
Cis (5 mg/kg)	42.13±3.87**	3.01±0.05*	187.76±5.54*	9.43±1.52**
Cis+Nar 25	45.65±4.76$^{\#}$	3.43±1.12	164.82±2.65$^{\#}$	7.54±0.62$^{\#}$
Cis+Nar 50	51.98±1.77$^{\#}$	4.08±0.17$^{\#}$	155.65±1.87$^{\#\#}$	7.98±1.65$^{\#}$
Cis+Nar 100	50.22±1.45$^{\#}$	4.15±0.42$^{\#}$	148.09±3.41$^{\#}$	7.43±0.77$^{\#}$
Statistical analysis	$F_{[7,73]}=12.25$, $p<0.001$	$F_{[7,73]}=8.42$, $p<0.001$	$F_{[7,73]}=7.49$, $p<0.001$	$F_{[7,73]}=13.05$, $p<0.001$

Results in each group represent mean±SEM ($n=10$)

*$p<0.05$, **$p<0.01$: cisplatin-exposed group (Cis) compared with control group (CT)

$^{\#}p<0.05$, $^{\#\#}p<0.01$: (Cis+Nar 25, Cis+Nar 50, Cis+Nar 100) groups compared with cisplatin group (Cis)

mRNA AChE gene expression level by 80 % in the hippocampus compared to the control group. Moreover, it was demonstrated that administration of Naringin with cisplatin (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100 mg/kg) significantly decreased the relative mRNA AChE expression level in the hippocampus when compared with the Cis group ($p<0.01$). The administration of naringin alone (Nar 25, Nar 50, or Nar 100 mg/kg body weight) did not affect gene expression of mRNA AChE in the hippocampus.

Naringin Prevents the Decrease in Na^+, K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase Activities Induced by Cisplatin in the Hippocampus

Results obtained for Na^+, K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase activities in the hippocampus are presented in Fig. 3. One-way ANOVA data revealed that exposure to cisplatin alone (Cis) decreased Na^+, K^+-ATPase, Ca^{2+}-ATPase, and Mg^{2+}-ATPase activities in the hippocampus [$F_{(7,\ 64)}$= 5.152, $p<0.0011$; $F_{(7,\ 63)}$=3.455, $p<0.0168$; $F_{(7,\ 63)}$=7.338, $p<0.001$), respectively, compared to the control group. The administration of naringin with cisplatin (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100 mg/kg) prevents this reduction observed in Cis group. Further, one-way ANOVA analysis showed that only Cis+Nar50 and Cis+Nar100 mg/kg groups prevent significantly this effect on the hippocampus compared to the Cis group. No significant differences ($p>0.05$) were observed in the Na^+, K^+, Ca^{2+}, and Mg^{2+}-ATPase activities in groups treated with naringin alone (Nar 25, Nar 50, or Nar 100 mg/kg body weight) compared to the control group.

Naringin Prevents the Alterations of Oxidative Biomarkers Induced by Cisplatin Exposure in the Hippocampus

Lipid peroxidation (MDA), protein carbonylation (PCO), and hydrogen peroxide formation in the hippocampus of aged rats treated with naringin alone (Nar) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (Cis; 5 mg kg^{-1} $week^{-1}$), and their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks are presented in Table 2. One way-ANOVA showed a statistically significant difference among the groups for MDA [$F_{(7,\ 63)}$= 9.127, $p<0.0028$], PCO [$F_{(7,\ 63)}$=7.832, $p<0.001$], and H_2O_2 level [$F_{(7,\ 63)}$=8.48, $p<0.0032$]. Statistical analysis showed that exposure to Cis caused a significant increase in MDA, PCO, and H_2O_2 levels as compared to the control group. When naringin was administered with Cis (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100 mg/kg), a significant reduction of these biomarkers in the hippocampus was observed compared to that in the Cis group. The administration of different doses of naringin alone (Nar 25, Nar 50, or Nar 100 mg/kg of body weight) did not show significant effects when compared with the control group (Table 2).

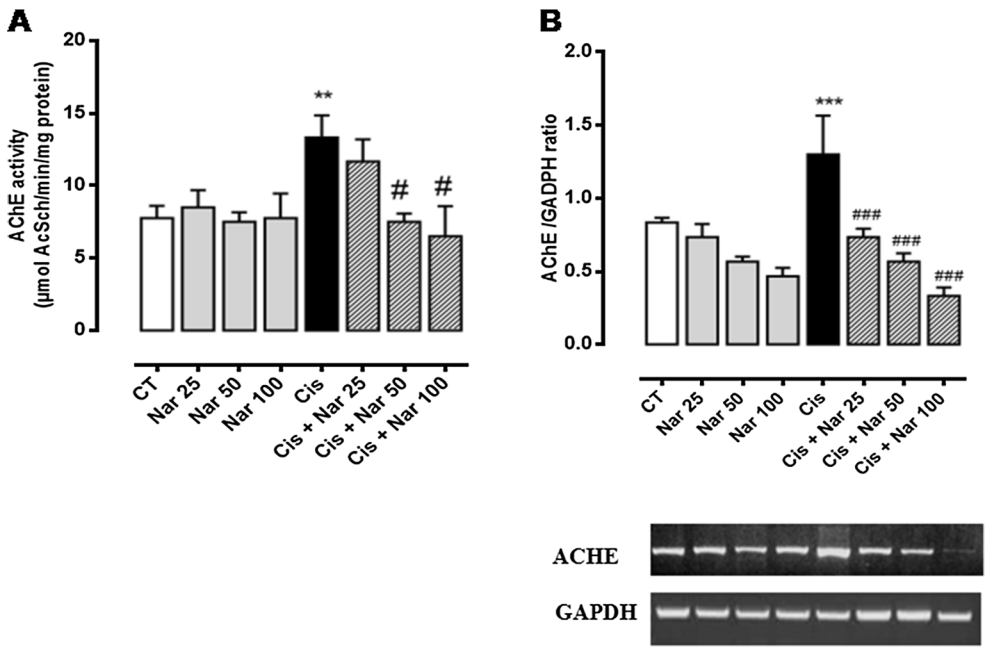

Fig. 2 Effect of oral administration of naringin alone (*Nar*) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (*Cis*; 5 mg kg^{-1} $week^{-1}$) or their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on acetylcholinesterase (*AChE*) activity (**a**) and mRNA expression of AChE (**b**) in the hippocampus of aged rats. *Bars* represent means± SEM of four to eight rats in each group. One-way ANOVA–Student followed by Tukey's post hoc test. $^{**}p<0.01$, $^{***}p<0.001$: cisplatin group (*Cis*) (*dark bar*) compared with control group (*CT*) (*white bar*). $^{\#}p<0.05$, $^{\#\#}\#p<0.001$: (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) groups (*stripped bars*) compared with cisplatin group (*Cis*) (*dark bar*)

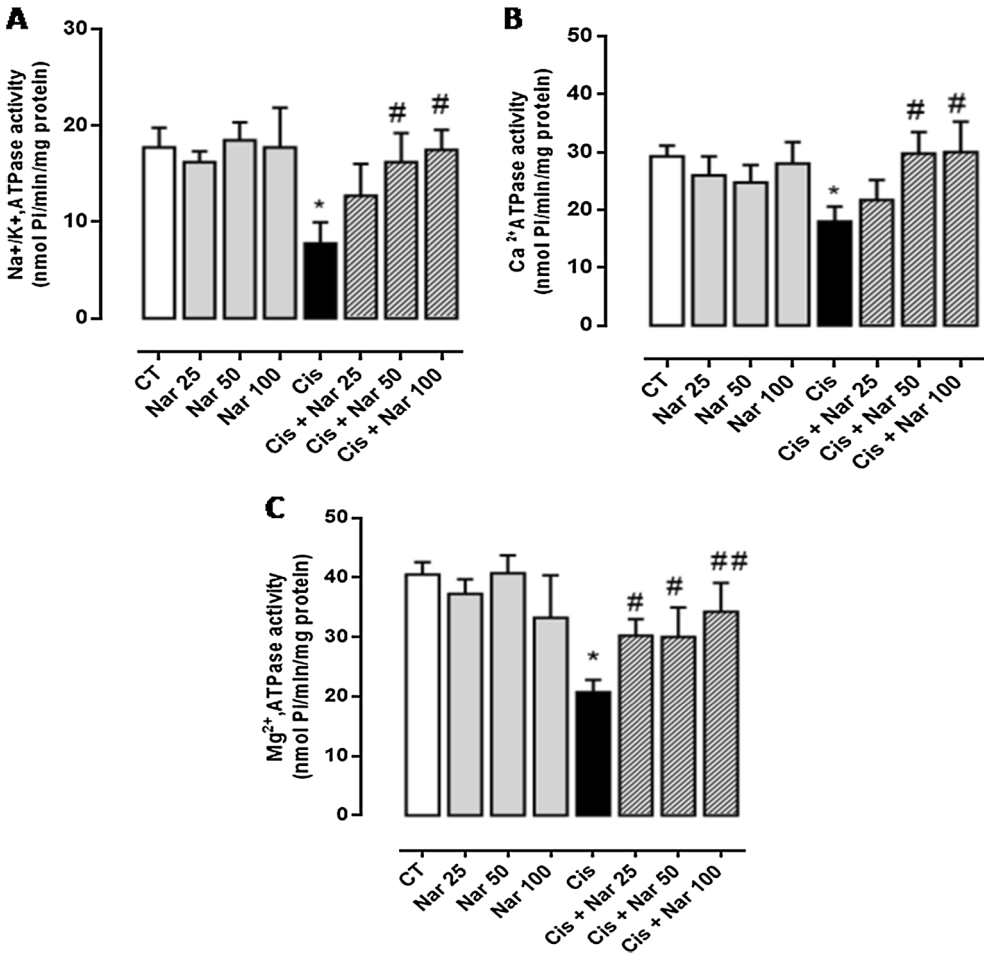

Fig. 3 Effect of oral administration of naringin alone (*Nar*) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (*Cis*; 5 mg kg^{-1} $week^{-1}$) or their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on Na^+/K^+, ATPase (**a**), Ca^{2+} ATPase (**b**), and Mg^{2+}, ATPase (**c**) activities in the hippocampus of aged rats. *Bars* represent means±SEM of eight rats in each group. One-way ANOVA–Student followed by Tukey's post hoc test. *$p<0.05$: cisplatin group (*Cis*) (*dark bar*) compared with control group (*CT*) (*white bar*). #$p<0.05$, ##$p<0.01$: (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) groups (*stripped bars*) compared with cisplatin group (*Cis*) (*dark bar*)

Naringin Prevents the Increase in Oxygen Reactive Species Levels Induced by Cisplatin Exposure in the Hippocampus

One-way ANOVA analyis on oxygen reactive species levels revealed a significant main effect of Cis exposure [$F_{(7,\ 61)}$= 13.56, $p<0.0023$] compared with the control group. The administration of naringin with cisplatin (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100 mg/kg) reduced significantly the increase in oxygen reactive species levels observed in the hippocampus of aged rats in the Cis group. The administration of different doses of naringin alone (Nar 25, Nar 50, or Nar 100 mg/kg of body weight) did not show a significant effects when compared with the control group (Fig. 4).

Effects of Naringin on Nitrite Formation and mRNA Expression of iNOS in the Hippocampus of Cisplatin-Treated Aged Rats

One-way ANOVA analysis revealed significant differences in gene expression of mRNA iNOS and NO formation in the hippocampus of aged rats, respectively [$F_{(7,\ 23)}$=5.44, $p<0.01$; $F_{(7,\ 61)}$=7.57, $p<0.01$] (Fig. 5). Compared to the control group, hippocampus expression of iNOS was significantly increased in the Cis group (Cis). The administration of naringin with cisplatin (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100 mg/kg) greatly inhibited the increase in iNOS mRNA expression observed in Cis group (Fig. 5b). We have observed also an increase in the levels of the NO in the Cis group (Cis) compared to the control group. Consistent with the down-regulation of iNOS, administration of naringin with cisplatin (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100 mg/kg) reduced the Cis-mediated production of NO (Fig. 5a). These results suggested that Nar suppressed the production of NO by inhibiting the expression of iNOS in the hippocampus of Cis-treated rats (Cis). Furthermore, no significant difference in the expression of iNOS and NO production were observed in naringin-treated group alone (Nar 25, Nar 50, or Nar 100 mg/kg) when compared with the control group.

Naringin Prevents the Alterations of the Nonenzymatic Antioxidant Induced by Cisplatin Exposure in the Hippocampus

Table 3 reports the changes of some nonenzymatic antioxidant parameters in the hippocampus of aged rats. Compared to the

Table 2 Effect of oral administration of naringin alone (Nar) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (Cis; 5 mg kg^{-1} $week^{-1}$) and their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on lipid peroxidation (MDA), protein carbonylation (PCO), and hyroperoxide generation (H_2O_2) in the hippocampus of aged rats

Groups	MDA (nmol/mg protein)	PCO (nmol/mg protein)	H_2O_2 (nmol/mg protein)
Control	15.21±1.24	0.97±0.33	11.87±1.65
Nar 25	17.12±3.21	1.05±0.04	10.43±2.11
Nar 50	14.05±1.02	0.83±0.02	12.65±1.87
Nar 100	14.32±0.78	0.74±0.03	10.44±1.66
Cis (5 mg/kg)	21.05±1.22**	1.43±0.02*	17.23±0.92*
Cis+Nar 25	19.73±1.83	1.30±0.27	13.03±0.33$^{\#}$
Cis+Nar 50	15.76±0.76$^{\#\#}$	1.05±0.02$^{\#\#}$	13.76±0.93$^{\#\#}$
Cis+Nar 100	14.45±1.42$^{\#\#}$	1.09±0.11$^{\#\#}$	12.56±1.42$^{\#\#}$
Statistical analysis	$F_{(7, 63)}$=9.12, p<0.001	$F_{(7, 63)}$=7.32, p<0.001	$F_{(7, 63)}$=12.05, p<0.01

Results in each group represent mean±SEM (n=10)

*p<0.01, **p<0.001: cisplatin exposed group (Cis) compared with control group (CT)

$^{\#}p$<0.05; $^{\#\#}p$<0.01: (Cis+Nar 25, Cis+Nar 50, Cis+Nar 100) groups compared with cisplatin group (Cis)

control group, a significant decrease in GSH [$F_{(7, 63)}$=9.12, p<0.0019] and ascorbic acid [$F_{(7, 63)}$=7.78, p<0.001] levels in the hippocampus were observed in the Cis group. The administration of naringin with cisplatin (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100 mg/kg) increased significantly these parmeters when compared with the Cis group. These biochemical variables did not differ noticeably between control and naringin-treated groups alone (Nar 25, Nar 50, or Nar 100 mg/kg).

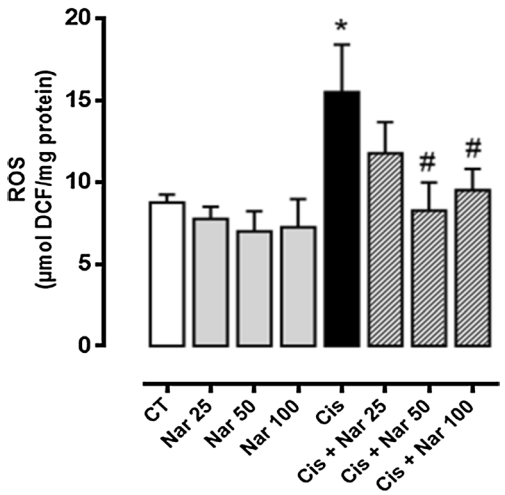

Fig. 4 Effect of oral administration of naringin alone (*Nar*) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (Cis; 5 mg kg^{-1} $week^{-1}$) or their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on intracellular reactive oxygen species (*ROS*) production by 2′-7′-dichlorofluorescein (*DCF*) levels in the hippocampus of aged rats. *Bars* represent means± SEM of eight rats in each group. One-way ANOVA–Student followed by Tukey's post hoc test. *p<0.05: cisplatin group (*Cis*) (*dark bar*) compared with control group (*CT*) (*white bar*). $^{\#}p$<0.05: (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) groups (*stripped bars*) compared with cisplatin group (*Cis*) (*dark bar*)

Naringin Prevents the Alterations of the Antioxidant Enzyme Activities Induced by Cisplatin Exposure in the Hippocampus

Table 4 shows the effect of oral administration of naringin at different doses (Nar 25, Nar 50, or Nar 100 mg/kg of body weight) alone or in combination with 5 mg kg^{-1} $week^{-1}$ of cisplatin (Cis) for five consecutive weeks on superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities in the hippocampus in aged rats. One-way ANOVA indicated a significant difference among the groups of antioxidant enzyme activities in the hippocampus. Statistical analysis showed that exposure to cisplatin (Cis) caused a significant decrease in SOD [$F_{(7, 63)}$=13.02, p<0.005], CAT [$F_{(7, 63)}$=8.325, p<0.0017], and GPx [$F_{(7, 63)}$=10.55, p<0.0015] activities when compared to the control group. The administration of naringin with cisplatin (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100 mg/kg) ameliorate the decrease in SOD, CAT, and GPx activities observed in the Cis group. The administration of different doses of naringin alone (Nar 25, Nar 50, or Nar 100 mg/kg of body weight) did not show a significant effect compared with the control group.

Discussion

Cisplatin-induced neurotoxicity is one of the major side effects that poses a serious medical challenge of paramount concern forcing its discontinuation during long-term treatment (Mc Donald et al. 2005). Lipid- and protein-rich brain tissue dependent on oxygen rich environment to function properly is at a greater risk of oxidative and nitrosative damage because of poor shield of antioxidant enzymes and low regeneration power as compared to other tissues in our body (Chirino and

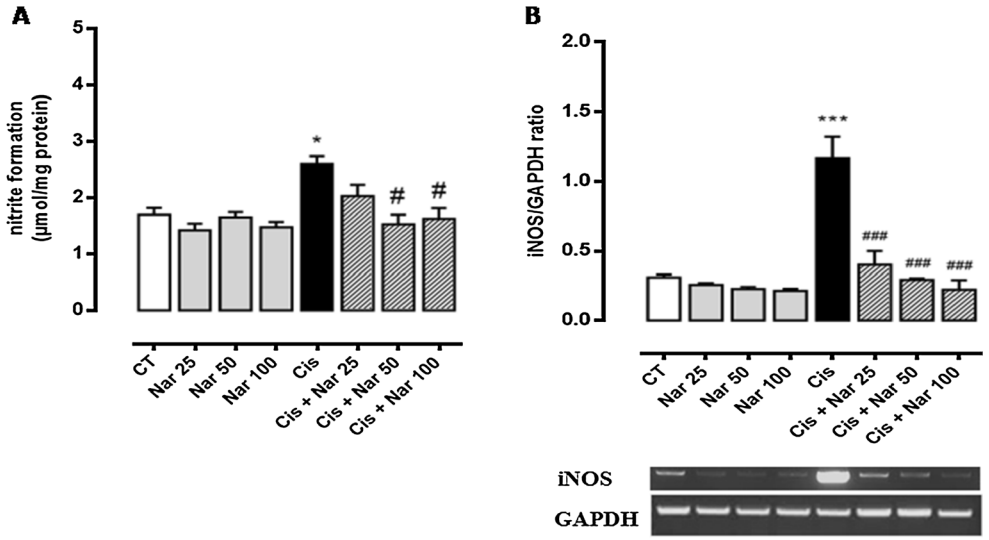

Fig. 5 Effect of oral administration of naringin alone (*Nar*) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (*Cis*; 5 mg kg^{-1} $week^{-1}$) or their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on nitrite formation (**a**) and mRNA expression of inducible nitric oxyde synthase iNOS (**b**) in the hippocampus of aged rats. *Bars* represent means±SEM of four to eight rats in each group. One-way ANOVA–Student followed by Tukey's post hoc test. *$p<0.05$, ***$p<0.001$: cisplatin group (*Cis*) (*dark bar*) compared with control group (*CT*) (*white bar*). $^{\#}p<0.05$, $^{\#\#\#}p<0.001$: (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) groups (*stripped bars*) compared with cisplatin group (*Cis*) (*dark bar*)

Pedraza-Chaverri 2009). Neurons, after the damage, cannot be healed properly, as they depend totally on the stem cells for their maintenance and limited regeneration. Thus, amelioration and/or prevention of neurotoxicity is an important clinical issue for a better life of the concerned patients. Different strategies have been used in the therapeutic usage of the drug to counter this side effect, but an effective strategy is yet to be acknowledged.

Because oxidative stress usually associates with cancer chemo- and radiotherapy, the complementary use of natural antioxidants could be a promising approach for reducing unfavorable adverse effects of such cancer modalities and improving treatment efficacy. In the present study, we demonstrated that a natural antioxidant, naringin, has great potential for complementary use in neuropathy by demonstrating its beneficial effects in preventing Cis-induced memory and learning impairment.

The open field (OF) and elevated plus maze (EPM) tests are a classic behavioral test with a strong aversive component and are used to evaluate learning and memory in rats and mice (Walsh and Cummins 1976; Pellow et al. 1985). In this study, we demonstrated that aged rats treated with cisplatin display significant cognitive deficits, including hippocampus-dependent learning and memory dysfunctions assessed by delayed alternation task in T-maze and open field tests accompanied simultaneously by neuronal alterations, decreased cholinergic function, and increased oxidative damage. Importantly, we show that the measure of central cholinergic dysfunction, oxidative damage, and neuronal alterations are significantly correlated with the impaired cognitive function in aged rats exposed to cisplatin, suggesting that cholinergic dysfunction and oxidative stress might be involved in the development of cognitive impairments induced by cisplatin treatment. These results are in agreement with other studies that have also verified cognitive impairment in Cis-exposed rats (Hill et al. 2010; Shabani et al. 2012) and in cancer patients (Vardy

Table 3 Effect of oral administration of naringin alone (Nar) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (Cis; 5 mg kg^{-1} $week^{-1}$) and their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on reduced glutathione (GSH) and ascorbic acid levels in the hippocampus of aged rats

Groups	GSH (nmol/mg protein)	Ascorbic acid (µg/mg protein)
Control	15.21±1.24	0.97±0.33
Nar 25	17.12±3.21	1.05±0.04
Nar 50	14.05±1.02	0.83±0.02
Nar 100	14.32±0.78	0.74±0.03
Cis (5 mg/kg)	21.05±1.22*	1.43±0.02*
Cis+Nar 25	19.73±1.83	1.30±0.27
Cis+Nar 50	15.76±0.76$^{\#}$	1.05±0.02$^{\#}$
Cis+Nar 100	14.45±1.42$^{\#}$	1.09±0.11$^{\#}$

Results in each group represent mean±SEM ($n=8$)

*$p<0.01$: cisplatin-exposed group (Cis) compared with control group (CT)

$^{\#}p<0.05$: (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) groups compared with cisplatin group (Cis)

Table 4 Effect of oral administration of naringin alone (Nar) at different doses of Nar 25, Nar 50, and Nar 100 (mg/kg of body weight), cisplatin alone (Cis; 5 mg kg^{-1} $week^{-1}$) and their combination (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) for five consecutive weeks on superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activities in the hippocampus of aged rats

Groups	SOD (U/mg protein)	CAT (μmol H_2O_2 consumed/min/mg protein)	GPx (nmol of GSH oxidized/min/mg protein)
Control	9.06±1.22	6.07±1.65	4.34±0.07
Nar 25	8.11±0.92	6.14±1.52	4.34±0.54
Nar 50	9.43±1.76	5.76±0.92	4.55±1.92
Nar 100	8.74±2.03	5.44±1.07	4.65±1.08
Cis (5 mg/kg)	5.43±1.67**	3.65±1.45*	1.01±0.01*
Cis+Nar 25	7.01±0.87$^{\#}$	7.11±1.99$^{\#}$	3.88±0.75$^{\#}$
Cis+Nar 50	8.42±1.79$^{\#}$	6.26±1.58$^{\#}$	3.96±0.44$^{\#}$
Cis+Nar 100	8.0±0.92$^{\#}$	6.17±0.86$^{\#}$	3.58±0.18$^{\#}$

Results in each group represent mean±SEM (n=8)

*p<0.05, **p<0.01: cisplatin-exposed group (Cis) compared with control group (CT)

$^{\#}$$p$<0.05: (Cis+Nar 25, Cis+Nar 50, and Cis+Nar 100) groups compared with cisplatin group (Cis)

et al. 2008). However, when Cis-exposed rats were orally treated with Nar (25, 50, or 100 mg/kg) for 5 weeks, behavioral parameters were similar to that found for aged rats from the control group. These findings indicate that treatment with naringin was able to prevent the learning and memory impairment induced by Cis exposure. Our results are in agreement with the literature; a number of studies have demonstrated that different doses of naringin, both orally and as an intragastric infusion, have the potential to ameliorate the cognitive impairment caused by different types of agents; D-galactose (Kumar et al. 2010a,b,c), ischemic reperfusion (Gaur et al. 2009), and pentylenetetrazol-induced seizures (Golechha et al. 2014). It is important to mention that naringin alone did not affect cognitive parameters.

The central cholinergic system, particularly in the hippocampus, plays an undisputed key role in the regulation of learning and memory, which are the primary constituents of cognitive behavior (Blokland 1995). In line with this role, the normal mRNA expression of AChE and its activity in the Cis-exposed groups treated with 25, 50, or 100 mg/kg of naringin in the hippocampus may be the major factor responsible for modulating learning and memory (Paul 2003; Havekesa et al. 2011). Further, AChE activity in the hippocampus was not influenced by naringin alone at the doses of 25, 50, or 100 mg/kg. We speculate that naringin may prevent the excitotoxicity promoted by Cis exposure, which might explain the finding that naringin is able to reduce AChE activity when it is stimulated, while it does not modify basal AChE activity. Although the majority of the naringin doses were effective in preventing the increase in AChE activity in the hippocampus promoted by the Cis exposure, the dose of 25 mg/kg was not effective in reversing this increase in the hippocampus. Corroborating our findings, a previous study also demonstrated that naringin, orally administered at different doses (50, 100, or 200 mg/kg) for 21 days, significantly attenuated cholinergic dysfunction observed in ICV-STZ rats (Sachdeva et al. 2014).

Oxidative stress and mitochondrial dysfunction are key pathogenic mechanisms involved in chemotherapy-induced peripheral neuropathy (CIPN) (Areti et al. 2014). Certain structural and functional attributes of central nervous system (CNS) make it more susceptible to accumulation of chemotherapeutics and some neurotoxins (Argyriou et al. 2012). Lack of an efficient vascular barrier and absence of lymph drainage make the CNS more prone to toxic chemical insults. In addition, mammalian nerves are known to be more susceptible to oxidative stress because of their high content of phospholipids, mitochondria rich axoplasm, and also due to weak cellular antioxidant defense. The excess generation of ROS and inducible NO synthase (iNOS) causes oxidative damage to cellular proteins, lipids, or DNA and subsequently inhibits their normal functions and disturbs homeostatis within the neuron, ultimately resulting in cell death via apoptosis (McDonald and Windebank 2002; Salvemini et al. 2011; Ta et al. 2013). In our study, the hippocampus of the Cis-treated rats showed an increase in malondialdehyde, protein carbonyls levels, and nitrite formation associated with an overexpression of iNOS, a marker of oxidative stress. Further, previous studies indicate that ROS and free radicals are involved in the inhibition of Ca^{2+}-ATPase (Rohn et al. 1996) and Na^{+}, K^{+}-ATPase (Franzon et al. 2003). The decrease in membrane-bound enzymes in the hippocampus of Cis-treated rats support these findings.

In regard to all pro-/antioxidant parameters evaluated in this study, ours results showed that naringin administration at different doses is effective in preventing the Cis-induced changes in the redox balance, thus contributing to prevent the increase in markers of cellular damage mediated by ROS. This improvement in learning and memory deficits, oxidative stress markers, and cholinergic dysfunction can be attributed to its actions as a potent antioxidant that result from

its direct scavenging activity of superoxide, hydroxyl radicals, metal chelating proprety (Gopinath et al. 2011), and its ability to improve membrane homeostasis (Kumar et al. 2010a,b,c). We suggest that naringin protection of membranes from ROS-mediated damage was of potential usefulness in the prevention of ion homeostasis breakdown and probably in the prevention and treatment of certain disease process involving the hippocampus. These results are supported by our previous laboratory findings, which demonstrated that naringenin, a metabolite of naringin, mediates the oxidative damages observed in the cerebral cortex of iron oveload rats (Chtourou et al. 2012) and in liver of hypercholeserolemic rats (Chtourou et al. 2015).

In summary, the present study shows that naringin ameliorated the cisplatin-induced cognitive deficits, cholinergic dysfunction, and oxidative damage in the hippocampus of aged rats. These results suggest that naringin and naringin-enriched diets should be an essential part of the therapeutic regimen to prevent against chemotherapy-induced peripheral neuropathy and cognitive deficits.

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