

RESEARCH ARTICLE

Genistein Improves 3-NPA-Induced Memory Impairment in Ovariectomized Rats: Impact of Its Antioxidant, Anti-Inflammatory and Acetylcholinesterase Modulatory Properties

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Abstract

Huntington's disease (HD) is a progressive neurodegenerative disorder. The pre-motor symptomatic stages of the disease are commonly characterized by cognitive problems including memory loss. 3-Nitropropionic acid (3-NPA) is a mitochondrial toxin that produces selective lesions in the brain similar to that of HD and was proven to cause memory impairment in rodents. Phytoestrogens have well-established neuroprotective and memory enhancing effects with fewer side effects in comparison to estrogens. This study investigated the potential neuroprotective and memory enhancing effect of genistein (5, 10 and 20 mg/kg), a phytoestrogen, in ovariectomized rats challenged with 3-NPA (20 mg/kg). These potential effects were compared to those of 17 β -estradiol (2.5 mg/kg). Systemic administration of 3-NPA for 4 consecutive days impaired locomotor activity, decreased retention latencies in the passive avoidance task, decreased striatal, cortical and hippocampal ATP levels, increased oxidative stress, acetylcholinesterase (AChE) activity, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expressions. Pretreatment with genistein and 17 β -estradiol attenuated locomotor hypoactivity, increased retention latencies in the passive avoidance task, increased ATP levels, improved the oxidative stress profile, attenuated the increase in AChE activity and decreased the expression of COX-2 and iNOS. Overall, the higher genistein dose (20 mg/kg) was the most effective. In conclusion, this study suggests neuroprotective and memory enhancing effects for genistein in a rat model of HD. These effects might be attributed to its antioxidant, anti-inflammatory and cholinesterase inhibitory activities.

Introduction

Huntington's disease (HD) is a progressive neurodegenerative disorder, characterized by cognitive, motor and emotional abnormalities [1]. It predominantly affects the striatum, cerebral

cortex and other areas of the brain involved in memory storage [2]. The pre-motor symptomatic stages of the disease are commonly characterized by cognitive problems [3,4]. This includes executive dysfunction, visuospatial deficits, perceptual deficits, memory loss and difficulty in learning new skills [5,6]. Structural and functional brain changes, including decreased acetylcholine levels, were correlated to cognitive deficits in HD patients [7–9].

3-Nitropropionic acid (3-NPA) is a fungal neurotoxin, which irreversibly inhibits succinate dehydrogenase enzyme in the mitochondrial tricarboxylic acid cycle. Subsequently, it blocks electron transport during oxidative phosphorylation, causing ATP levels in the brain to fall [10–14]. It produces selective lesions in different brain areas, including the striatum, cortex and hippocampus, in a very similar manner to that of HD. It is therefore considered as a good experimental model, which closely resembles some of human pathological and behavioral aspects of HD [15–17]. Systemic administration of 3-NPA has been proposed to produce memory impairment in rodents, as one of the symptoms of HD, which can be further related to an increase in oxidative stress [18].

Classically, estrogen is considered a reproductive hormone but then it was found to have a broader spectrum of action and can be defined as the ovarian steroid hormone that elicits complex, tissue-specific responses [19]. A growing body of evidence was shown over the several last decades revealing the regulatory and neuroprotective role for estrogen on the central nervous system. Moreover, experimental studies and clinical observations have highlighted that estrogen has positive influence on learning and memory in rats [20] and cognitive functions within the mammalian brain [21]. Unfortunately, these potential benefits of estrogen therapy for the brain are counterbalanced by the negative life-threatening risks, due to its proliferative and oncogenic effects, on non-neuronal cells responsive to estrogen such as the breast and the endometrium [22,23]. Therefore, researchers' attention were drawn to a class of drugs known as selective estrogen receptor modulators (SERMs) including the naturally occurring phytoestrogens.

Genistein is one of the estrogenic compounds naturally occurring in plants and shares structural features with the potent estrogenic compound, 17 β -estradiol, which makes it able to bind to estrogen receptors (ER). Genistein binds differentially to human ER- α and - β with preference to ER- β , to which it was found to have seven to eight times more binding affinity [24,25]. Genistein has the therapeutic potential to reduce cognitive decline and neurodegeneration associated with menopause through diminishing oxidative stress [26]. Moreover, high dietary consumption of genistein has been linked to a number of potential health benefits including memory improvement in male and female volunteers [27]. Many factors make genistein a potential neuroprotective and memory enhancing drug. These include its protective effect against oxidative stress-induced apoptosis [28], and its preferential agonistic effect on ER- β , which is abundant in brain regions associated with learning and memory (neocortex, hippocampus, and nuclei of the basal forebrain) [29]. ER- β was found to be needed in regulating the stability of synaptic connections and memory [30,31]. On the other hand, genistein is described as a potent agent in both prophylaxis and treatment of hormone-dependent cancers, such as those of breast and endometrium. Therefore eliminating the risk associated with the classical estrogen replacement therapy [32,33].

This study was designed to investigate the potential cognitive-enhancing effect of genistein in 3-NPA-induced HD-like phenotype in ovariectomized rats as well as the underlying mechanism.

Materials and Methods

1. Animals

Female albino rats (initial weight 180–200 g) were purchased from the National Institute of Research, Cairo, Egypt. They were housed in plastic cages at constant temperature ($21 \pm 2^\circ\text{C}$),

with alternating 12 h light/dark cycle. Animal chow and water were provided *ad libitum*. All animal treatments adhered strictly to institutional and international ethical guidelines of the care and use of laboratory animals. The experimental protocol was approved by Ain Shams University Faculty of Pharmacy Ethical Committee for the use of animal subjects (Permit no. PhD,13).

2. Chemicals

3-NPA, thiobarbituric acid (TBA), acetylthiocholine, 5, 5'-Dithiobis [2-nitrobenzoic-acid] (DTNB) and 1,10,3,30-tetramethoxypropane were purchased from Sigma chemicals (St. Louis, MO, USA). Genistein was purchased from (Winherb Med Sci Co Ltd, China). 17 β -estradiol was purchased from Steraloid (Newport, RI, USA). Ketamine hydrochloride injection was purchased as vials (Rotexmedica, Trittau, Germany). The other reagents were of the highest pure grade commercially available.

3. Experimental design

Female albino rats were anaesthetized using i.p. ketamine hemisulfate (100 mg/kg) and received injections of the analgesic carprofen (5 mg/kg, s.c.) then bilaterally ovariectomized under aseptic condition [34]. Animals were left 3 weeks to recover from surgery; this period is usually enough for the cessation of the estrous cycle [35]. During the recovery period, cleaning of the cages and wounds disinfection were performed on daily basis. Then animals were randomly allocated into 8 groups (n = 8) including the sham operated group (subjected to bilateral incisions without removing the ovaries). Groups (1) and (2) are sham operated and control groups (received the respective vehicles); (3) 3-NPA-treated group; (4) reference group (17 β -estradiol- and 3-NPA-treated group); (5 to 7) genistein- and 3-NPA-treated groups; (8) genistein alone-treated group. 3-NPA was dissolved in saline (pH was adjusted to 7.4 with 0.1 N NaOH) and administered intraperitoneally (i.p) at a dose of 20 mg/kg body weight for 4 consecutive days. 17 β -estradiol (2.5 mg/kg body weight) and genistein (5, 10 and 20 mg/kg body weight) were dissolved in DMSO and administered subcutaneously and i.p, respectively, for 8 days, beginning 4 days before and continued for 4 days one hour before 3-NPA injections. Doses of 17 β -estradiol and genistein were chosen according to Tunéz et al. [35] and Bagheri et al. [36] respectively. On day 8, 3.5 hours after the last 3-NPA injection, animals were sacrificed by decapitation and skulls were split on iced phosphate buffer saline. Whole brains were fixed in 10% formol saline for histopathological examination and immunohistochemical quantitation. In another series of experiments, rats were subjected to the same treatment protocol as previously described and sacrificed then striata, cortices and hippocampi were dissected out. Ten percent (w/v) homogenates in 0.1 M phosphate buffer (pH 7.4) were prepared.

According to the results of the behavioral and histological examinations, the effective doses of genistein were selected for completion of the study.

4. Behavioral experiments

4.1. Locomotor activity detection. Activity monitor (Opto-Varimex-Mini Model B, Columbus Instruments, OH, USA) was used to measure the locomotor activity of animals. Such activity was measured based on the traditional infrared photocell principle where interruption of 15 infrared beams (wavelength = 875 nm, scan rate = 160 Hz, diameter = 0.32 cm, spacing = 2.65 cm) reflected total activity of the animal. The total locomotor activities of animals are expressed as counts/5 min.

4.2. Step-through passive avoidance paradigm. A step-through passive avoidance apparatus was used (Ugo Basile, Italy). It consisted of a Plexiglas box divided into two compartments. One compartment is white and illuminated by a light fixture, featuring a 24-V, 10-W bulb,

fastened to the compartment lid. The second compartment is dark and made of black Perspex panels. The two compartments are separated by an automatically operated sliding door. The apparatus included a steel-rod grid floor, which consisted of 40 parallel bars (0.3cm in diameter, set 1.2cm apart). The bars of the dark compartment floor are wired to a constant current high-precision eight-pole scrambling circuit located in the controller. Rats that showed immobility from the previous experiment were excluded from this test. The rest were subjected to a training session before treatments of day 5 (before genistein and 17 β -estradiol administration) and 2 test sessions at days 6 (before genistein and 17 β -estradiol administration) and 8 (3.5 hours after the last 3-NPA dose).

Training session: each rat was trained by gently placing it in the light compartment then when it stepped through the dark compartment putting the 4 paws on the grid floor, the door automatically closed and an electric shock of 0.5 mA was delivered for 2 s. Rats that failed to step through within a cut-off time of 90s were excluded.

Test sessions: each rat was introduced to the light compartment and the latency to step-through to the dark compartment was recorded as a passive avoidance behavior indicating memory acquisition, with an upper cut-off time of 300s. No electric shock was delivered during test sessions.

5. Histopathological examination

Brain samples were fixed in 10% formol saline for twenty four hours, washed with tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56°C in hot air oven for twenty four hours. Paraffin bees wax tissue blocks were prepared for coronal sectioning at 4 microns thickness by a rotary microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin (H) & eosin (E) stain for routine examination by the light electric microscope [37].

6. ATP levels

ATP level detection was performed according the kit instructions (ApoSensor ATP assay kit, Biovision, Milpitas Boulevard, USA). This method depends on the bioluminescent detection of ATP using luciferase to catalyze the formation of light from ATP and luciferin, and the light can be detected using illuminometer. ATP levels were expressed as $\mu\text{g/g}$ tissue.

7. Biomarkers of oxidative stress

7.1. Lipid peroxidation products. The level of malondialdehyde (MDA), the major thiobarbituric acid-reactive species (TBARS) as a measure of lipid peroxidation, in cortices and hippocampi was assessed according to the method of Mihara and Uchiyama [38] where for each 0.5 ml homogenate, 3 ml of the 1% O-Phosphoric acid solution and 1 ml of the 0.6% TBA solution were added, mixed then placed in a boiling water bath for 45 min. After cooling, the pink color formed is extracted by n-butanol and detected at 2 wave lengths (535 and 520) and absorbance difference was calculated. MDA levels were expressed as nanomoles per gram tissue (nmol/g tissue).

7.2. Reduced glutathione (GSH) assay. According to the kit instructions (Biodiagnostics, Egypt), 0.5 ml of the tissue homogenate (10% (w/v) in 0.1 M phosphate buffer, pH 7.4) was added to a tube with 0.5 ml of 10% trichloroacetic acid. The tubes were shaken gently and intermittently for 15 min, followed by centrifugation at 3000 xg for 10 min. An aliquot of the resulting supernatant (0.2 ml) was added to a tube containing 1.7 ml phosphate buffer and 0.1 ml Ellman's reagent then the absorbance was read at 412 nm within 5 min [40]. GSH levels were expressed as $\mu\text{mol/g}$ tissue.

7.3. Catalase activity. Catalase activity was assayed according to the instructions of the kit (Biodiagnostics, Egypt). The homogenates were centrifuged at 4000 xg at 4°C for 15 min then; 0.05 ml of supernatant was added to a known quantity of H_2O_2 . The reaction was stopped after exactly 1 min with a catalase inhibitor. In the presence of peroxidase, remaining H_2O_2 reacts with 3, 5-dichloro-2 hydroxybenzene sulfonic acid and 4-aminophenazone to form a chromophore of pink color detected at 510 nm. Its intensity is inversely proportional to the amount of catalase in the sample [39]. Catalase activities were expressed as U/g tissue.

8. Acetylcholinesterase (AChE) activity

The inhibitory activity of genistein on cholinesterases, mainly AChE, was measured according to the method described by Ellman et al. [41], with slight modifications. The assay was performed by adding 2.9 mL of 0.1 mM sodium phosphate buffer (pH 8.0) to 50 μ l of the tissue homogenate (10% (w/v) in 0.1 M phosphate buffer, pH 7.4) and incubating the mixture at 37°C for 5 min. After incubation, 40 μ l of acetylthiocholine iodide (154.38 mM) and 10 μ l of DTNB (10 mM) were added to the reaction mixture and the formation of thionitrobenzoic acid was recorded at 412 nm for 150 s at 30 s intervals using UV spectrophotometer. The AChE activity was quantified by measuring the concentration of thionitrobenzoic acid (extinction coefficient 1.36×10^4 /molar/cm). AChE activities were expressed as nM/min/mg tissue

9. Immunohistochemical detection of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2)

According to Johansson et al. [42] and Yuan et al. [43], after deparaffinization of brain paraffin sections (4-mm-thick), endogenous peroxide was quenched with 3% hydrogen peroxide in deionized water for 10 min. Nonspecific binding sites were blocked by incubating the sections in 10% goat serum for 10 min. The sections were then incubated with polyclonal rabbit anti-iNOS and anti-COX-2 (Dilution 1:25) overnight at 4°C, followed by incubation with biotinylated goat-anti-rabbit IgG at room temperature for 10 min. After 3 times of 3 min PBS rinses, sections were incubated for 10 min with streptavidin horseradish peroxidase. The antibody binding sites were visualized by incubation with diaminobenzidine- H_2O_2 solution. Sections incubated with PBS instead of the primary antibody were used as negative controls. Antibodies for iNOS and COX-2 were purchased from Thermo Fisher Scientific, UK, with catalogue numbers RB-9242-R7 and RB-9072-P respectively. Brown granules in cytoplasm or nuclei were recognized to be positively stained. Computerized image analysis to calculate the optical density (OD) and area percent (A %) was performed using imageJ software (version 1.48).

10. Statistical analysis

Locomotor activity data were expressed as mean \pm standard error of the mean (SEM) and analyzed by two-way ANOVA followed by Bonferroni post hoc test. Passive avoidance non-parametric data were expressed as medians and quartiles and analyzed by Kruskal–Wallis test followed by Dunn' test as a post hoc test. Parametric data were expressed as mean \pm SEM and analyzed by one-way ANOVA followed by Tukey test as a post hoc test. All statistical analyses were performed using the GraphPad Prism software (version 5.01, Inc., 2007, San Diego California USA). Probability values of less than 0.05 were considered statistically significant.

Results

1. Effects of genistein on the locomotor activity of 3-NPA-treated ovariectomized rats

Two-way ANOVA revealed significant effects of treatments on locomotor activity [$F(7,56) = 3.65$, $P < 0.001$] and days [$F(3,56) = 124.9$, $P < 0.0001$], however the interaction was shown to be insignificant [$F(21.56) = 1.28$, $P = 0.193$] (Fig. 1). Further analysis by Bonferroni test showed that all treatments had no effect on locomotor activity at days 1, 5 and 6. Systemic administration of 3-NPA caused significant reduction in locomotor activity compared to the sham and control groups at day 8 of the experiment. However, pretreatment with 17β -estradiol (2.5 mg/kg) and genistein (10 and 20 mg/kg) significantly restored locomotor activity. The effect of the lower dose of genistein (5 mg/kg) was not statistically significant from 3-NPA-treated group. There was no significant difference between 17β -estradiol and genistein (10 and 20 mg/kg) also genistein alone did not cause any significant changes in locomotor activity if compared to the sham and control groups.

2. Effects of genistein on step-through passive avoidance of 3-NPA-treated ovariectomized rats

Kruskal–Wallis test showed no significant difference among groups for the first retention latency at day 6 of the experiment [$H(7, 56) = 4.1$, $P = 0.76$]. However, at day 8, the test revealed significant effect on the second retention latency [$H(7, 56) = 19.17$, $P < 0.05$] (Fig. 2). Further statistical analysis with Dunn's test showed that 3-NPA (20 mg/kg) resulted in a shorter latency to step through compared to the sham and control groups, while pretreatment with 17β -estradiol (2.5 mg/kg) and genistein (10 and 20 mg/kg) significantly attenuated 3-NPA-induced amnesia. The effect of the lower genistein dose (5 mg/kg) was not statistically significant from the 3-NPA-treated group. There was no significant difference between 17β -estradiol and genistein (10 and 20 mg/kg) also genistein alone did not cause any significant changes in step-through latency if compared to the sham and control groups.

3. Histopathological examination of the cortical and hippocampal tissues

Examination of the H & E stained brain sections showed that systemic administration of 3-NPA (20 mg/kg) caused focal hemorrhage in the cortex and neurodegeneration, encephalomalacia, plus focal hemorrhage in the hippocampus. Pretreatment with 17β -estradiol (2.5 mg/kg) and genistein (10 and 20 mg/kg) relatively restored brain histological features, where genistein 10 mg/kg pretreated group only showed focal gliosis in the cortex with no alteration in the hippocampus and genistein 20 mg/kg pretreated group showed only congested blood vessel in the cortex. While the lowest genistein dose (5 mg/kg) showed focal gliosis in the cortex and failed to protect the hippocampal tissue. Sham, control and genistein alone-groups showed no histological alterations in the cortical and hippocampal tissues (Figs. 3 & 4).

4. Effects of genistein on striatal, cortical and hippocampal ATP level of 3-NPA-treated ovariectomized rats

One-way ANOVA showed significant effects of treatments on striatal, cortical and hippocampal ATP levels [$F(6,35) = 12.6$; $P < 0.0001$, $F(6,35) = 8.89$, $P < 0.0001$; $F(6,35) = 6.032$, $P < 0.0001$, respectively] (Table 1). Rats treated with 3-NPA showed significant reduction in striatal, cortical and hippocampal ATP levels as compared to sham and control groups. Pretreatment with 17β -estradiol (2.5 mg/kg) and genistein (20 mg/kg) significantly attenuated this reduction. However, pretreatment with genistein (10 mg/kg) only showed significant increase of hippocampal ATP levels. There were no significant difference between rats pretreated with 17β -estradiol and genistein.

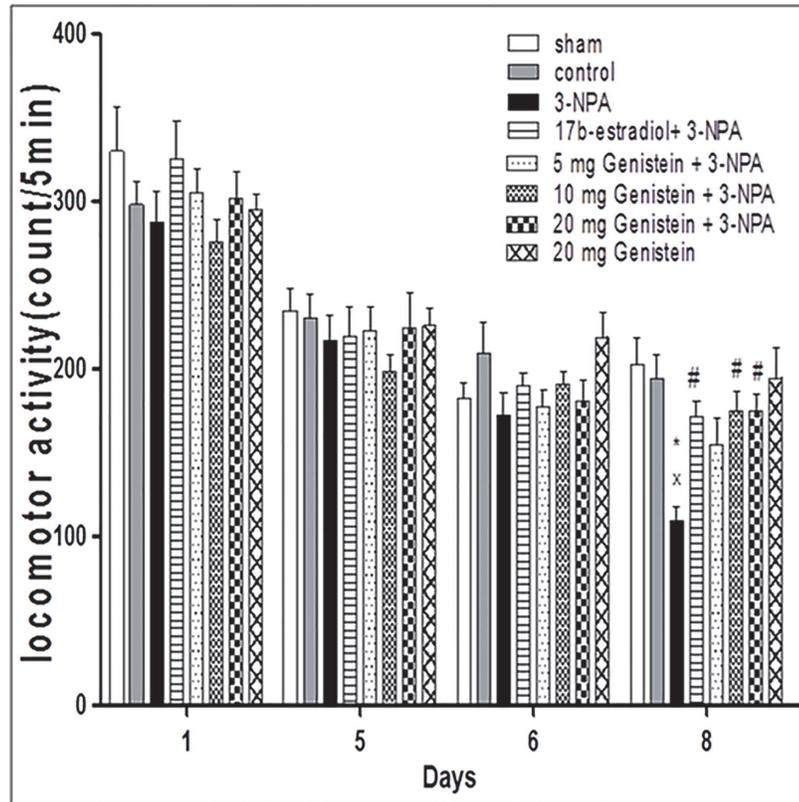


Fig 1. Effects of 3-NPA and/or genistein on locomotor activity in ovariectomized rats. 3-NPA was administered i.p. (20 mg/kg) for 4 consecutive days. 17β-estradiol (2.5 mg/kg body weight, s.c) and genistein (5, 10 and 20 mg/kg body weight, i.p) were administered for 8 days, beginning 4 days before and continued for 4 days one hour before 3-NPA injections. Data are presented as means ±S.E.M. (n = 8). * statistically significant compared to sham group at P < 0.05, * statistically significant compared to control group at P < 0.05, # statistically significant compared to 3-NPA-treated group at P < 0.05 (two-way ANOVA followed by Bonferroni test).

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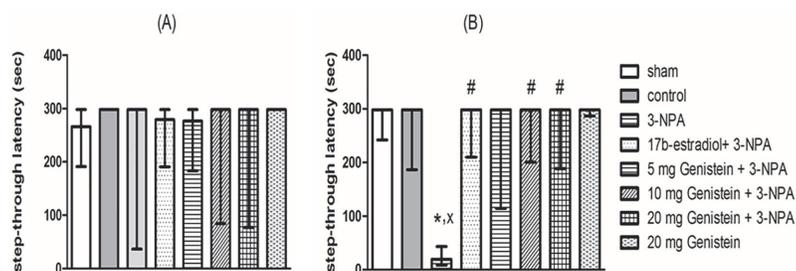


Fig 2. Effects of 3-NPA and/or genistein on step through passive avoidance in ovariectomized rats. 3-NPA was administered i.p. (20 mg/kg) for 4 consecutive days. 17β-estradiol (2.5 mg/kg body weight, s.c) and genistein (5, 10 and 20 mg/kg body weight, i.p) were administered for 8 days, beginning 4 days before and continued for 4 days one hour before 3-NPA injections. Training was performed on day 5. First and second retention latencies were assessed at days 6 (A) and 8 (B). Data are presented as medians and quartiles (n = 8). * statistically significant compared to sham group at P < 0.05, * statistically significant compared to control group at P < 0.05, # statistically significant compared to 3-NPA-treated group at P < 0.05 (Kruskal-Wallis nonparametric test followed by Dunn's test).

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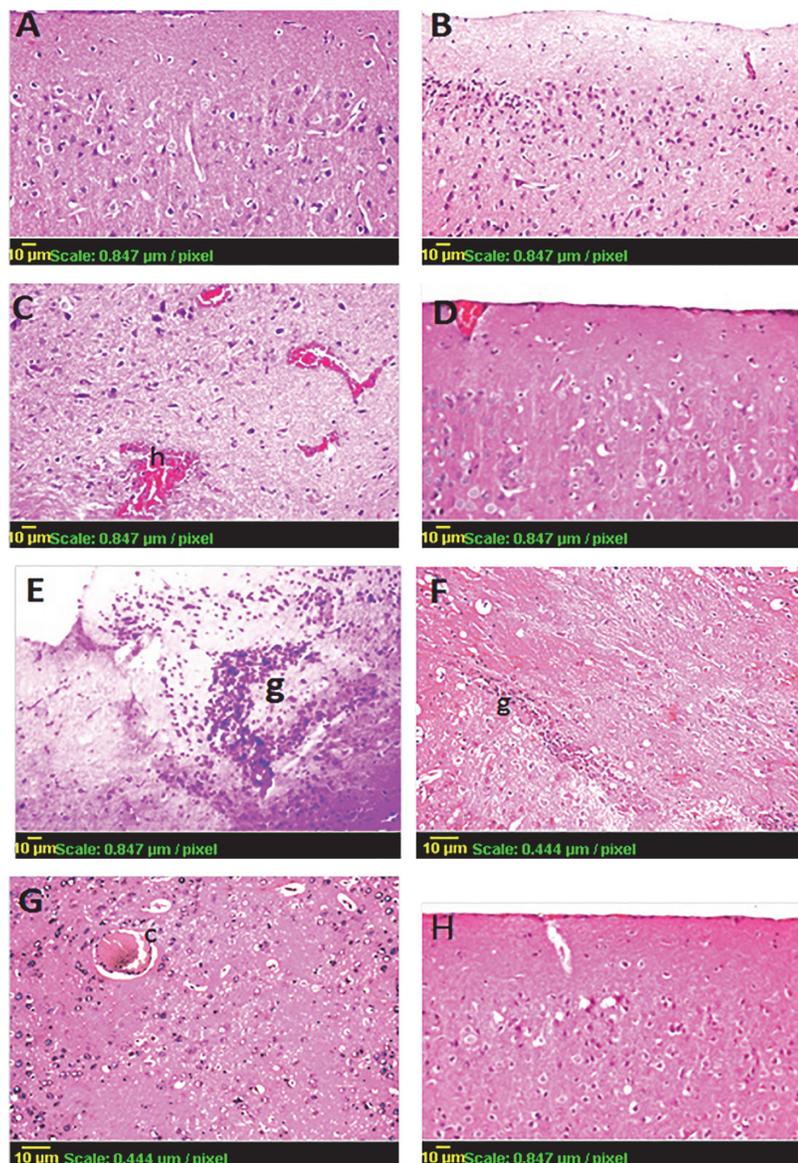


Fig 3. H & E staining of the cortices of rats belonging to the sham group (A), control group (B), 3-NPA-treated group (C), 17 β -estradiol- and 3-NPA-treated group (D), genistein (5 mg/kg)- and 3-NPA-treated group (E), genistein (10 mg/kg)- and 3-NPA-treated group (F), genistein (20 mg/kg)- and 3-NPA-treated group (G) and genistein alone-treated group (H): A, B, D and H showed no histological alterations, C showed severe hemorrhage (h), E showed focal gliosis (g) F showed focal gliosis (g) and G showed congested blood vessel (C).

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5. Effects of genistein on striatal, cortical and hippocampal oxidative status (lipid peroxidation, reduced glutathione level and catalase activity) in 3-NPA-treated ovariectomized rats

One-way ANOVA showed significant differences among groups on striatal, cortical and hippocampal MDA levels [F(6,35) = 13.7, $P < 0.0001$, F(6,35) = 9.6, $P < 0.0001$; F(6,35) = 16.4, $P < 0.001$, respectively], GSH levels [F(6,35) = 6.6, $P < 0.0001$, F(6,35) = 2.35, $P < 0.05$; F(6,35) = 5.7, $P < 0.0001$] and catalase activities [F(6,35) = 16.5, $P < 0.0001$, F(6,35) = 6.5,

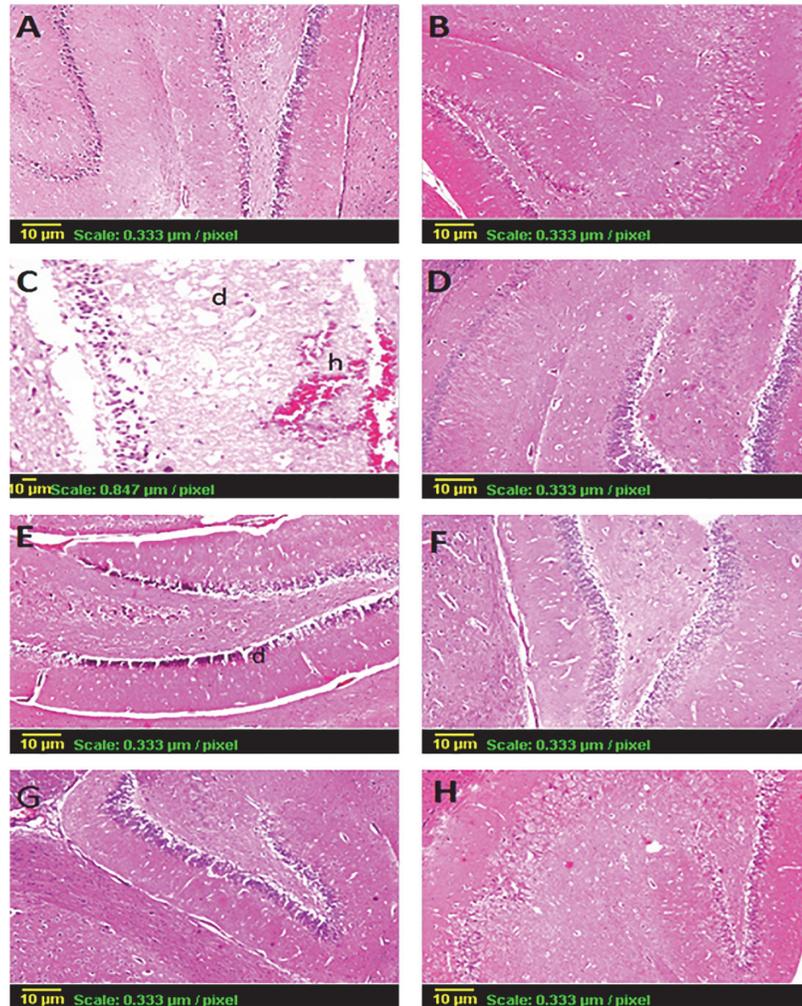


Fig 4. H & E staining of the hippocampi of rats belonging to the sham group (A), control group (B), 3-NPA-treated group (C), 17 β -estradiol- and 3-NPA-treated group (D), genistein (5 mg/kg)- and 3-NPA-treated group (E), genistein (10 mg/kg)- and 3-NPA-treated group (F), genistein (20 mg/kg)- and 3-NPA-treated group (G) and genistein alone-treated group (H): A, B, D, F, G and H showed no histological alterations, C showed severe neurodegeneration (d) and hemorrhage (h) and E showed some degenerated hippocampal cells (d).

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$P < 0.0001$; $F(6,35) = 15.4$, $P < 0.0001$] (Table 2). Results showed that administration of 3-NPA at a dose of 20 mg/kg s.c. for 4 consecutive days significantly increased lipid peroxidation, depleted GSH and reduced catalase enzyme activity as compared to sham and control groups. However, pretreatment with genistein (10 mg/kg) decreased lipid peroxidation in the striatum, cortex and hippocampus, restored glutathione levels in the striatum and attenuated the decrease in catalase activity in the cortex and hippocampus compared to 3-NPA-treated group. Pretreatment with genistein (20 mg/kg) decreased lipid peroxidation in the striatum, cortex and hippocampus, restored striatal and hippocampal glutathione levels and attenuated the decrease in catalase activity in the three brain areas compared to 3-NPA-treated group. It is obvious from the results that the effect of genistein on the hippocampus is more prominent. Pretreatment with 17 β -estradiol (2.5 mg/kg) significantly reduced lipid peroxidation and attenuated the decrease in catalase activity in the three brain areas and restored only hippocampal GSH levels as compared to

Table 1. Effects of genistein on striatal, cortical and hippocampal ATP levels in 3-NPA-treated ovariectomized rats.

Groups	ATP level (µg/g tissue)		
	Striatum	Cortex	Hippocampus
Sham	4.9±0.36	4.2±0.21	5±0.44
Control	3.8 ±0.19	3.7±0.15	4±0.28
3-NPA	1.7 ^{*,x} ±0.11	2.2 ^{*,x} ±0.17	2.1 ^{*,x} ±0.26
17β-estradiol+ 3-NPA	3.1 [#] ±0.26	3.6 [#] ±0.35	4.1 [#] ±0.4
Genistein(10mg/kg)+ 3-NPA	2.8±0.29	2.9±0.22	4.2 [#] ±0.39
Genistein(20mg/kg)+ 3-NPA	3.2 [#] ±0.17	3.6 [#] ±0.33	4.6 [#] ±0.36
Genistein (20mg/kg)	4.5±0.41	3.8±0.31	5.1±0.38

3-NPA was administered i.p. (20 mg/kg) for 4 consecutive days. 17β-estradiol (2.5 mg/kg body weight, s.c) and genistein (10 and 20 mg/kg body weight, i.p) were administered for 8 days, beginning 4 days before and continued for 4 days one hour before 3-NPA injections. Data are presented as means ±S.E.M. (n = 6).

^x statistically significant compared to sham group at P < 0.05

^{*} statistically significant compared to control group at P < 0.05

[#] statistically significant compared to 3-NPA-treated group at P < 0.05 (One-way ANOVA followed by Tukey test).

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3-NPA treated group. Moreover, ovariectomy caused significant increase in TBARs and the two doses of genistein significantly decreased hippocampal lipid peroxidation compared to 17β-estradiol.

Table 2. Effects of genistein on striatal, cortical and hippocampal TBARs and GSH levels and catalase activities in 3-NPA-treated ovariectomized rats.

Groups	TBARs (nmol/g tissue)			GSH (µmol/ g tissue)			Catalase activity (U/g tissue)		
	Striatum	Cortex	Hippocampus	Striatum	Cortex	Hippocampus	Striatum	Cortex	Hippocampus
Sham	79±6	91.8±5	104±8	2.14±0.11	2.4±0.25	1.17±0.14	74.3±4.4	56.3±3.3	59.5±4.3
Control	115 ^x ±10	149 ^x ±7	156 ^x ±8	1.97±0.08	2.14 ±0.15	1.2±0.11	60.1±5.9	46.5±3.6	48.3±1.8
3-NPA	173 ^{x,*} ±6	207 ^{*,x} ±19	229 ^{*,x} ±21	1.2 ^{*,x} ±0.1	1.5 ^{*,x} ±0.1	0.72 ^{*,x} ±0.03	32.5 ^{x,*} ±3	31.5 ^{*,x} ±1.9	26.7 ^{*,x} ±2.7
17β-estradiol+ 3-NPA	104 [#] ±5	130 [#] ±13	162 [#] ±4	1.6±0.15	2.06 ±0.24	1.33 [#] ±0.05	60.2 [#] ±3.8	55.6 [#] ±2.8	48.8 [#] ±2.3
Genistein(10 mg/kg)+ 3-NPA	106 [#] ±9	130 [#] ±9	100 [#] ±5	1.81 [#] ±0.14	2±0.06	1.02±0.05	47.3±2.7	47 [#] ±2.6	57.3 [#] ±0.6
Genistein(20 mg/kg)+ 3-NPA	97 [#] ±5	129 [#] ±9	104 [#] ±9	1.86 [#] ±0.11	1.9±0.16	1.15 [#] ±0.05	58.6 [#] ±3.2	51.1 [#] ±3.2	55.2 [#] ±2.1
Genistein (20 mg/kg)	89±8	94±6	107±5	2.01±0.13	2.19±0.2	1.11±0.1	66.2±4	49.3±4.3	56.1±1

3-NPA was administered i.p. (20 mg/kg) for 4 consecutive days. 17β-estradiol (2.5 mg/kg body weight, s.c) and genistein (10 and 20 mg/kg body weight, i.p) were administered for 8 days, beginning 4 days before and continued for 4 days one hour before 3-NPA injections. Data are presented as means ±S.E.M. (n = 6).

^x statistically significant compared to sham group at P < 0.05

^{*} statistically significant compared to control group at P < 0.05

[#] statistically significant from 3-NPA-treated group at P < 0.05

⁺ statistically significant compared to 17β-estradiol-pretreated group at P < 0.05 (One-way ANOVA followed by Tukey test).

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6. Effects of genistein on striatal, cortical and hippocampal AChE activity of 3-NPA-treated ovariectomized rats

One-way ANOVA showed significant effects of treatments on striatal, cortical and hippocampal AChE activity [$F(6,35) = 11.06$; $P < 0.0001$, $F(6,35) = 9.46$, $P < 0.0001$; $F(6,35) = 9.07$, $P < 0.0001$, respectively] (Fig. 5) Rats treated with 3-NPA showed significant increase in striatal, cortical and hippocampal AChE activity as compared to sham and control groups. Pretreatment with 17β -estradiol (2.5 mg/kg) and genistein (10 and 20 mg/kg) significantly attenuated this elevation. Ovariectomy caused non-significant increase in AChE activity.

7. Effects of genistein on cortical and hippocampal iNOS and COX-2 expressions of 3-NPA-treated ovariectomized rats

Immunohistochemical staining of cortical and hippocampal COX-2- and iNOS-positive cells immunized with goat-anti-rabbit IgG is shown in Figs. 6–9. There were only faint immunoreactive staining detected in the brain areas of the sham and control and groups. Sections from 3-NPA-treated rats showed patches of COX-2 and iNOS positive cells spread over the whole areas whereas fewer positive cells were detected in sections from rats pretreated with 17β -estradiol (2.5 mg/kg) and genistein (10 and 20 mg/kg). Note that the higher genistein dose was more effective in reducing COX-2 and iNOS expression. These photomicrographs greatly correlate with the values of the mean OD and A % shown in (Tables 3 and 4).

Discussion

The present study was conducted to examine the potential neuroprotective and memory enhancing effects of the phytoestrogen, genistein on 3-NPA-induced HD model. These effects were compared to a reference structurally similar drug- 17β -estradiol-with known neurotrophic [35, 44] and cognitive enhancing activities [21]. The choice of genistein was based on previous studies that demonstrated its neuroprotective activity in experimental models of Alzheimer's disease, Parkinson's disease and ischemic stroke [45–47] in addition to memory enhancing effects [36,48]. Systemic administration of 3-NPA (20 mg/kg) caused a significant reduction of spontaneous motor activity following the fourth injection. Such compromise in locomotor

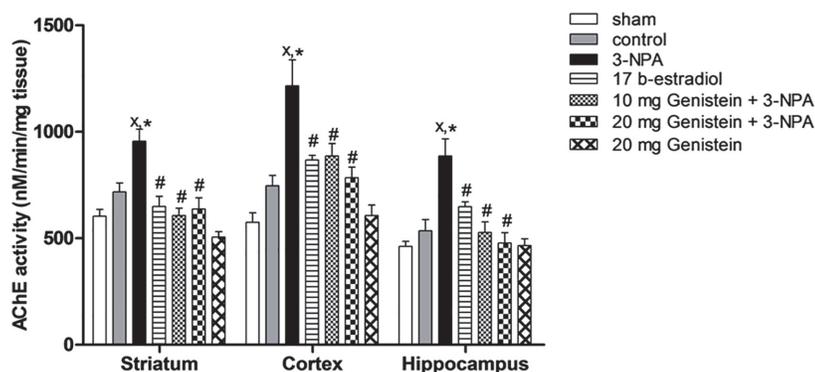


Fig 5. Effects of 3-NPA and/or genistein on striatal, cortical and hippocampal AChE activity of ovariectomized rats. 3-NPA was administered i.p. (20 mg/kg) for 4 consecutive days. 17β -estradiol (2.5 mg/kg body weight, s.c) and genistein (10 and 20 mg/kg body weight, i.p) were administered for 8 days, beginning 4 days before and continued for 4 days one hour before 3-NPA injections. Data are presented as means \pm S.E.M. (n = 6). ^X statistically significant compared to sham group at $P < 0.05$, ^{*} statistically significant compared to control group at $P < 0.05$, [#] statistically significant compared to 3-NPA-treated group at $P < 0.05$ (one-way ANOVA followed by Tukey test).

doi:10.1371/journal.pone.0117223.g005

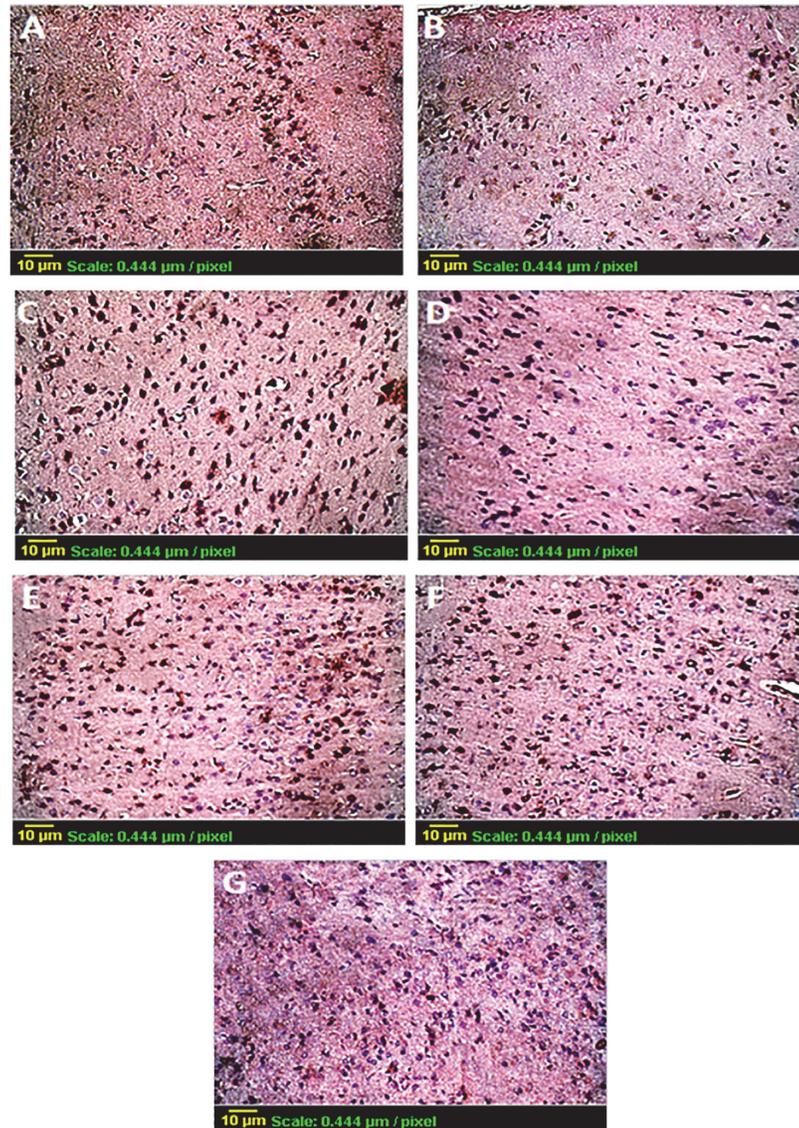


Fig 6. Immunohistochemical staining of cortical COX-2-positive cells immunized with goat-anti-rabbit antibodies of the sham group (A), control group (B), 3-NPA-treated group (C), 17 β -estradiol- and 3-NPA-treated group (D), genistein (10 mg/kg)- and 3-NPA-treated group (E), genistein (20 mg/kg)- and 3-NPA-treated group (F) and genistein alone-treated group (G).

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behavior reflects a combination of lowered energy levels and consequent short term changes in neural processing [49]. The current observation is also in agreement with other reports which showed that rats exhibited significant hypoactivity following exposure to 3-NPA [50–53]. Pre-treatment with 17 β -estradiol (2.5 mg/kg) and genistein (10 and 20 mg/kg) significantly attenuated this locomotor hypoactivity. Previous studies showed that genistein and 17 β -estradiol prevented motor dysfunction in rat models of Parkinson's disease [46, 54,55]. In addition, there is a considerable decrease in locomotor activity trend if compared to day one, which could be attributed to the decrease in the exploratory behavior of the rats as they are placed in the same cage for more than once [56].

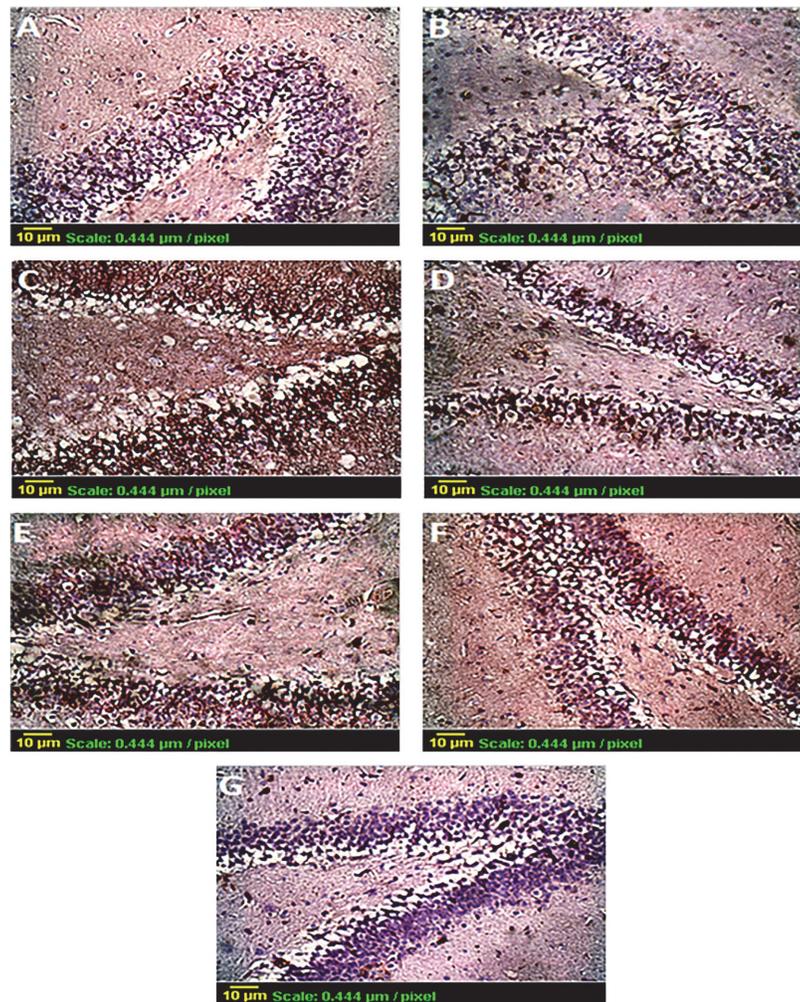


Fig 7. Immunohistochemical staining of hippocampal COX-2-positive cells immunized with goat-anti-rabbit antibodies of the sham group (A), control group (B), 3-NPA-treated group (C), 17 β -estradiol- and 3-NPA-treated group (D), genistein (10 mg/kg)- and 3-NPA-treated group (E), genistein (20 mg/kg)- and 3-NPA-treated group (F) and genistein alone-treated group (G).

doi:10.1371/journal.pone.0117223.g007

Evaluation of the cognitive status of ovariectomized rats using step-through passive avoidance paradigm showed that systemic administration of a single 3-NPA dose was not enough to induce memory deficit in rats, manifested as normal retention latency. However, after 4 consecutive doses of 3-NPA, there was a significant reduction in the retention latency suggesting impaired memory retention. This is in agreement with a previous study which demonstrated that systemic administration of 4 injections of 3-NPA (15 mg/kg) induced reduction in retention latencies in the passive avoidance test and memory impairment in sleep-deprived mice [57]. Pretreatment with 17 β -estradiol and genistein (10 and 20 mg/kg) significantly improved memory retention represented in prolonged retention latencies. A previous experiment showed that pretreatment with 17 β -estradiol (10 $^{\circ}$ g/kg) significantly inhibited ouabain-induced amnesic effect tested by passive avoidance task [58]. Another study showed that genistein (10 mg/kg) significantly improved retention and recall aspects of learning and memory in passive avoidance task in a rat model of Alzheimer's disease [36].

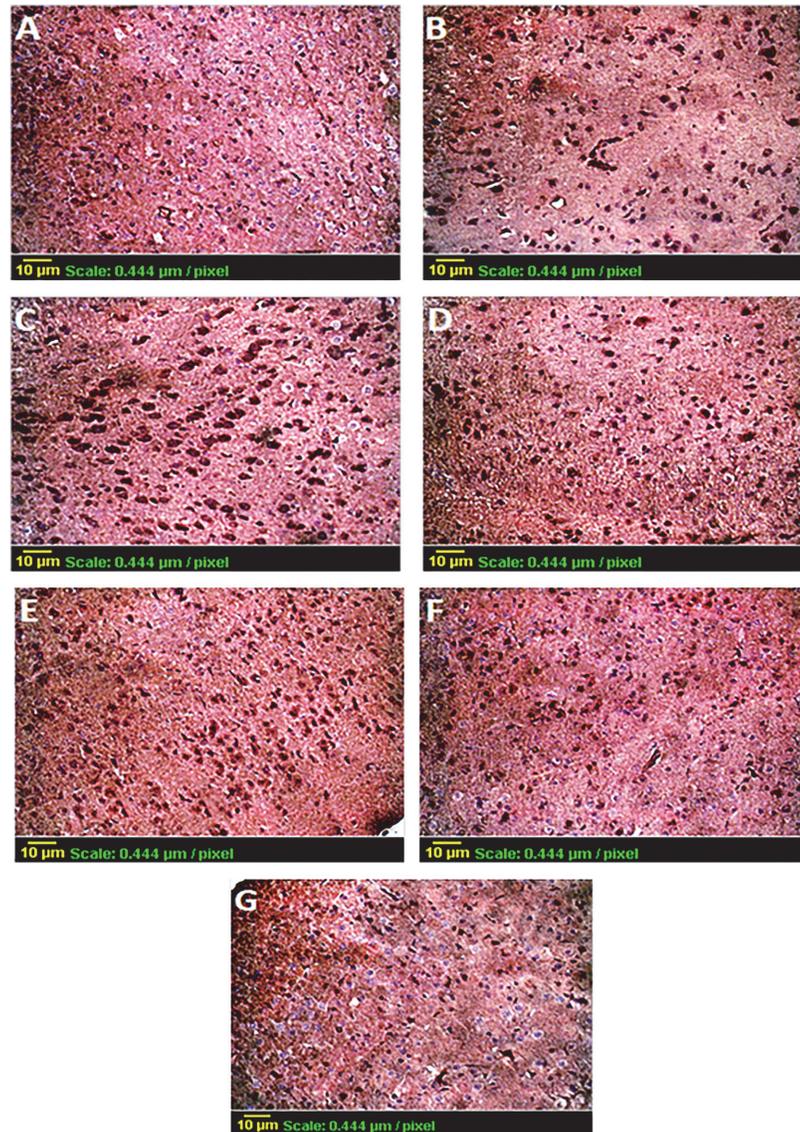


Fig 8. Immunohistochemical staining of cortical iNOS-positive cells immunized with goat-anti-rabbit antibodies of the sham group (A), control group (B), 3-NPA-treated group (C), 17 β -estradiol- and 3-NPA-treated group (D), genistein (10 mg/kg)- and 3-NPA-treated group (E), genistein (20 mg/kg)- and 3-NPA-treated group (F) and genistein alone-treated group (G).

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Histological examination showed marked hippocampal neurodegeneration appeared as encephalomalacia, porous brain tissue, as well as cortical focal gliosis and hemorrhage of the brain of rats treated with 3-NPA. It is well reported that 3-NPA produce lesions in hippocampal neurons associated with cognitive performance [52,59]. Moreover, several studies demonstrated passive avoidance deficit in rodents following dorsal hippocampal lesion which is proven to play a crucial role in acquisition, consolidation and retrieval of rat's passive avoidance response [60]. The current study showed that pretreatment with 17 β -estradiol and genistein (10 and 20 mg/kg) relatively restored the cortical and hippocampal histological structure. This neuroprotective effect of genistein could be attributed to its antiapoptotic effect where it

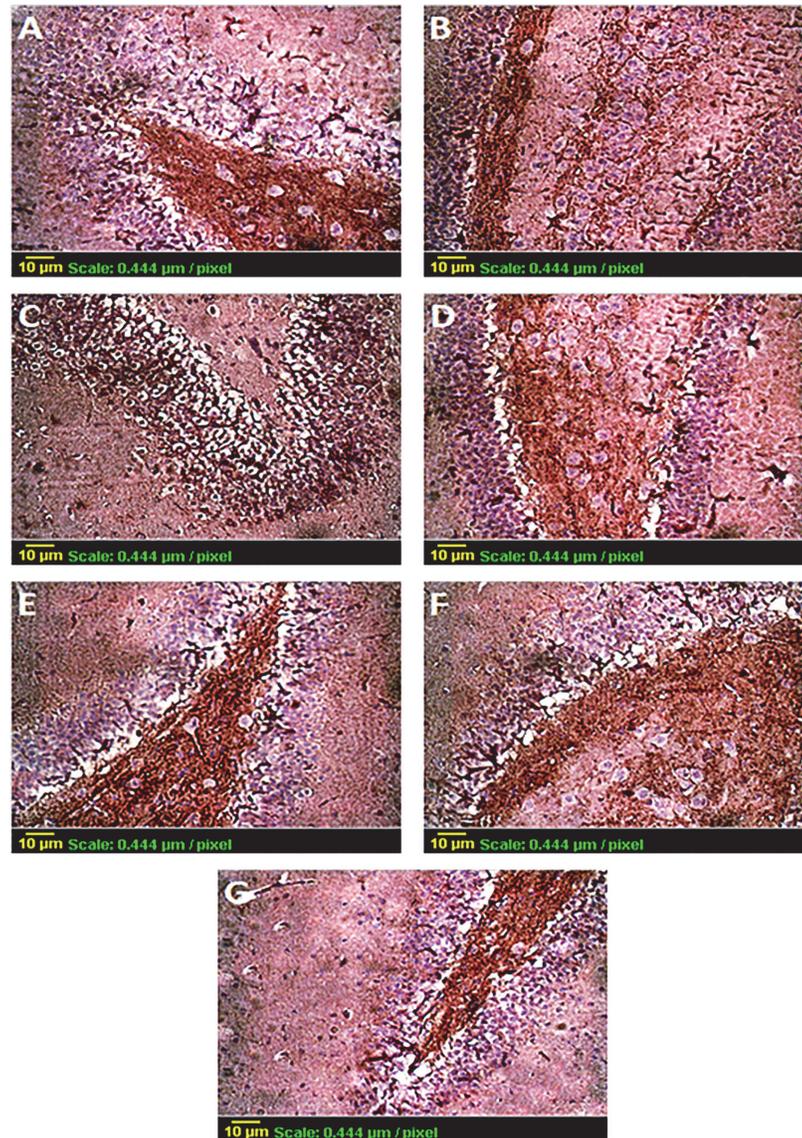


Fig 9. Immunohistochemical staining of hippocampal iNOS-positive cells immunized with goat-anti-rabbit antibodies of the sham group (A), control group (B), 3-NPA-treated group (C), 17 β -estradiol- and 3-NPA-treated group (D), genistein (10 mg/kg)- and 3-NPA-treated group (E), genistein (20 mg/kg)- and 3-NPA-treated group (F) and genistein alone-treated group (G).

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protected against H₂O₂-induced apoptosis via the regulation of Bcl-2/Bax expression and the modulation of cell survival signaling such as phosphatidylinositol 3-kinase pathway [61].

The lowest genistein dose (5 mg/kg) showed no improvement in the behavioral and pathological features when challenged with 3-NPA (20 mg/kg) for 4 consecutive days. Therefore, this dose was excluded and the Biochemical assays were performed with the two higher doses to further investigate the most effective dose and to cite the underlying mechanisms involved in the behavioral and pathological alterations caused by 3-NPA and/or genistein.

A decreased cellular ATP level can be a cause or a consequence of cell death [62]. Concerning 3-NPA toxicity, decreased ATP levels can be considered a cause of cell death since it blocks the transport of electrons in oxidative phosphorylation [10,14]. And also a consequence of cell

Table 3. OD and A% values of COX-2 immunohistochemically stained cortical and hippocampal sections.

Groups	OD		A%	
	Cortex	Hippocampus	Cortex	Hippocampus
Sham	1.36±0.098	1.26±0.049	22.23±1.5	23.4±2.3
Control	1.2±0.07	1.32±0.033	26.8±1.07	28.6±0.87
3-NPA	1.95 ^{*x} ±0.15	1.78 ^{*x} ±0.03	38 ^{*x} ±1.15	56.7 ^{*x} ±1.45
17 β-estradiol+3-NPA	1.44 [#] ±0.089	1.53±0.04	28 [#] ±1.2	34.2 [#] ±1.2
Genistein (10 mg/kg)+3-NPA	1.6±0.078	1.59±0.1132	30.2 [#] ±1.4	44.7 [#] ±2.57
Genistein (20 mg/kg)+3-NPA	1.4 [#] ±0.105	1.39 [#] ±0.087	28.3 [#] ±0.88	37 [#] ±3.1
Genistein (20 mg/kg)	1.15±0.05	1.19±0.009	19.3 [*] ±1.43	20.9±1.09

3-NPA was administered i.p. (20 mg/kg) for 4 consecutive days. 17β-estradiol (2.5 mg/kg body weight, s.c) and genistein (10 and 20 mg/kg body weight, i.p) were administered for 8 days, beginning 4 days before and continued for 4 days one hour before 3-NPA injections. Data are presented as means ±S.E.M. (n = 3).

^x statistically significant compared to sham group at P < 0.05

^{*} statistically significant compared to control group at P < 0.05

[#] statistically significant compared to 3-NPA-treated group at P < 0.05 (One-way ANOVA followed by Tukey test).

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death since apoptosis is an energy-dependent process requiring increased synthesis of particular proteins responsible for killing the cell therefore consuming ATP [63]. Estrogen was shown to have beneficial effects on the entire bioenergetics system of the brain and ATP production; an effect that has a positive impact on cognitive functions [64,65]. A previous study also demonstrated that genistein protected rat embryo cortical neurons from ATP loss and reduced the loss of neuronal viability induced by glucose deprivation [66]. Results of the current study showed that 3-NPA caused significant reduction in striatal, cortical and hippocampal ATP, while pretreatment with 17β-estradiol or genistein (20 mg/kg) significantly attenuated this

Table 4. OD and A% values of iNOS immunohistochemically stained cortical and hippocampal sections.

Groups	OD		A%	
	Cortex	Hippocampus	Cortex	Hippocampus
Sham	1.24±0.08	0.98±0.006	12.7±1.15	21.5±1.2
Control	1.16±0.042	1.25±0.1	19.6±1.7	27±1.35
3-NPA	1.58 ^{*x} ±0.032	1.6 ^{*x} ±0.029	40.5 ^{*x} ±1.07	48.5 ^{*x} ±1.32
17 β-estradiol+3-NPA	1.34 [#] ±0.025	1.08 [#] ±0.04	28.2 [#] ±1.6	28.2 [#] ±2.1
Genistein (10 mg/kg)+3-NPA	1.3 [#] ±0.011	1.15 [#] ±0.008	31.5 [#] ±1.3	35.6 [#] ±1.76
Genistein (20 mg/kg)+3-NPA	1.18 [#] ±0.052	1.08 [#] ±0.055	21.6 [#] ±1.7	31.1 [#] ±1.6
Genistein (20 mg/kg)	1.24±0.032	0.99±0.056	16.4±1.13	18.3±1.6

3-NPA was administered i.p. (20 mg/kg) for 4 consecutive days. 17β-estradiol (2.5 mg/kg body weight, s.c) and genistein (10 and 20 mg/kg body weight, i.p) were administered for 8 days, beginning 4 days before and continued for 4 days one hour before 3-NPA injections. Data are presented as means ±S.E.M. (n = 3).

^x statistically significant compared to sham group at P < 0.05

^{*} statistically significant compared to control group at P < 0.05

[#] statistically significant compared to 3-NPA-treated group at P < 0.05

[°] statistically significant compared to genistein (10 mg/kg)-pretreated group at P < 0.05 (One-way ANOVA followed by Tukey test).

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reduction in the striata, cortices and the hippocampi. However, pretreatment with genistein (10 mg/kg) only showed significant increase of hippocampal ATP levels.

It is worthy noted that oxidative stress is a corner stone in mediating behavioral impairment and memory deficit in age-related neurodegenerative disorders [67]. This theory was supported by previous studies on the neurotoxic effects of 3-NPA [52, 68, 69], as well as the current study, where systemic 3-NPA administration caused significant increase in cortical and hippocampal lipid peroxidation and decrease in GSH levels and catalase activity. 17β -estradiol is known to have a potent neuroprotective activity which is in part due to its antioxidant effect [70–72]. Similarly, genistein, previously showed strong antioxidant activity [36,48,73]. These were consistent with the findings of the current study, where, pretreatment with 17β -estradiol (2.5 mg/kg) and genistein (20 mg/kg) significantly reduced oxidative stress. It was also noted that genistein may have stronger antioxidant activity than 17β -estradiol demonstrated significantly in the hippocampus. There were also significant increase in the levels of cortical and hippocampal TBARS in the control group compared to the sham which is attributed to the decrease in endogenous estrogen in the control group due to ovariectomy.

The decline of the cortical and hippocampal cholinergic activity occurs continuously with aging and this is associated with cognitive dysfunctions [74]. Therefore, cholinesterase activity, mainly that of AChE, was assessed. Results showed significant increase in striatal, cortical and hippocampal AChE activity in 3-NPA-treated group. Pretreatment with 17β -estradiol and genistein significantly attenuated this increase. A previous study reported that 17β -estradiol can modulate AChE activity [75]. Genistein (15 and 30 mg/kg) and 17β -estradiol (100 μ g/kg) also previously showed AChE inhibitory effect in ovariectomized rats [48]. Moreover, Genistein (12.5 mg/kg) decreased AChE activity in a rat model of schizophrenia [76]. Ovariectomy caused a non-significant increase in AChE activity which highlights that 4 weeks following ovariectomy may be not enough to affect memory; and this correlates with the results of passive avoidance.

Neuroinflammatory response was proven to propagate neurodegeneration [77,78]. A previous study suggested that the inflammatory response and production of nitric oxide by iNOS could be involved in the toxicity of amyloid beta_{25–35} with different implications for spatial memory [79]. Also 3-NPA induced inflammatory response through increasing COX-2 and iNOS expression [80]. Therefore, the effects of the treatments on the expression of inflammatory mediators, COX-2 and iNOS, were assessed. Immunohistochemical staining of iNOS and COX-2 showed that 3-NPA treatment increased COX-2 and iNOS in both the cortex and hippocampus and this effect was significantly reduced via pretreatment with 17β -estradiol and genistein. Results showed that the larger dose of genistein (20 mg/kg) was more effective. These results are supported with previous studies that demonstrated the anti-inflammatory effect of genistein and 17β -estradiol in Alzheimer's disease through decreasing COX-2 and iNOS expression in cultured astrocytes [81] and the effect of genistein in inhibiting hemolysate-induced iNOS and COX-2 expression in primary astrocytes [82].

Conclusion

Genistein improved locomotor activity, memory retention, cell viability and restored the histological abnormalities caused by 3-NPA in ovariectomized rats. This activity could be—at least partly—explained based on its antioxidant, anti-inflammatory and attenuating the increase of cholinesterase activity.

Author Contributions

Conceived and designed the experiments: ABA AEK. Performed the experiments: ETM AE. Analyzed the data: MGT. Contributed reagents/materials/analysis tools: ETM AE. Wrote the paper: ETM AE MGT.

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