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Neuroprotective Effects of Genistein in Mongolian Gerbils: Estrogen Receptor–*β* Involvement

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Abstract. Genistein is a naturally occurring plant-derived phytoestrogen, present in the human diet, known to possess some beneficial effects. The present study investigated the effect of genistein on neuroprotection evaluated through electroencephalographic and behavioural correlates in a model of global cerebral ischemia in gerbils. Over the dose range tested, genistein (3 and 10 mg/kg), given 5 min after recirculation antagonized the ischemia-induced electroencephalographic total spectral power decrease 7 days after ischemia; fully prevented ischemia-induced hyperlocomotion evaluated 1 day after ischemia; reversed ischemia-induced memory impairment evaluated through both nest building behaviour and object recognition test; decreased malondialdehyde overproduction in the brain, evaluated 7 days after reperfusion; and fully promoted the survival of pyramidal cells in the CA_1 hippocampal subfield. The selective antagonist for estrogen receptor–*β* (ER*β*), 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol (PHTPP) given 30 min before carotid occlusion, fully prevented the neuroprotective effect of genistein at the dose of 3 mg/kg. These results demonstrate the neuroprotective effect of genistein through the activation of ER*β* and provide further grounds for the growing interest concerning the true potential of phytoestrogens as compounds to beneficially affect brain injury without having the disadvantages of estrogens.

Keywords: ischemia, electroencephalography (EEG), phytoestrogen, CA_1, estrogen receptor–*β*

Introduction

Phytoestrogens are naturally occurring plant-derived compounds that are present in the human diet and are considered 'selective estrogen receptor (ER) modulators'. Because estrogen replacement therapy is presently not indicated for the primary or secondary prevention of cardiovascular diseases in post-menopausal women, phytoestrogens have emerged as a promising alternative means of cardiovascular neuroprotection (1 – 3), to decrease age-related cognitive decline (4) and bone loss (5), and protect against breast cancer (6). Genistein, 4′,5,7-trihydroxyisoflavone, is the major isoflavone found in soy and belongs to an important class of phytoestrogens. Its structure resembles human estrogens and is capable of binding to ERs, thus producing a weak estrogenic effect (7). Genistein has many potential physiological effects, including control of bone loss (8), anticarcinogenic effect (9, 10), and cardioprotective activities (11, 12). In vitro and in vivo studies have also demonstrated neuroprotective effects of soy isoflavones. Genistein can protect primary neurons from glutamate toxicity (13, 14), thapsigargin-induced apoptosis (15), and *β*-amyloid toxicity (16).

Inoue et al. (17) found that treatment with genistein reduced cytochrome C release and the number of damaged neurons in the CA_1 region after transient forebrain ischemia in the mouse embryonic hippocampal neurons. In an in vivo mouse model of singlet oxygen-induced cerebral stroke, genistein showed antioxidant activity (18). Furthermore, a high-soy diet reduced stroke injury in female and male rats (19, 20). Acute treatment of genistein (0.1, 1, or 10 mg/kg) in vivo protected against hippocampal neuronal loss induced by the systemic ad-

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ministration of kainic acid to adult Wistar ovariectomized female rats (21). Accordingly, genistein protected neurons from transient global cerebral injury in rat hippocampus by attenuating oxidative stress, lipid peroxidation, and the signaling cascade leading to apoptotic cell death (19).

Overall, the above quoted findings revealed a clear neuroprotective effect of genistein against ischemic injury. However, no study reported the potential therapeutic effect of genistein since the soy phytoestrogen was always given before the ischemic insult or 24 h after injury but with a previous repeated treatment. In this study, we hypothesized that genistein would be neuroprotective in vivo against global cerebral ischemia in gerbils even when acutely administered 5 min after the reperfusion. To investigate the mechanism of action, we used the selective antagonist for estrogen receptor–β (ERβ), 4-[2-phenyl-5,7-bis(trifluoromethyl) pyrazolo[1,5-*a*]-pyrimidin-3-yl]phenol (PHTPP), since genistein is a mild-selective agonist for ERβ, with more than 7-fold higher binding affinity for ERβ than ERα (22). To quantify the ischemic damage, from 1 h to 7 days after reperfusion we measured different parameters known to be influenced by global cerebral ischemia: electroencephalography (EEG) total spectral power, spontaneous motor activity, memory function, and hippocampal CA_1 neuronal count. We measured motor activity on day 1, since ischemia-induced hyperlocomotion is maximal 24 h after occlusion (23), and determined memory function on day 2 according to Wappler et al. (24). Since the decrease in EEG has been related to pronounced damage of neurons on day 7 (25 – 27), EEG spectral power and neuronal counts were measured at this time. Anti-oxidant mechanisms, by assaying lipoperoxide content, were also investigated.

Materials and Methods

Animals

Male Mongolian gerbils (*Meriones unguiculatus*) (Charles River, Calco, Como, Italy) weighing 60 – 80 g were housed singly under standard laboratory conditions: air-conditioned room (22 ± 2°C), 12-h light / 12-h dark illumination cycle, and free access to standard food and water. The gerbils were allowed to acclimatize themselves to the environment for a period of 1 week prior the surgical implantation of cortical electrodes. All gerbils were submitted to EEG electrodes implantation and then divided in different groups on the basis of the treatment received. Each animal was submitted to all tests. All the experimental procedures followed the guidelines established by the European Communities Council Directive of 1986 (86/609/EEC) and were approved by the Italian Council on Animal Care by the Italian Government decree No. 36/2007. All efforts were made to minimize the number of animals used and their suffering.

Surgical procedure

Gerbils were anesthetized with an i.p. injection of chloral hydrate (450 mg/kg; Sigma, St. Louis, MO, USA) dissolved in saline and given at a volume of 10 ml/kg. Four electrodes (Bilaney, Dusseldorf, Germany) for EEG recordings were implanted, as described elsewhere (28), on the right and left of the parieto-occipital cortex according to the coordinates (anterior +2 mm, posterior −3 mm, lateral 2 mm from the bregma) of a brain atlas (29). A further electrode was inserted into the nasal bone as ground. The five electrodes were connected to a pedestal (Bilaney) and fixed with an acrylic cement (Palavit; New Galetti & Rossi, Milan, Italy). After the chronic implantation of the electrodes, 1 week was allowed for recovery from surgery before experiments started.

EEG recording

Freely moving, awake gerbils were acclimatized in a sound-attenuated Faraday chamber and then their EEG was recorded for 1 h before the induction of cerebral ischemia and 1 h after recirculation to determine the basal total spectral power. Spectral powers between 0 and 25 Hz were evaluated using a resolution of 0.2 Hz. The signals were recorded and processed for fast Fourier Transform spectral analysis by means of PC software (Power-Lab; ADinstruments, Pty., Ltd., Castle Hill, Australia). EEG recordings were also made during 10 min ischemia and 1 h, 1, 3, and 7 days after. During 1 h recording, mean spectral power was calculated every 5 min.

Induction of global cerebral ischemic injury

After basal EEG recordings, each gerbil was again lightly anesthetized with 2,2,2-tribromoethanol (200 mg/kg, 10 ml/kg; Sigma-Aldrich, Milan, Italy). Body temperature was kept at 37°C throughout surgery with a heating lamp during 10-min ischemia, which was induced by common carotid occlusion as previously described (28). The ischemia was verified qualitatively by the complete flattening of the EEG. As the control, a group of animals (sham-operated) was submitted to the same surgical procedure except for carotid clamping.

Treatment schedule

Gerbils submitted to ischemia were divided into 5 groups of 6 animals each, receiving acutely s.c.: vehicle + vehicle; vehicle + genistein (3 and 10 mg/kg) (Sigma-Aldrich); PHTPP + vehicle; PHTPP + genistein (3 mg/kg). Genistein was given 5 min after recirculation

and PHTPP (10 mg/kg) (Tocris Cookson Ltd., Bristol, UK) was injected 30 min before carotid occlusion. Vehicle was given 30 min before or 5 min after ischemia. Both drugs were dissolved in an appropriate vehicle (ethanol, cremophor, saline, 1:1:18) for genistein and in saline for PHTPP and injected in a volume of 10 ml/kg. The sham-operated group received the same volume of vehicle.

Spontaneous motor activity

Spontaneous motor activity was evaluated as previously described (30) in an activity cage (Ugo Basile, Varese, Italy) placed in a sound-attenuating room. The cage was fitted with two parallel horizontal infrared beams located 2 cm from the floor. Cumulative horizontal movement counts were counted for 30 min, every 5 min, 24 h after recirculation.

Nesting behavior

Nest building behavior was assessed for 5 days following the ischemic episode, placing, every day for 5 days, a new paper towel into each cage. Paper shredding was scored on a 4-point scale adapted from (31): 0 = none; 1 = pieces >4 cm^2; 2 = pieces between 2 and 4 cm^2; 3 = pieces <2 cm^2.

Novel object recognition

On the second day after ischemia each gerbil was examined in the novel object recognition task according to Wappler et al. (24) with slight modifications. The test was conducted in a open plastic arena (60 × 50 × 30 cm) and was divided into two phases: familiarization (T_1) and novel object recognition (T_2). During the initial familiarization stage, two identical objects were placed into the arena equidistant from the walls and each other. The gerbils were placed in the centre of the arena between the two objects and allowed to explore them freely for 5 min. Object recognition was scored when the animal was within 0.5 cm of an object with its nose toward the object. Exploration was not scored if a gerbil reared above the object with its nose in the air or climbed on an object. After 30 min delay (inter-trial interval) spent in the home-cage, each gerbil was replaced into the test arena for the T_2 for another 5 min. During the second trial, one of the familiar objects (the most explored in T_1) was replaced by a novel object. Scoring of object recognition was performed in the same manner as during the familiarization phase. From gerbil to gerbil the role (familiar or new object) as well as the relative position of the two objects were counterbalanced and randomly permuted. The objects for the gerbil to discriminate consisted of black plastic cylinders, red plastic Lego stacks of different shape, and brown plastic tin. The arena was cleaned with 10% acetic acid after each trial. The basic measure was the time (in s) taken by the gerbil to explore the objects in the two trials. The performance was evaluated as the time exploring objects during T_2 and by calculating a discrimination index (N − F/N + F), where N = time spent exploring the new object during T_2 and F = time spent exploring the familiar object during T_2 according to (32).

Estimation of neuronal damage

Seven days after the ischemic injury, all the gerbils were anaesthetized with an overdose of 5% chloral hydrate and transcardially perfused with 4% paraformaldehyde (Sigma-Aldrich) for the histological determination as previously described (33). Brains were removed and placed in the same fixative overnight and then embedded in paraffin wax. Five serial 5-μm coronal hippocampal sections were cut at 1.5-, 1.7-, and 1.9-mm caudal to the bregma and stained with cresyl violet (Sigma-Aldrich). Neurons with a normal appearance in the pyramidal cell layer of the CA_1 sector were counted blind manner (from coded slides) in each section for each group.

Oxidative stress evaluated by determining lipid peroxide levels

Animals were sacrificed 7 days after ischemia, and the brains excluding cerebellum were removed and immediately stored at −20°C until the lipid peroxide assay. Lipid peroxide levels were determined by measuring malondialdehyde (MDA) in the brain homogenate prepared using an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany; Model T25, shaft 18N) in a ratio 1:5 ($w \cdot v^{-1}$) potassium phosphate (50 mmol/l)–EDTA (0.1 mmol/l) buffer, pH 7.4. The lipid peroxide level was established spectrophotometrically at 532 nm by means of the thiobarbituric acid test with 0.156 $\mu mol \cdot l^{-1} \cdot cm^{-1}$ being used as the extinction coefficient and expressed as nmol MDA/mg wet weight tissue as previously described (34).

Data analyses

Data are presented as the mean ± S.E.M. Total EEG spectral data were expressed as the mean of the percent difference vs. the pre-ischemic value. EEG, motor activity, latency time, and neuronal count were analyzed by one-way analysis of variance (ANOVA) for multiple comparisons, followed by Tukey's test. Nesting building was evaluated by two-way analysis of variance followed by Bonferroni's test. The accepted level was $P < 0.05$. All statistical analyses were done using software Prism, version 5 (GraphPad, La Jolla, CA, USA).

Results

The physiological parameters, such as food and water consumption and body weight, were stable throughout the study in all groups (data not shown).

Effect of genistein on EEG

A quantitative EEG analysis of gerbils given different treatments is given in Fig. 1, in which significant between-group differences were observed in terms of percent vs. pre-ischemic value of mean total spectral power evaluated on day 7 ($P < 0.0001$). *Post hoc* analysis showed that in comparison with sham-operated values, the vehicle group had a decrease of EEG power of about 70% ($P < 0.001$). Genistein, at both dosages, significantly ($P < 0.001$) antagonized the ischemia-induced EEG flattening. Pretreatment with PHTPP, which *per se* (10 mg/kg) did not induce any change in comparison with the vehicle group, completely antagonized the protective effect induced by genistein (3 mg/kg) ($P < 0.001$).

Spontaneous motor activity

Surgery did not influence spontaneous motor activity since sham-operated gerbils exhibited a number of horizontal counts similar to controls (3463 ± 497 and 2334 ± 154, respectively) (Fig. 2). Vehicle-treated ischemic gerbils displayed a significant increase ($P < 0.001$) in motor activity as compared to sham and control animals. Genistein (3 and 10 mg/kg) produced significant ($P < 0.001$) reduction in motor activity as compared to vehicle-treated ischemic injured gerbils. PHTPP (10 mg/kg), which *per se* did not affect ischemia-induced hyperactivity, reversed the protective effect of genistein (3 mg/kg) ($P < 0.001$).

Effect of genistein on nesting behavior

For sake of brevity, only data on days 1, 3, and 5 are reported (Fig. 3). Control non-ischemic gerbils, which did not receive any treatment, built a complete nest within 1 day following novel exposure to nesting material. A slight but non-significant decrease in nesting was observed in sham-operated groups, suggesting a merely transient anesthetic effect. In contrast, at 24 h after carotid occlusion, vehicle-treated gerbils displayed a significant decrease ($P < 0.001$) in nesting compared to non-ischemic control and sham-operated animals. This impairment continued at least for 5 days. Starting from day 3 after recirculation, treatment with genistein (3 and 10 mg/kg) significantly reversed the ischemia-induced impairment ($P < 0.01$). Pretreatment with PHTTP, which *per se* did not modify ischemia-induced nesting deficit, significantly blocked genistein-induced neuroprotection starting from day 3 ($P < 0.05$).

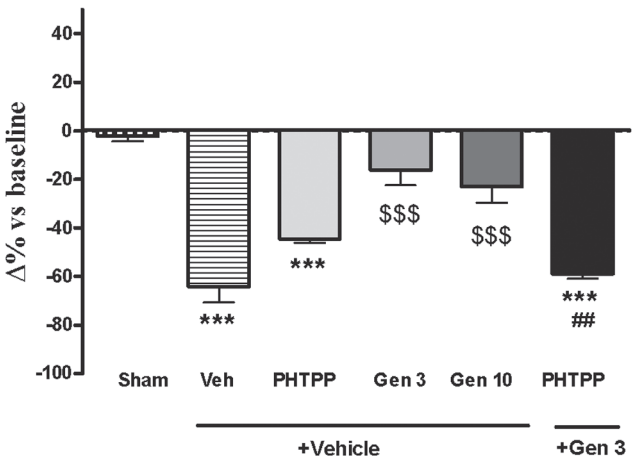

Fig. 1. Cortically derived EEG total spectral power, evaluated on day 7 after recirculation in terms of the difference (Δ%) from the pre-ischemic value in freely moving, awake gerbils given vehicle (Veh); genistein (Gen) (3 and 10 mg/kg), s.c., 5 min after recirculation; or PHTPP (10 mg/kg), s.c., 30 min before bilateral carotid occlusion, alone or with genistein (3 mg/kg). Each column represents the mean ± S.E.M. of 6 animals. ***$P < 0.001$, compared with sham-operated animals; $^{\$\$\$}P < 0.001$, compared with the vehicle group; $^{\#\#}P < 0.01$, compared with genistein at both dosages (one-way ANOVA followed by Tukey's test).

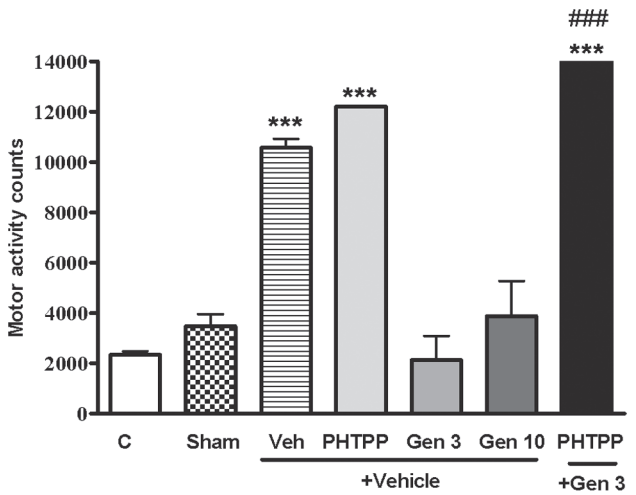

Fig. 2. Effect of vehicle (Veh) and genistein (Gen) (3 and 10 mg/kg) given s.c. 5 min after recirculation on spontaneous motor activity evaluated for 30 min and 24 h after recirculation, in gerbils. PHTPP (10 mg/kg) was given 30 min before genistein. C = non ischemic control group. Each column represents the mean ± S.E.M of horizontal counts of six animals. ***$P < 0.001$, compared with all the remaining groups; $^{\#\#\#}P < 0.001$, compared with genistein at both dosages (one-way ANOVA followed by Tukey's test).

Object recognition

During the training session (T_1), no significant differences among the groups in the mean total amount of time spent exploring the two objects, were found (data not shown). A significant ($P < 0.003$) treatment effect on the mean discrimination index was observed during the T_2 session (Fig. 4). *Post hoc* comparison showed that vehicle-treated gerbils spent significantly less time exploring a novel object than exploring a familiar object that they had been exposed to 5 min previously in comparison with control and sham-operated gerbils, resulting in a mean discrimination index of 0.13 ± 0.11 for the vehicle group vs. 0.83 ± 0.04 for the controls and 0.48 ± 0.05 for sham-operated groups. Treatment with genistein at both dosages significantly reversed ischemia-induced memory deficit ($P < 0.01$). Pre-treatment with PHTPP significantly ($P < 0.001$) antagonized the genistein-induced neuroprotective effect at a dose that per se did not alter the ischemic memory damage.

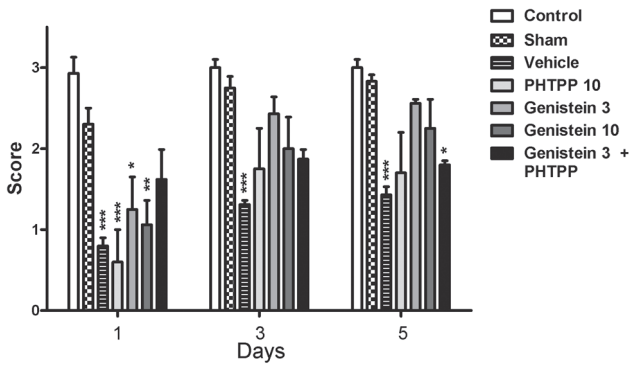

Fig. 3. Effect of genistein (3 and 10 mg/kg) on nest building behavior after ischemia. Nest building was assessed each day for 5 days following the ischemic episode. Daily nest building score (mean ± S.E.M.).*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, compared with control and sham-operated group (two-way ANOVA followed by Bonferroni's test).

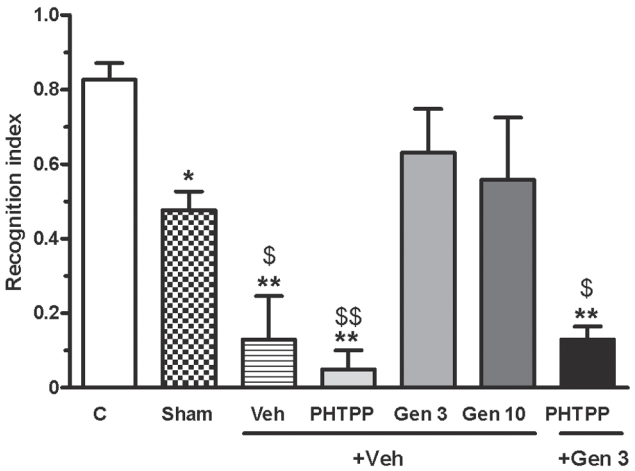

Fig. 4. Recognition of novel object 2 days following ischemic insult or sham-operation. Discrimination index (mean ± S.E.M.) between familiar and new object in sham-operated and ischemic animals, treated with vehicle (Veh) or genistein (3 and 10 mg/kg) (Gen) given s.c. 5 min after recirculation. PHTPP (10 mg/kg) was given 30 min before genistein (3 mg/kg) or vehicle. *$P < 0.05$, **$P < 0.01$, compared with the control (C); $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, compared with the sham-operated group (one-way ANOVA followed by Tukey's test).

Effect of genistein on neuronal damage

Histological examination of the hippocampus at 7 days after reperfusion, following 10 min of occlusion and 5 min of treatment, showed that severe alterations in the CA_1 region occurred in the ischemic group compared to the sham-operated animals (Fig. 5). Most of the pyramidal cells were darkly stained and shrunken. A comparison of the number of neuronal cells counted (using ANOVA) revealed significant differences between the groups ($P < 0.001$) (Fig. 6). A *post hoc* analysis revealed a decreased number of neuronal cells in the vehicle- and PHTPP alone–treated gerbils compared with the sham-operated group ($P < 0.001$). Genistein, at both dosages, completely prevented this loss of cells ($P < 0.001$). Pre-treatment with PHTPP significantly antagonized the genistein-induced neuroprotective effect ($P < 0.001$).

Lipid peroxidation (MDA)

Gerbils subjected to global injury and treated with vehicle or PHTPP exhibited a significant ($P < 0.01$) increase (181.2 ± 5.41 for vehicle and 175.3 ± 16.66 nmol/mg of wet tissue for PHTPP-treated gerbils) in MDA levels as compared with sham-operated animals (89.69 ± 19.62 nmol/mg of wet tissue) (Fig. 7). Significant reduction in brain MDA overproduction was observed with genistein treatment (3 and 10 mg/kg: 74.04 ± 10.93 and 60.71 ± 11.04 nmol/mg of wet tissue, respectively). Pre-treatment with PHTPP significantly antagonized the reduction in MDA levels (154.9 ± 8.31 nmol/mg of wet tissue) in comparison with genistein alone (3 mg/kg).

Discussion

The present study demonstrates the neuroprotective effect of the soy isoflavone genistein in gerbils when given after the ischemic insult, thus suggesting its possible therapeutic efficacy. It is interesting to note that genistein was effective when given once in a range of doses (3 – 10 mg/kg) previously found to ameliorate painful neuropathy (35) and to reduce the risk of breast cancer formation in rats (36) and mice (37). Our findings confirm those obtained with genistein, given before in-

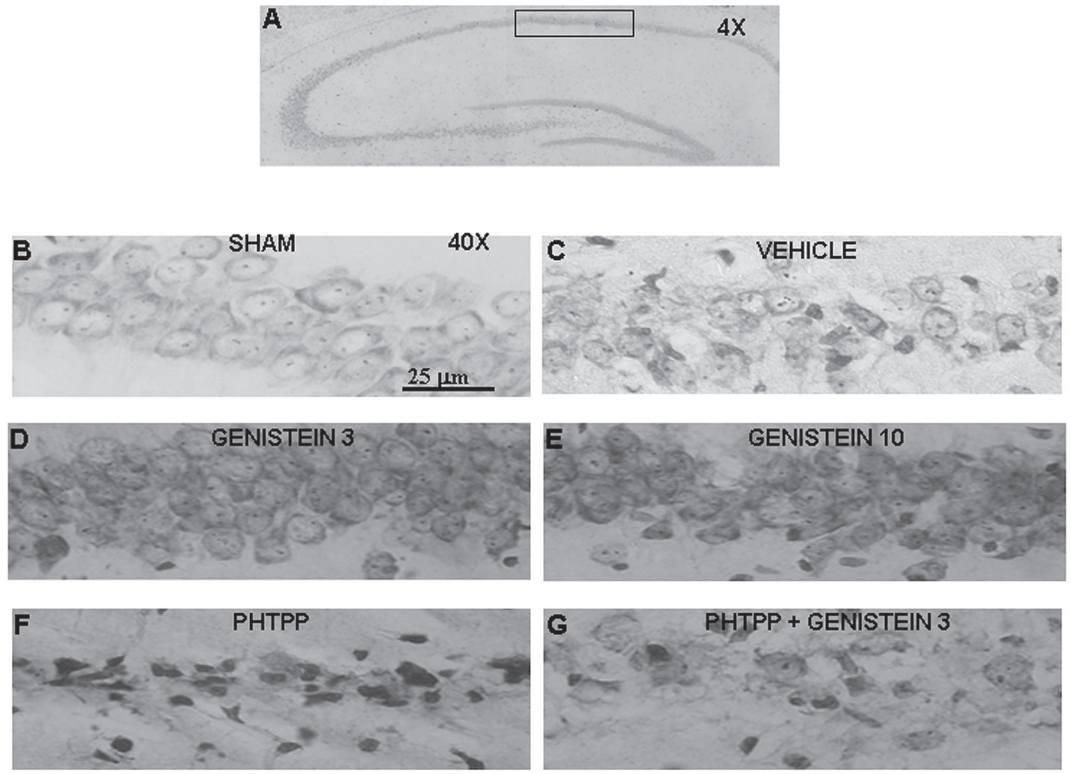

Fig. 5. Photomicrographs of the hippocampal CA_1 region of gerbils with or without 10-min ischemia 7 days after recirculation. A) CA_1 hippocampal region. B) Sham-operated animal (control group without ischemia). C) Ischemic animal treated with vehicle, 5 min after recirculation. Ischemic animal treated with genistein at 3 (D) or 10 (E) mg/kg; PHTPP at 10 mg/kg (F); and PHTPP at 10 mg/kg with genistein at 3 mg/kg (G). Genistein was given s.c. 5 min after recirculation and PHTPP was given 30 min before carotid occlusion.

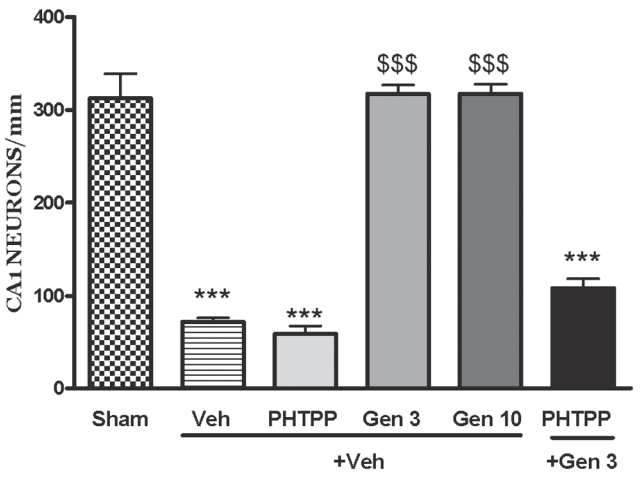

Fig. 6. Effect of genistein (3 and 10 mg/kg) and PHTPP (10 mg/kg) alone or with genistein 3 mg/kg on neuronal counts 7 days after reperfusion in the CA_1 region of the hippocampus of sham-operated or ischemic gerbils. Genistein was given s.c. 5 min after recirculation and PHTPP was given s.c. 30 min before carotid occlusion. Each column represents the mean ± S.E.M. of five hippocampal sections from the same coronal plane for each animal. N = 5 for each group. $^{***}P < 0.001$, compared with sham-operated animals; $^{\$\$\$}P < 0.001$, compared with the vehicle (one-way ANOVA, followed by Tukey's test).

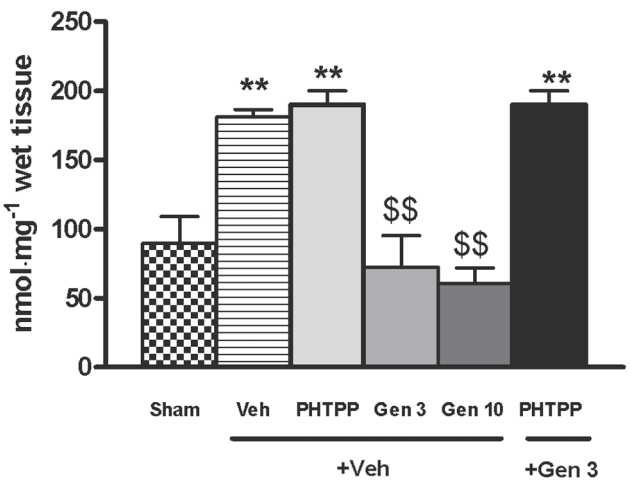

Fig. 7. Genistein reduced malondialdehyde (MDA) content, a marker of oxidative stress due to lipid peroxidation, on day 7. Data are expressed as the mean ± S.E.M. of 5 animals for each group. $^{**}P < 0.01$ vs. sham, $^{\$\$}P < 0.01$ vs. veh (one-way ANOVA followed by Tukey's test).

jury in focal cerebral ischemia in the mouse (18) and global cerebral ischemia in rats (19) and gerbils (38). The neuroprotection was quantified in terms of complete recovery of total spectral power, spontaneous motor activity, memory function, and hippocampal CA_1 neuronal density. The neuroprotectant effect of genistein appeared due to its anti-oxidant properties, as shown by the reduction of oxidative stress in terms of decrease in MDA overproduction in the brain. Oxygen free radical–induced lipid peroxidation has been strongly suggested to play an important role in the pathogenesis of delayed neuronal damage after global ischemia (39, 40). Severe interference with cerebral blood flow has a pronounced effect on electrical activity, which is reflected in EEG flattening. It has been reported that hippocampal injury is not observed until 3 – 7 days after insult in the gerbil global ischemic model (41); this prompted us to perform a histological evaluation on day 7 after ischemia. The decrease in EEG spectral power, observed 7 days after ischemia, has been related to severe neuronal damage (delayed death), since the cells are replaced by astrocytes in the hippocampal CA_1 subfield (42). Global cerebral ischemia, even for a short period of time, results in selective neurodegeneration in vulnerable brain regions such as CA_1 region of the hippocampus. Particularly, pyramidal neurons of the CA_1 field are among the most vulnerable cells to ischemic–reperfusion injury. Severe loss of CA_1 hippocampal neurons have been shown to occur after transient global cerebral ischemia as a consequence of immediate, maturational, and delayed neuronal death (42 – 44). Genistein, at both dosages, fully protected against ischemia-induced EEG flattening and CA_1 neuronal death, indicating that the compound may work against the cascade of pathological events that lead to neuronal death. This finding agrees with the only available data about the protective effect on EEG flattening induced by 30-min carotid occlusion in rats by estradiol given chronically for 2 weeks before injury (45). Accordingly, chronic or acute 17β-estradiol treatment reduced damage in some injury paradigms in rats and gerbils of both sexes (46).

The protective effect of genistein confirms previous findings where 2-day treatment of the phytoestrogen was associated with significant cardioprotective effects in ovariectomized rats (47). In addition, genistein has also been proposed as a neuroprotectant against neurodegenerative diseases such as Parkinson's (48) and Alzheimer's (49) diseases and for neuropathic pain (35). Other phytoestrogens such as daidzein and the daidzein metabolite equol protected embryonic rat primary cortical neurons from ischemic-like injury in vitro at doses typical of circulating concentrations in human populations (14). Daidzein administration in vivo reduced ischemia/reperfusion-induced myocardial damage via inhibition of NF-κB activation (50).

The survival of CA_1 neurons obtained 7 days after ischemia with the soy isoflavone may account for the protection against EEG flattening in genistein-treated gerbils.

We observed the restoration of ischemia-induced hyperactivity 1 day after ischemia/reperfusion in gerbils because ischemia-induced hyperactivity is a reliable sign of brain neurotransmission disturbance and a useful behavioural test for the neuroprotective evaluation of different pharmacological treatments (51, 52). Genistein (3 – 10 mg/kg) significantly reduced hyperactivity 1 day after ischemia/reperfusion in this study. Ischemia-induced hyperactivity has been correlated with hippocampal neuronal damage and reduction in the animal's ability to habituate or to form spatial maps (40, 53).

Ischemic gerbils were protected against memory deficit in terms of innate and spatial memory. Nesting behaviour proved to be a reliable marker of hippocampal ischemic damage, as previously reported (54). Ischemic gerbils suffer deficits which result in delays in habituation and spatial mapping upon exposure to a novel testing environment. The present study clearly demonstrates that genistein fully recovered nest-building behavior, which was dramatically reduced following ischemic damage.

Some evidence points to the hippocampus as a critical brain structure involved in spatial memory loss (55). Moreover, the hippocampus is selectively vulnerable to ischemic stroke and principally involved in spatial learning (56). For the Mongolian gerbil, being a desert animal, the object recognition test appears to be a suitable test for estimating spatial memory. The object recognition test provides a couple of advantages; for example, it does not require a long training period and does not induce a high level of arousal or stress. Moreover, it is not based on the usual positive and negative reinforcements such as food and electric shock, which can interfere with drug effects and also with memory performance. Also in this case genistein fully reversed the inability of ischemic gerbils to recognize the novel object. In the present study, we found that 10 min of transient global cerebral ischemia induced selective neuronal damage in the CA_1 region at 7 days of reperfusion. Treatment with genistein markedly reduced the degree of neuronal cell death, suggesting that it provided neuroprotection against cerebral injury in the gerbil. These results were consistent with those reported in both in vitro and in vivo mouse models of stroke (19, 38) in which treatment with genistein (15 mg/kg, i.p.) significantly attenuated neuronal death.

Genistein also decreased MDA overproduction in the brain, evaluated 7 days after recirculation. These results suggest that genistein protects neurons from transient

global cerebral injury in gerbil hippocampus by attenuating oxidative stress. The mechanism, however, by which genistein was neuroprotective against delayed neuronal death has not been fully elucidated. Delayed neuronal death in the CA_1 region of the hippocampus following transient cerebral ischemia has been widely attributed to an intracellular Ca^{2+} overload (57), free radical–related damage (58), and glutamate-receptor–mediated neurotoxicity (59). Oxidative stress predominates in the pathophysiology of ischemic/reperfusion injury and exerts its deleterious effects by oxidizing various cellular components. Peroxidation of lipid bi-layer, a marker of oxidative stress, can be estimated by measuring the MDA levels. Consistent with the report that MDA levels in the brain tissue may be an indicator of lipid peroxidation (60), we have observed significant increase in MDA content in ischemic gerbils. MDA increased levels were significantly reduced by genistein treatment after cerebral ischemic injury.

The protective effect of genistein appears receptor-mediated since its beneficial effect in the different behavioral, biochemical, and histological parameters was prevented by pre-treatment with PHTPP, the selective ERβ ER antagonist. This finding confirms the mild-selective agonist property of genistein for ERβ with more than 7-fold higher binding affinity for ERβ than ERα (22), thus avoiding unwanted severe ERα agonist side effects such as cancer promotion. An ER-mediated neuroprotection in a model of global cerebral ischemia has been shown in female rats pre-treated with the ERβ agonist diarylpropionitrile but not with the ERα agonist propylpyrazole triol (61) and in vitro in a model of oxygen glucose deprivation (62).

Both ERs are expressed in the rodent hippocampus but ERβ is more prevalent regulating hippocampal synaptic plasticity and improving hippocampus-dependent cognition (63). Increased ERβ immunoreactivity in the post-ischemic monkey hippocampus has also been found (64).

It is known that following cerebral ischemia neuronal survival depends not only by neurons but also by glial cells, vascular smooth muscle, and endothelial cells (65). Neurons, microglia, astrocytes, and immunocells express ERβ (66 – 69). The antioxidant effect of genistein may be due its ability to up-regulate antioxidant genes (70, 71). In line with our results, other recent studies have established that the induction of antioxidant detoxification genes is mediated by the direct binding of phytoestrogens to ERβ, with the subsequent gene transcription being mediated by binding to antioxidant or electrophilic response elements (72). Finally, ERβ has substantial effects on mitochondrial function, particularly during insults where it can affect transcription of critical mitochondrial genes (73). In this context, the observed antioxidant activity of genistein could slow neural tissue ischemic damage or even help its repair.

Genistein is known to be a specific inhibitor of tyrosine kinase (74) and previous findings demonstrated an increase in tyrosine kinase activity following transient ischemia in the gerbil brain (75) mainly in the hippocampus. Such increase was seen within 1 min after the initiation of reperfusion. In this regard, genistein microinjected into hippocampus has been found to inhibit, through the inhibition of tyrosine kinase, ischemia-induced delayed neuronal death only when given 30 or 0 min before carotid occlusion in gerbils, while it had a little effect when given 5 – 60 min after ischemia. This discrepancy with our findings where genistein was active when given 5 min after reperfusion could be ascribed to the different route of administration and dosage. In any case, since the tyrosine kinase may be an initiating factor in the pathway mediating the ischemic cell death (38), the protective effect of genistein shown in our experiments could be independent of its activity against tyrosine kinase.

In conclusion, the present study demonstrates the protective effect of genistein, when acutely given after 10-min carotid occlusion in gerbils, against neuronal death. This effect appears mediated through the activation of ERβ even if a variety of other mechanisms cannot be excluded. These findings provide further grounds for the growing interest concerning the true potential of phytoestrogens as compounds to beneficially affect brain injury without having the disadvantage of estrogens (76). Further studies are needed to verify if the beneficial effects can be seen after prolonged injection intervals.

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