

Naringenin Mitigates Iron-Induced Anxiety-Like Behavioral Impairment, Mitochondrial Dysfunctions, Ectonucleotidases and Acetylcholinesterase Alteration Activities in Rat Hippocampus

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Received: 4 March 2015/Revised: 21 May 2015/Accepted: 25 May 2015/Published online: 7 June 2015
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Abstract Studies demonstrated that the iron chelating antioxidant restores brain dysfunction induced by iron toxicity in animals. Earlier, we found that iron overload-induced cerebral cortex apoptosis correlated with oxidative stress could be protected by naringenin (NGEN). In this respect, the present study is focused on the mechanisms associated with the protective efficacy of NGEN, natural flavonoid compound abundant in the peels of citrus fruit, on iron induced impairment of the anxiogenic-like behaviour, purinergic and cholinergic dysfunctions with oxidative stress related disorders on mitochondrial function in the rat hippocampus. Results showed that administration of NGEN (50 mg/kg/day) by gavage significantly ameliorated anxiogenic-like behaviour impairment induced by the exposure to 50 mg of Fe-dextran/kg/day intraperitoneally for 28 days in rats, decreased iron-induced reactive oxygen species formation and restored the iron-induced decrease of the acetylcholinesterase expression level, mitochondrial membrane potential and mitochondrial complexes activities in the hippocampus of rats. Moreover, NGEN was able to restore the alteration on the activity and expression of ectonucleotidases such as adenosine triphosphate diphosphohydrolase and 5'-nucleotidase, enzymes which hydrolyze and therefore control extracellular ATP and adenosine concentrations in the synaptic cleft. These results may contribute to a better understanding of the neuroprotective role of NGEN, emphasizing the influence of including this

flavonoid in the diet for human health, possibly preventing brain injury associated with iron overload.

Keywords Iron · Anxiety · AChE · Oxidative stress · Hippocampus · NTPDase · Naringenin

Introduction

Iron is an essential nutrient that plays an important role as a cofactor in a number of physiologically crucial processes [1]. Excessive iron deposition is known to generate massive reactive oxygen species (ROS) and increase the level of oxidative stress through the Fenton reaction [2]. These ROS, notably the hydroxyl radical, would consequently damage the structure of cell membranes, proteins and nucleic acids leading to apoptosis and necrosis in brain regions [3, 4]. Iron accumulation in neurons, astrocytes, and microglia has been reported in the basal ganglia as well as in the cerebral cortex and hippocampus regions affected in neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), and multiple sclerosis (MS) [5]. Excess iron in the brain appears to alter anxiety-like behavior and mood [5]. Anxious responses, determined by the elevated plus maze, are observed in adult rats receiving daily intraperitoneal injections of iron [6]. Other behavioral impairments have been found in rats fed on a carbonyl iron diet containing 20,000 ppm iron [7]. These findings support the idea that the imbalanced iron metabolism plays a pivotal role in modulating anxiety. The hippocampus is a critical brain area for learning, memory and many other behavioral processes. Adult hippocampus relies on iron availability for many essential functions, but is highly vulnerable to iron-induced oxidative stress [8].

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Iron has been found to be required for long-term potentiation in hippocampal CA1 neurons and it is known to participate in the stimulation of calcium release through ROS produced via the Fenton reaction and triggering the activation of cellular signaling pathways [9, 10]. These results support a coordinated action between iron and calcium in synaptic plasticity and raise the possibility that elevated iron levels may contribute to neuronal degeneration through excessive intracellular calcium increase caused by iron-induced oxidative stress [11]. Previous studies in rodents have shown that oral administration of iron during the period of rapid brain development produces iron accumulation in the hippocampus. Da Silva et al. [12] observed that iron-induced oxidative stress may be related to mitochondrial dysfunction, resulting in an alteration in the expression of fusion and fission proteins, which might ultimately lead to unbalanced mitochondrial dynamics and impaired energy production reducing the ATP levels in the brain of rats [12]. The impairment of mitochondrial function and reduction of ATP levels and extracellular adenosine accumulation are pathological conditions found in neurodegenerative diseases such as Alzheimer's disease (AD), which is closely linked to the decline of cognitive processes [13]. The hippocampal ATP is essential in regulating glial interactions with neurons and glial regulation of synaptic transmission. ATP is released with the neurotransmitter and it acts upon purinergic receptors in perisynaptic glia. The glial cells in turn release many neuromodulatory substances to regulate the postsynaptic or presynaptic function. The astrocytes can then communicate among themselves by sending ATP signals through astrocytic networks to perhaps affect another synapse to modulate neuro-transmission at a distant site [14]. Extracellular nucleotides (ATP and ADP) may be hydrolyzed by members of the ecto-nucleoside triphosphate diphosphohydrolase family (E-NTPDases), and AMP may be hydrolyzed by the ecto-5'-nucleotidase to produce adenosine. In this way, E-NTPDases control the availability of ligands for both nucleotide and nucleoside receptors and consequently the duration of receptor activation. Therefore, this is an enzymatic pathway with the double function of eliminating one signaling molecule, ATP, and generating another, adenosine. These enzymes may also exert a protective function by keeping extracellular ATP/ADP and adenosine within physiological concentrations [15]. Due to the role of iron in oxidative stress, iron chelators have been used as a neuroprotective strategy in different *in vitro* and *in vivo* neurotoxic models. Deferoxamine (DFO), one such chelator, acts by binding Fe^{3+} and thereby preventing iron ions from catalyzing redox reactions that lead to free radical formation. In this sense, the very high affinity of the natural product desferrioxamine, has been explored to treat circulatory iron overload [13]. Growing evidence from *in vitro*,

in vivo studies and clinical trials has shown that dietary polyphenols are strongly associated with a reduced risk of nervous diseases. Naringenin, a naturally occurring flavanone in grapefruits, citrus fruits and tomatoes has been demonstrated to elicit a wide range of activities such as antioxidant, anti-inflammatory and chemopreventive properties [16]. Importantly, its lipophilic character favors a good blood–brain barrier (BBB)-permeability, which suggests that naringenin could play a significant role in important functions of the CNS especially under pathological conditions. For example, naringenin exerts protective effect against cerebral ischemic injury, attenuates β -amyloid toxicity [17], induces the activation of MAP kinases, modulates glutamate uptake [18] and protects against neurodegeneration with cognitive impairment caused by the intracerebroventricular-streptozotocin in diabetic oxidative damage rat model [19]. However, the mechanisms underlying the neuroprotective effects of naringenin on cholinergic and purinergic neurotransmission in animal models of iron exposure remain unknown. In this context, considering the neuroprotective actions of naringenin and the importance of NTPDase, 5'-nucleotidase and acetylcholinesterase (AChE) for CNS functioning, the aim of the present study was to evaluate the activity of these enzymes in hippocampus from iron-induced rats treated with naringenin, in order to investigate the potential therapeutic use of this compound in hippocampal dysfunction associated with iron overload.

Experimental Procedures

Chemicals and Reagents

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

Animal Treatments and Experimental Protocol

Male rats of Wistar strain aged 10 weeks weighing around 250 g were obtained from the Central Pharmacy (SIPHAT, Tunisia). They were fed on pellet diet, purchased from the Industrial Society of rodent diet (SICO, Sfax, Tunisia). All animal procedures were conducted in strict conformity with the local Institute Ethical Committee Guidelines for the Care and Use of laboratory animals of our Institution. Tissue samples for this study were obtained from rats treated in the same manner as in the previously described animal model [20]. Maximum effort was made to minimize the number of used animals. Briefly, rats were randomly divided into 4 experimental groups ($n = 30/\text{group}$): the first group of rats served as the control, received *ad libitum*

distilled water with intraperitoneal (i.p.) injections of iron (Fe) and orally NGEN of buffered saline and propylene glycol/saline [25/75 (v/v)] vehicle solutions, respectively. The second group (Fe) was randomized to receive repetitive i.p. injections of 50 mg of Fe-dextran/kg/day dissolved in buffered saline, 5 days per week. Animals in the third group (FeNGEN) were given repetitive (ip) injections of iron dextran 24 h after the oral administration of NGEN (50 mg/kg bw). The fourth group (NGEN) was given a single of NGEN dissolved in propylene glycol and saline 25/75 (v/v) orally administered on a daily basis. In the last day of treatment (after 28 days), animals were subjected to training and behavior parameter estimation.

Behavioral Assessment

Marble Burying Test (MBT)

The marble burying test was used to measure an anxiety-induced behavioral response to environmental challenge. It was performed in the last week of the experiment, during the light phase of a stress-free period. The method was adapted from previous studies [21]. Briefly, four glass marbles (2.5 cm in diameter) were placed along a side-wall of each home-cage, and behavior of rats was observed during a 30-min test period. The following parameters were recorded: the number of rats showing burying behavior, and the number of buried marbles (at least two thirds of the surface covered with sawdust). Marble burying behavior reflected an active effort of a rat to hide the unfamiliar object in sawdust bedding, and therefore, it may indicate anxiety-like behavior [21].

Open Field Test (OFT)

The open-field test was used to investigate locomotor activity and exploratory behavior. Briefly, the rats were transferred to an open field measuring 40 × 45 × 50 cm with the floor divided into 16 (4 × 4) equal squares highlighted by black lines. Animals were placed in the central case to explore the field freely for 5 min. Latency to start locomotion, line crossings, rearings, and the numbers of fecal pellets produced were counted. The number of crossings and rearings were used, respectively, as measures of locomotor activity and exploratory behavior, whereas the latency to start locomotion and the number of fecal pellets were used as measures of anxiety [22]. At the end of each test, the apparatus was thoroughly cleansed with cotton wool dipped in 30 % ethanol.

Elevated Plus Maze Test (EPM)

The anxiolytic-like behavior was evaluated using the task of the elevated plus maze as described by Cohen et al. [23].

Briefly, the maze apparatus is composed of 4 arms of the same size, with two closed arms (50 × 10 cm, walls 40 cm) and two open (50 × 10 cm). Each rat was placed individually at the centre 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. The apparatus was thoroughly cleaned with 30 % ethanol after each session.

Biochemical Analysis of Hippocampus Homogenate

After the behavioral tests, animals in different groups were sacrificed by cervical decapitation to avoid stress conditions. The brain tissue was immediately removed and dissected over ice-cold glass slides and the hippocampus region was collected. The Hippocampi were homogenized in a glass potter in a solution of 10 mM Tris-HCl, with pH 7.4, on ice, at a proportion of 1:10 (w/v) and centrifuged at 10,000×g for 15 min at 4 °C. The resulting homogenate was used to determine the acetylcholinesterase (AChE) activity, oxidative stress parameters, antioxidant enzymes activities and endogenous non-enzymatic antioxidant content.

Reactive oxygen species (ROS) levels in supernatants were measured according to Shinomol and Muralidhara [24] using 2',7'-dichlorofluorescein diacetate (DCFH-DA) in a 96-well plate. DCF fluorescence intensity was recorded using a CFX96 (Bio-Rad Laboratories, Inc., Hercules, CA, USA) fluorescence plate reader with an excitation wavelength of 485 nm and emission detection at 530 nm. Results were expressed as nmoles DCF/mg of protein, using a standard curve with DCF.

The extent of lipid peroxidation by measuring thiobarbituric acid reactive substances (TBARS) in terms of malondialdehyde (MDA) formation was measured using a microplate reader at 532 nm according to the method of Draper and Hadley [25]. The MDA values were calculated using 1,1,3,3-tetraethoxypropane as the standard and expressed as nmoles of MDA/mg protein.

Protein carbonyls were determined using 2,4 dinitrophenylhydrazine and the basis of the assay involved the reaction between protein carbonyl and dinitrophenylhydrazine to form a protein hydrazone [26]. The absorbance was measured spectrophotometrically at 370 nm, using the molar extinction coefficient of DNPH, $\epsilon = 22,000/\text{M cm}$ and the results were expressed as nmoles of carbonyl/mg protein.

Reduced glutathione (GSH) levels were estimated in terms of non-protein thiols according to the method previously described [27]. Results were expressed as nmoles GSH/mg protein.

Nitric oxide production was determined according to the method of Green et al. [28]. Absorbance was measured

spectrophotometrically 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 nmoles/mg protein.

Catalase (CAT) activity was assayed by the decomposition of hydrogen peroxide according to the method of Aebi [29]. 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 $\mu\text{mol H}_2\text{O}_2$ consumed/min/mg protein.

Total superoxide dismutase activity (SOD) was estimated spectrophotometrically according to Beauchamp and Fridovich [30]. Units of SOD activity were expressed as the amount of enzyme required to inhibit the reduction of NBT by 50 %, and the activity was expressed as U/mg protein.

Glutathione peroxidase activity (GPx) was measured according to Flohe and Gunzler [31]. The enzyme activity was expressed as nmoles of GSH oxidized/min/mg protein.

Acetylcholinesterase (AChE) activity was measured using a colorimetric assay according to the methods of Ellman et al. [32]. The protocol was modified for use with 96-well microplates as previously described [33] using acetylthiocholine iodide (AcSCh) as a substrate. The release of the thiol compound (thiocholine), which produces the color-forming compound TNB after reaction with DTNB was measured at 412 nm and the results are expressed as $\mu\text{moles AcSCh/min/mg protein}$.

Mitochondrial Parameter Assessment

Mitochondria were isolated from the hippocampus as described previously [34]. Briefly, the hippocampus were immediately excised, weighed and homogenized in ice-cold isolation buffer with EGTA (225 mM Mannitol, 75 mM sucrose, 0.1 % BSA, 1 mM EGTA, pH 7.2) using a Potter homogenizer with Teflon pestle. The homogenate was centrifuged at $1300\times g$ for 10 min, and the supernatant was re-centrifuged at $14,000\times g$ for 15 min at 4 °C. The crude mitochondrial pellet was separated and washed with buffer and further centrifuged at $7000\times g$ for 15 min at 4 °C. The final pellet containing mitochondrial rich fraction was resuspended in isolation buffer without EGTA. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) reduction was used to assess the activity of the mitochondrial respiratory chain in isolated mitochondria as described by Liu et al. [35]. NADH dehydrogenase (complex I) activity was measured spectrophotometrically as described by King [36] and results were expressed as nmoles NADH oxidized/min/mg of protein using the molar extinction coefficient of reduced cytochrome c at 550 nm (1.96/mM cm). Succinate-cytochrome c oxidoreductase (complex II–III) activity was assayed according to the method

described by Clark et al. [37] and results were expressed as nmoles of oxidized cytochrome c reduced/min/mg of protein using the molar extinction coefficient of potassium ferricyanide (1000/M cm). Cytochrome oxidase (complex IV) was assayed according to Sottocasa et al. [38] and results were expressed as nmoles of cytochrome c oxidized/min/mg of protein using the molar extinction coefficient of cytochrome c (1.96/mM cm). F1–F0 ATP synthase (complex V) was assayed using the method of Griffiths and Houghton [39]. The released phosphate was analyzed on the basis of the reaction of the inorganic phosphate with ammonium molybdate as described by Fiske and Subbarow [40] and results were expressed as nmoles of ATP hydrolyzed/min/mg protein. The mitochondrial membrane potential ($\Delta\Psi_m$) was measured using rhodamine 123 (Rho123) in a fluorescence plate reader at the excitation and emission wavelength of 490 and 535 nm, respectively [41] and results were expressed as the percentage of control group.

Ectonucleotidase Activities in Synaptosomes

Synaptosomes from pooled hippocampus (three/group/isolation) of the same group were isolated as described previously [42]. Briefly, the hippocampi were gently homogenized in 10 volumes of an ice-cold medium (0.32 M sucrose, 5 mM HEPES–Tris, pH 7.4) with a Teflon-glass homogenizer, using a discontinuous percoll gradient. The pellet was resuspended in an isosmotic solution and the final protein concentration was adjusted to 0.4–0.6 mg/ml.

The ectonucleoside triphosphate diphosphohydrolase (NTPDase) enzymatic assay of the synaptosomes was carried out in a reaction medium containing 5 mM KCl, 1.5 mM CaCl_2 , 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μl according to Schetinger et al. [43]. The ecto-5'-nucleotidase (CD73) activity was determined essentially by the method of Heymann et al. [44] in a reaction medium containing 10 mM MgSO_4 and 100 mM Tris–HCl buffer, pH 7.5, in a final volume of 200 μl .

Analysis of Gene Expression by Semi-quantitative RT-PCR

Expression of the ectonucleoside triphosphate diphosphohydrolase (NTPDase1–3), ecto-5'-nucleotidase (CD73) and acetylcholinesterase (AChE) genes in the hippocampus tissues of experimental rats ($n = 4/\text{group}$) were measured using a reverse transcriptase 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}

A280 ratios. The cDNA was produced from 2 µg of total 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 denaturing the enzyme for 10 min at 70 °C. cDNA (2 µl) was used as a template for PCR according to the recommended protocol using the PCR Master Mix (TaKaRa Taq™ DNA Polymerase) and primer sequences used for the gene amplification are given in Table 1 [45, 46]. The amplification profile consisted of an initial denaturation at 94 °C for 5 min followed by denaturation at 94 °C for 30 s, annealing from 59 to 66 °C and extension at 72 °C for 1 min. Expression of the housekeeping gene β-Actin served as the control. The number of amplification cycles was determined using individual primer sets to maintain exponential product amplification (25–30 cycles). Amplicons [or Mixed Amplicons (1:1) for NTPDase1–3, ecto-5′-nucleotidase (CD73)] 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 Image J (National Institute of Health, MD, USA). All signals were normalized to mRNA levels of the house keeping gene, β-Actin, and expressed as a percentage of control.

Results

Effect of Iron Exposure and Naringenin Co-treatment on Anxiolytic-Like Behavior

The marble burying test (MBT), Open field test (OFT) and Elevated plus maze test (EPM) were conducted to test the anxiety-like behavior of rats. In MBT test, as seen in

Fig. 1, Fe-treated rats showed a significant increase in the number of buried marbles compared to control animals ($p < 0.05$). NGEN was able to decrease the number of buried marbles in FeNGEN-treated rats. No significant differences were found between the control and NGEN groups in the activity level.

Figure 2 shows the effect of iron exposure and naringenin co-treatment on the anxiolytic-like behavior in the elevated plus-maze task. Statistical analysis of testing (one-way ANOVA) showed that rats exposed to iron spent more time in closed arms and entered the closed arms more frequently compared with the other groups indicating a possible behavior induced by Fe (Fig. 2a, b). Moreover, NGEN (50 mg/kg) was able to prevent this increase of time in closed arms and the number of entries in closed arms induced by Fe (Fig. 2a, b). Figure 2c shows that Fe

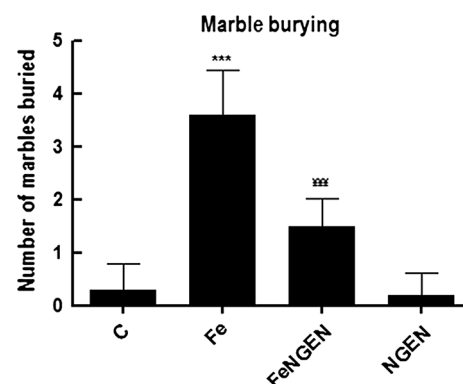


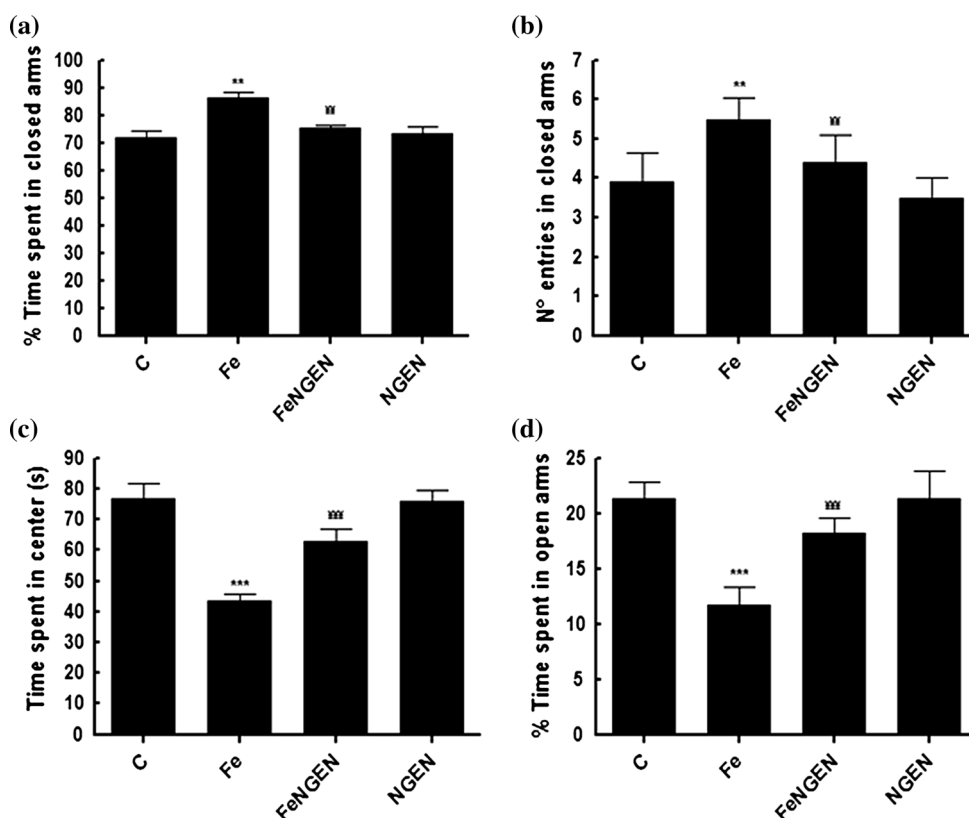
Fig. 1 Effect of iron-exposed (Fe) rats and treated with naringenin (NGEN) or their combination (FeNGEN) on marble burying test. Values are expressed as mean ± SD of 10 rats per group. Fe group versus control group: *** $p < 0.001$. Fe + NGEN group versus Fe group ^{###} $p < 0.001$

Table 1 Primer sequences used for semi-quantitative RT-PCR reactions

Gene name	Primer sequence	Accession number	Product size (pb)
β-Actin	Forward: 5′-GGAGATTACTGCCCTGGCTCCTA-3′	NM_031144.3	150
	Reverse: 5′-GACTCATCGTACTCCTGCTTGCTG-3′		
NTPDase1	Forward: 5′-GATCATCACTGGGCAGGAGGAAGG-3′	XM_008760464.1	543
	Reverse: 5′-AAGACACCGTTGAAGGCACACTGG-3′		
NTPDase2	Forward: 5′-GCTGGGTGGGCCGGTGGATACG-3′	NM_172030.1	331
	Reverse: 5′-ATTGAAGCCCGGGACGCTGAC-3′		
NTPDase3	Forward: 5′-CGGGATCCTTGCTGTGCGTGGCATTCTT-3′	NM_178106	267
	Reverse: 5′-TCTAGAGGTGCTCTGGCAGGAATCAGT-3′		
CD73	Forward: 5′-CCCGGGGGCCACTAGCACCTCA-3′	NM_021576.2	405
	Reverse: 5′-GCCTGGACCACGGGAACCTT-3′		
AChE	Forward: 5′-CCTGTGCGGGCAAATTG-3′	NM_172009.1	112
	Reverse: 5′-CTGGATCCCTCGCTGAA-3′		

β-Actin, beta actin; NTPDase, ectonucleoside triphosphate diphosphohydrolase; CD73, ecto-5′-nucleotidase; AChE, acetylcholinesterase

Fig. 2 Effect of iron-exposed (Fe) rats and treated with naringenin (NGEN) or their combination (FeNGEN) on anxiolytic-like behavior test in the elevated plus maze. **a** The % time in closed arm, **b** the number of entries in closed arms, **c** time in center and **d** the % time in the open arms. Values are expressed as mean \pm SD of 10 rats per group. Fe group versus control group: *** $p < 0.001$, ** $p < 0.01$. FeNGEN group versus Fe group: $^{***}p < 0.001$, $^{**}p < 0.01$



decreased the time in center, and NGEN was also able to prevent this anxiogenic effect induced by Fe.

Table 2 shows the number of crossings or rearings, latency to start locomotion, and defecation quantified in the experimental rats during the behavior study of the open field test. The control and the NGEN treated animals show normal locomotor activity due to less anxiety and stress while the Fe-treated group showed an increased number of crossings and rearings with a lower latency time to start locomotion compared to control rats (Table 2). The findings also revealed that these statistic variations were significantly reduced following NGEN co-administration, thus confirming its neuroprotective effects. There is no significant difference observed in a number of fecal bowls in all the groups (Table 2).

Naringenin Reduced Iron-Induced Oxidative Stress Markers in the Hippocampus

Tables 3 and 4 show the changes in the oxidative stress marker levels in the hippocampus tissue of control and experimental rats. The Fe exposure resulted in a significant increase ($p < 0.001$) in the hippocampal ROS, MDA, NO_2^- , PCO and the depletion of reduced GSH, SOD, GPx and catalase as compared to the control group. However, co-administration of NGEN with Fe significantly attenuated oxidative stress (reduced the elevated ROS, MDA,

PCO, NO_2^- concentration and restored SOD, GPx, catalase and GSH levels) when compared to the group induced by iron exposure.

Naringenin Restored Iron-Induced Mitochondrial Enzyme Complexes Activities and Mitochondrial Membrane Potential

Results on the activity of enzyme complexes involved in the energy metabolism of mitochondria assessed in the hippocampus of rats exposed to iron, naringenin or exposed simultaneously to iron and NGEN are presented in Fig. 3a. A significant decrease ($p < 0.001$) in the activity of complex I, complex II–III, complex IV and complex V in the hippocampus mitochondria was observed following Fe exposure in rats as compared to controls. Simultaneous treatment with NGEN and Fe in rats was found to ameliorate Fe-induced damage and significantly restored ($p < 0.001$) the activity of these enzymes when compared to Fe-treated rats. No significant effect on the activity of any of the mitochondrial complexes in the hippocampus was observed in rats treated with NGEN as compared to control rats. Moreover, in Fig. 3b, we evaluated the effect of iron on the mitochondrial membrane potential ($\Delta\Psi_m$) by the measurement of the uptake of the cationic fluorescent dye rhodamine 123. $\Delta\Psi_m$ is a highly sensitive indicator of the mitochondrial inner membrane condition. The

Table 2 Effects of naringenin (NGEN) on open-field behavior test: in latency to start locomotion, number of rearings, number of crossings, and defecation in iron-treated rats (Fe) or their combination (FeNGEN)

Parameters	C	Fe	FeNGEN	NGEN
Latency (s)	6.60 ± 0.70	3.50 ± 0.97***	5.90 ± 0.9 ^{YYY}	6.90 ± 0.90
Number of crossings	60.90 ± 2.65	73.80 ± 3.50***	64.30 ± 2.70 ^{YYY}	59.50 ± 3.0
Number of rearings	23.80 ± 0.63	33.40 ± 2.36***	26.80 ± 1.4 ^{YYY}	22.90 ± 1.6
Number of fecal pellets	2.1 ± 0.56	1.6 ± 0.7	1.80 ± 0.60	2.00 ± 0.80

Values are expressed as mean ± SD of 10 rats per group

Fe group versus control group: *** $p < 0.001$

FeNGEN group versus Fe group: ^{YYY} $p < 0.001$

Table 3 Effects of different treatments on oxidative stress parameters: Reactive oxygen species (ROS), lipid peroxidation (MDA), protein carbonyl content (PCO), reduced glutathione (GSH) and nitrite (NO_2^-) formation in the hippocampus of control (C) and rats treated with iron (Fe), naringenin (NGEN) or their combination (FeNGEN)

Parameters and treatments	Control	Fe	FeNGEN	NGEN
ROS (nmoles DCF/mg protein)	555.0 ± 20	810.50 ± 24***	670.0 ± 35 ^{###}	525 ± 30
MDA (nmoles/mg protein)	15.20 ± 1.70	25.50 ± 2.20***	19.60 ± 1.50 ^{###}	14.50 ± 1.80
PCO (nmoles/mg protein)	1.60 ± 0.15	5.20 ± 0.30***	3.40 ± 0.20 ^{###}	1.45 ± 0.25
NO_2^- (nmoles/mg protein)	1.50 ± 0.12	3.85 ± 0.20***	1.90 ± 0.20 ^{###}	1.40 ± 0.15
GSH (nmoles/mg protein)	0.72 ± 0.10	0.20 ± 0.08***	0.55 ± 0.06 ^{###}	0.75 ± 0.07

Values are expressed as mean ± SD of 8 rats in each group

Fe group versus control group: *** $p < 0.001$

FeNGEN group versus Fe group: ^{###} $p < 0.001$

Table 4 Effects of different treatments on antioxidant enzyme activities (CAT, GPx and SOD) in the hippocampus of controls (C) and rats treated with iron (Fe), naringenin (NGEN) or their combination (FeNGEN)

Parameters and treatments	Control	Fe	FeNGEN	NGEN
SOD (U/mg protein)	4.65 ± 0.4	1.15 ± 0.6***	3.45 ± 0.52 ^{###}	4.8 ± 0.48
CAT ($\mu\text{mol H}_2\text{O}_2$ consumed/min/mg protein)	24.50 ± 1.20	12.50 ± 0.90***	19.25 ± 2.30 ^{##}	23.10 ± 2.0
GPx (nmoles of GSH oxidized/min/mg protein)	42.82 ± 2.50	23.70 ± 3.70***	33.70 ± 2.60 ^{###}	40.40 ± 3.10

Values are expressed as mean ± SD of 8 rats in each group

Fe group versus control group: *** $p < 0.001$

FeNGEN group versus Fe group: ^{###} $p < 0.001$; ^{##} $p < 0.01$

results showed that $\Delta\Psi_m$ were significantly ($p < 0.001$) decreased in the Fe-treated rats, compared with that of the control group. In contrast, NGEN co-treatment was found to partially abolish this reduction in the FeNGEN group indicating its protective effect (Fig. 3).

Naringenin Prevented the Ectonucleotidase Enzymatic Activity and Gene Expression Alterations Induced by Fe

As shown in Fig. 4a, the effects of Fe were investigated on ectonucleotidase enzymes activity, rates of ATP, ADP, and AMP hydrolysis were determined in synaptosome samples

obtained from hippocampus of rats. Fe administration decreased significantly ectonucleotidase activity in the hippocampus as compared to control rats ($p < 0.05$). However, NGEN (50 mg/kg) co-treatment significantly improved ectonucleotidase activity as compared to that of Fe-treated group ($p < 0.05$).

We have also evaluated the relative expression of ectonucleotidases by semi-quantitative RT-PCR. Co-administration of NGEN attenuated significantly ($p < 0.05$) the augmentation of NTPDase1, NTPDase2 and NTPDase3 expression due to iron-treatment. Whereas, no significant differences in mRNA expression for 5'-nucleotidase (CD73) was observed in all experimental groups (Fig. 4b).

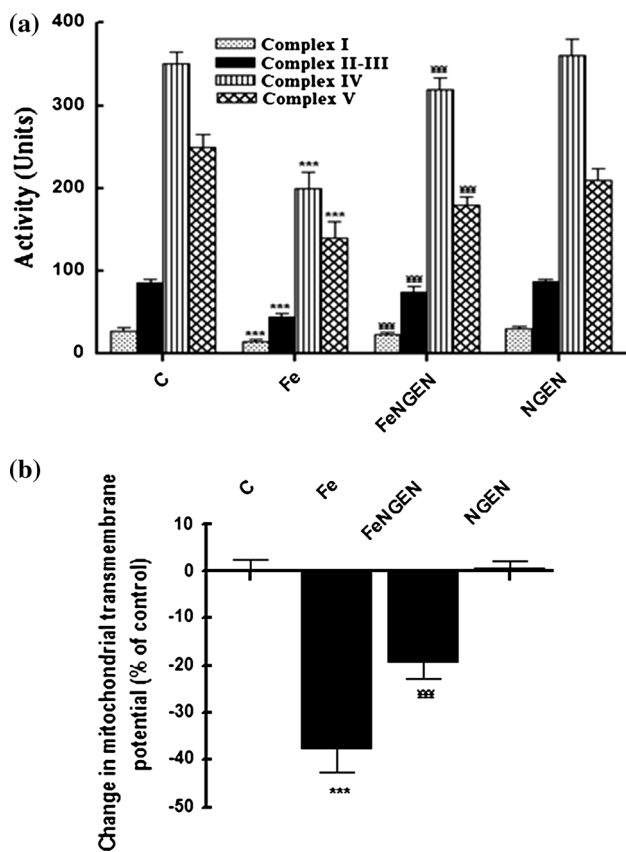


Fig. 3 Effect of iron (Fe), naringenin (NGEN) and their simultaneous treatment (FeNGEN) on the activity of mitochondrial NADH dehydrogenase (complex I), succinate-cytochrome c oxidoreductase (complex II–III), cytochrome c reductase (complex IV), and ATP synthase (complex V) (a) and mitochondrial membrane potential (b) in rat hippocampus. Values are expressed as mean \pm SD of 6 (a) to 8 (b) rats per group. Fe group versus control group: *** $p < 0.001$. FeNGEN group versus Fe group: $^{***}p < 0.001$

Naringenin Prevented the Alterations Induced by Fe in AChE Activity in the Hippocampus

As shown in Fig. 5a, the effect of iron was investigated on AChE activity in the hippocampus of rats. The exposure to Fe decreased significantly ($p < 0.001$) the AChE activity in the hippocampus when compared with control, while NGEN (50 mg/kg) co-treatment significantly improved AChE activity as compared to that of the Fe-treated group ($p < 0.01$). In addition, we also evaluated the relative expression of AChE by semi-quantitative RT-PCR. The co-administration of NGEN upregulated AChE expression in the FeNGEN group when compared to Fe-treated group. NGEN alone did not increase AChE expression under control levels (Fig. 5b).

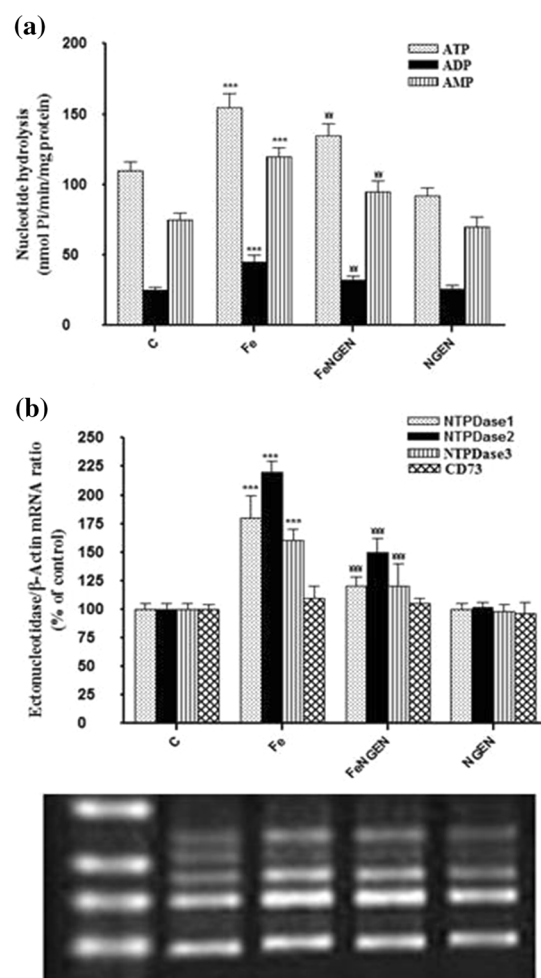


Fig. 4 Effect of iron (Fe), naringenin (NGEN) and their simultaneous treatment (FeNGEN) on ectonucleotidase activity (nmol Pi/mg protein/min) using ATP, ADP and AMP as substrate in synaptosomes from hippocampus of rats (a) and hippocampal gene expression of ecto-nucleotidases: NTPDase1, NTPDase2, NTPDase3 and 5'-nucleotidase (CD73) with the respective representative images (below) (b). Values are expressed as mean \pm SD of 4 (b) to 6 (a) rats per group. Fe group versus control group: *** $p < 0.001$. FeNGEN group versus Fe group: $^{**}p < 0.01$, $^{*}p < 0.05$

Discussion

In the present study, we evaluated the effects of naringenin (NGEN) on behavioral and hippocampal biochemical, cholinergic and purinergic neurotransmission changes induced by iron administration, a well-documented animal model of cognitive impairment [47, 48]. The involvement of iron in Fenton chemistry is a large contributor of oxidative stress. There is speculation that iron plays a large role in the pathophysiology of some common neurodegenerative disorders like Alzheimer's disease (AD), Parkinson's disease, Huntington's disease, and

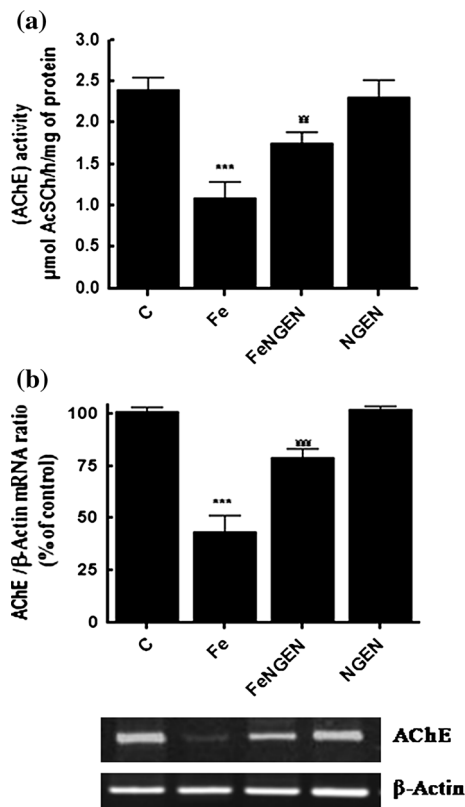


Fig. 5 Effects of different treatments on acetylcholinesterase activity (a) and gene expression (b) in the hippocampus of controls (C) and rats treated with iron (Fe), naringenin (NGEN) or their combination (FeNGEN). Values are expressed as mean \pm SD of 4 (b) to 8 (a) rats per group. Fe group versus control group: *** $p < 0.001$. FeNGEN group versus Fe group: ^{yyy} $p < 0.001$, ^{yy} $p < 0.01$

amyotrophic lateral sclerosis [49]. Iron overload in rats enhances reactive oxygen species (ROS) [50], in brain regions such as the cerebral cortex and the hippocampus, areas known to be affected in AD [51]. Importantly, our previous studies exhibited that iron induced cholinergic deficits in the cerebral cortex in rats are protected by simultaneous treatment with naringenin [20]. Since the hippocampus is a crucial brain area associated with learning and memory functions. A lot of studies have also revealed that ROS-mediated oxidative stress as an important contributor to behavior impairment [52]. In the current study, the effects of NGEN on iron-induced anxiolytic-like behaviors were investigated using the elevated plus-maze (EPM), the open field test (OFT) and the marble burying test (MBT). Our results showed that a high-dose iron administration for four-weeks cause an increase in the anxiogenic-like behavior accompanied by a marked elevation of the ROS level in the hippocampus, and that co-administration of NGEN significantly decreased hippocampal ROS levels and greatly prevented the onset of anxious behaviors that were seen in the iron treated

animals, such as a decreased number of closed arms entries in the EPM and numbers of marbles buried in the MBT. In fact, some studies have shown that iron exposure increases anxiety. Maaroufi et al. [53] showed that male Wistar rats that received a single intraperitoneal injection 3.0 mg/kg daily along five consecutive days of ferrous sulfate underwent significant iron accumulations in the hippocampus and caused a higher anxiety in rats. Moreover, Perez et al. [54] proved that male Wistar rats that received iron (10.0 mg/kg) from the beginning of pregnancy until weaning had an increased anxiety-like behavior. Although the mechanism by which these metals are able to alter behavior in the elevated plus-maze is still not fully established, it is suggested that there is a link with hippocampal serotonergic and dopaminergic neurons as well as the involvement of AChE and Na^+ , K^+ -ATPase activities in the anxiety alterations [55]. On the other hand, this study reveals that the treatment with NGEN is able to prevent the anxiogenic effect induced by iron exposure. The findings related to anxiety are in agreement with previous reports that have shown that NGEN produces a variety of anxiolytic-like behavioral effects [19–56]. Among the mechanisms by which the NGEN produces these effects is the modulation of neurotransmitter systems associated with anxiety and depression-like GABAergic and serotonergic systems [57]. Therefore, according to our results, the present study is in accordance with the anxiogenic effect caused by iron exposure as well as the anxiolytic-like effect reported for NGEN. To our knowledge, this is the first work that reports the beneficial actions of NGEN against iron-mediated anxiolytic-like behavior and memory impairment in rats. Another important aspect to be discussed here is the effect of iron on the cholinergic, nitroergic and purinergic signaling involved in the neurobiology of stress-related anxiety [58, 59]. The purinergic system employs extracellular nucleotides as signaling molecules. Adenine nucleotides (ATP, ADP and AMP) comprise an important class of signaling molecules co-released with others, such as glutamate, GABA, and acetylcholine in different subpopulations of neurons in CNS. Extracellular nucleotides (ATP and ADP) may be hydrolyzed by members of the ecto-nucleoside triphosphate diphosphohydrolase family (E-NTPDases), and AMP may be hydrolyzed by the ecto-5'-nucleotidase to produce adenosine [60]. Several studies have demonstrated that ectonucleotidases and cholinesterases have an important role in neurotransmission and their activities are altered in several pathologies as diabetes mellitus [61], multiple sclerosis [62], cancer [63], as well as the animal model for aluminum and cadmium intoxication in rats [64]. In line with this, the results of the present study demonstrated a significant decrease in AChE activity in the hippocampus from Fe-exposed rats. According to our results, Pohanka [65]

showed a decrease in the human AChE activity *in vitro*. Several mechanisms have been proposed to explain the effects of iron on AChE activity, de Lima et al. [66] suggested that iron may alter the AChE activity most likely due to alterations in the protein structure and the function of brain cells, leading to an indirect effect on AChE activity at synaptic clefts. In addition, Ibrahim et al. [67] suggested that the inactivation of the AChE enzyme could be a result of the effects of hydroxyl radical (OH[•]) and nitric oxide (NO) and confirmed that the change of the catalysis rate in the presence of NO was likely due to structural alterations of the AChE active site, rather than the binding site. Moreover, increased NO production is proposed to be one of the main mechanisms by which the purinergic transmission exerts its behavioral effects [58]. This view is in agreement with the results of the current study, in which an impairment of the activity of cell surface-located extracellular nucleosides hydrolyzing enzymes was found in synaptosomes from the hippocampus of iron-treated rats. Interestingly, we also observed that NGEN co-treatment prevented the increase in NO, NTPDase and 5'-nucleotidase activities in hippocampal synaptosomes of iron treated rats. This effect is interesting because the decrease in ATP, ADP, and AMP hydrolysis can maintain the levels of extracellular ATP molecules, an important excitatory neurotransmitter in purinergic nerve synapses.

The potential pro-oxidant property of iron is an important mechanism that has been proposed to explain the undesirable effects of iron and considering the results obtained in this study related to ROS, NO, AChE and NTPDase activities, we further investigated the effects of iron exposure on parameters of oxidative stress as well as on the mitochondria enzyme complexes, antioxidant enzyme activities and endogenous non-enzymatic antioxidants in order to understand the possible mechanisms causing the iron induced neurotoxicity and the hippocampus alterations. Moreover, these data can further exhibit the possible mechanism by which NGEN exerts its neuroprotective effects. The underlying mechanism in iron neurotoxicity is primarily its effect on the mitochondria which play an important role in the energy metabolism by oxidative phosphorylation involving enzyme complexes [68, 69]. The role of NADH dehydrogenase (complex I), succinic dehydrogenase (complex II), ubiquinone-cytochrome c oxidoreductase (complex III) and cytochrome oxidase (complex IV) is critical to orchestrate the flow of high energy electrons to molecular oxygen along the electron transport chain. Flow of electrons from the respiratory chain and pumping of protons in these complexes generate proton gradient/membrane potential which is effectively utilized by complex V (ATP-synthase) to generate ATP by stimulating ADP phosphorylation and

channels back the protons to the mitochondrial matrix [70]. The alteration in the integrity of these enzyme complexes, responsible for oxidative metabolism may lead to mitochondrial dysfunctions and cytopathy [71]. Decrease in the activity of complexes I, II–III, and IV in the hippocampus following iron exposure as observed in the present study is consistent with earlier studies [69, 72, 73]. During the process of oxidative phosphorylation, mitochondria are the potential source of oxidative stress due to the generation of reactive oxygen species [74]. Enhanced mitochondrial ROS generation in the biological system may target the lipid bilayers and cause biochemical alterations. Further, a decrease in the membrane potential could affect the opening of mitochondrial permeability transition pores associated with enhanced apoptosis due to increased ROS generation [75]. As a delicate balance between ROS generation and the anti-oxidant system is required for the normal physiological functions of mitochondria, the intrusion of iron may cause an inadequate interaction with other anti-oxidant proteins affecting the REDOX state and cause mitochondrial dysfunctions [76]. While the intensity in the mitochondrial membrane potential in the hippocampus following iron exposure decreased, the change was significant and could possibly contribute in oxidative stress. Increased generation of ROS in the hippocampus following iron exposure as observed in the present study could be associated with decreased mitochondrial membrane potential and is consistent with earlier reports [68, 76]. ROS production may decrease the effectiveness of the antioxidant system and affects numerous cellular components [13]. In fact, the elevation in ROS production found in our study was accompanied by an increase in MDA, protein carbonyl. In parallel with this, we also found alterations in the antioxidant system including a decrease in GSH content in the hippocampus, as well as the reduction of the antioxidative enzyme activities (SOD, CAT and GPx). We showed that the co-administration of NGEN (50 mg/kg) to iron exposed rats led to a reduction of reactive oxygen species and the neuroprotective properties of NGEN exhibited various pathways of action: (1) an inhibition of lipid peroxidation and membrane stabilization, (2) a decreased glutathione depletion, (3) an ability to scavenge and quench reactive oxygen species based on the strong antioxidant capacity of the flavonoids and (4) a direct modulation of antioxidant enzyme synthesis [18–20].

In conclusion, to our knowledge, this is the first work that reported the possible neuroprotective mechanisms of naringenin against the neurotoxicity and consequent emotional disorders in iron exposed rats. Our findings suggest that naringenin can modulate cholinergic neurotransmission resting membrane potential of neurons by modulating the enzymatic and non-enzymatic antioxidant defense system, preserving the cellular integrity. These effects,

consequently improved the anxiogenic-like behavior observed in iron exposure, possibly by amending AChE and E-NTPDases activities. Thus, this study may contribute to a better understanding of the neuroprotective role of naringenin, emphasizing the influence of this polyphenol and other antioxidants in the diet for human health, possibly preventing brain injury associated with iron overload.

Acknowledgments The present work was supported by the grants of DGRST (Appui a la Recherche Universitaire de base, ARUB, UR11ES70) Tunisia. The authors are also grateful to Kamel MAA-LOUL, English professor at the Faculty of Sciences of Sfax, for having proofread the manuscript.

Conflict of interest None of the authors has any conflict of interest.

Ethical standard All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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