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Genistein ameliorates learning and memory deficits in amyloid $\beta_{(1-40)}$ rat model of Alzheimer's disease

Running title: Genistein and learning and memory deficits

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Abstract

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder characterized by increased β -amyloid ($A\beta$) deposition and neuronal dysfunction leading to impaired learning and recall. Ageing, heredity, and induced oxidative stress are among proposed risk factors. The increased frequency of the disease in women also suggests a role for estrogen in development of AD. In the present study, effects of the phytoestrogen genistein (10 mg/kg) on learning and memory impairments was assessed in intrahippocampal $A\beta_{(1-40)}$ -injected rats. The estrogen receptor antagonist fulvestrant was injected intracerebroventricularly in a group of $A\beta$ -lesioned rats. The $A\beta$ -injected animals exhibited the following: lower spontaneous alternation score in Y-maze tasks, impaired retention and recall capability in the passive avoidance test, and fewer correct choices and more errors in the RAM task. Genistein, but not genistein and fulvestrant, significantly improved most of these parameters. Measurements of oxidative stress markers in hippocampal tissue of $A\beta$ -injected rats showed an elevation of malondialdehyde (MDA) and nitrite content, and a reduction of superoxide dismutase (SOD) activity. Genistein significantly attenuated the increased MDA content but did not affect the nitrite content or SOD activity. These results indicate that genistein pretreatment ameliorates $A\beta$ -induced impairment of short-term spatial memory in rats through an estrogenic pathway and by inducing attenuation of oxidative stress.

Keywords: Alzheimer's disease; Beta-Amyloid; Genistein; Learning and memory

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder that gradually impairs memory and the ability to learn, make judgments, communicate with others, and carry out daily activities (reviewed by (Stuchbury & Munch, 2005). It has been estimated that about 5% of the population older than 65 years is affected by AD (reviewed by(Shah et al., 2008). There is also evidence that the number of dementia cases in Europe will reach almost 6 million in 2010 and around 14 million in 2050 (Mura, Dartigues, & Berr, 2010), and 80% of those individuals may suffer from AD (Jellinger & Attems, 2010). Furthermore, a report from the United States has predicted that the annual incidence of the disease will increase from 337,000 cases in 1995 to 959,000 in 2050 (Hebert, Beckett, Scherr, & Evans, 2001). This projection certainly indicates the magnitude of the problem in terms of the high number of suffering patients, the pressure on their relatives, and the negative socioeconomic outcomes of AD.

In the course of the disease, short-term memory is affected first due to neuronal dysfunction and degeneration in the hippocampus and amygdala. Neuropathological hallmarks of AD include deposition of β -amyloid ($A\beta$)-containing senile plaques and the presence of intracellular neurofibrillary tangles in hippocampal and cerebral cortical regions (reviewed by (Munch et al., 1998; Retz, Gsell, Munch, Rosler, & Riederer, 1998). The pathogenic mechanisms underlying AD include impaired cholinergic function, increased oxidative stress, induction of the amyloid cascade (i.e., $A\beta$ deposition and plaque formation), expression of inflammatory mediators, deficiencies in steroid hormones, and appearance of glutamate-mediated excitotoxicity (reviewed by (Shah et al., 2008). Of all these, the amyloid cascade hypothesis suggesting a pivotal role for $A\beta$ in the pathogenesis of AD is the mechanism that is accepted by most investigators in this field (reviewed by (Klafki, Staufenbiel, Kornhuber, & Wiltfang, 2006). As mentioned, increased oxidative stress due to lipid peroxidation, protein oxidation, and the formation of hydrogen peroxide may also be involved in $A\beta$ -induced neurotoxicity (reviewed by (Butterfield & Lauderback, 2002). Indeed, in studies based on that assumption, it was found that antioxidant therapy prevented the learning and memory deficits induced by $A\beta$ in rats (Yamada et al., 1999), and it also delayed the clinical progression of the disease in humans (Sano et al., 1997). Anti-

inflammatory agents and hormone therapy are examples of other approaches for treating or slowing the progression of the disease. At present, symptomatic treatments with acetylcholine esterase inhibitors in addition to treatment with the NMDA receptor antagonist memantine have been approved for patients with mild to moderate forms of AD. Nonetheless, as Gongadze and colleagues (2008) have mentioned, there is still an enormous need to develop novel therapeutic strategies that target the underlying pathogenic mechanisms in AD. Without progress in establishing new strategies to prevent or delay the onset of the disease, the population that is affected will increase significantly during next decades.

In the search for new drugs to treat age-related neurodegenerative diseases such as AD, attention has been focused to some extent on the potential neuroprotective effect of flavonoids (Bastianetto, Yao, Papadopoulos, & Quirion, 2006). Soy isoflavones, which are referred to as phytoestrogens and include genistein, can bind to estrogen receptors (ERs) and affect estrogen-mediated processes (Molteni, Brizio-Molteni, & Persky, 1995). Pan and colleagues (2000) showed that oral administration of soy phytoestrogens could improve working memory in ovariectomized retired breeder rats. Significant improvements in short-term and long-term memory have also been observed in human subjects eating a high-soy diet for 10 weeks (File et al., 2001). In addition, genistein has been found to have a neuroprotective effect on cortical cell lines (Sonee, Sum, Wang, & Mukherjee, 2004), on dopaminergic neurons in a mouse model of Parkinson's disease (Liu, Chen, Xie, & Wong, 2008), and in a 6-hydroxydopamine hemiparkinsonian rat model (Baluchnejadmojarad, Roghani, Nadoushan, & Bagheri, 2009). Estrogen also has a neuroprotective effect, as shown by several *in vivo* and *in vitro* experiments. Although estrogen is beneficial to patients with AD, it has limited clinical application due to its proliferative and oncogenic effects on non-neuronal cells (Zeng, Chen, & Zhao, 2004). Accordingly, genistein may prove to be an alternative to estrogen in the treatment of AD (Bang et al., 2004). Also of interest, soy isoflavones display protective action against several chronic diseases and against disorders associated with postmenopausal estrogen deficiency (Clarkson, Anthony, Williams, Honore, & Cline, 1998). Recent studies have shown that, like estradiol, genistein can prevent AD-associated inflammation (Valles et al., 2010), rescue neurons from A β -induced cell death (Valles et al., 2008), and exert an anti-apoptotic effect on cultured A β -treated cortical neurons (Yu et al., 2009). Genistein is also capable of scavenging free radicals (Ho, Li,

Zhao, & Qian, 2003; Ruiz-Larrea et al., 1997). In addition, genistein protects neurons from transient global cerebral ischemia-reperfusion injury in rat hippocampus by attenuating oxidative stress, lipid peroxidation, and apoptotic cell death (Liang et al., 2008). Based on these findings, we examined the beneficial effect of genistein and the involvement of estrogen receptors and oxidative stress in relation to learning and memory deficits in an intrahippocampal A β -injected rat model of AD.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (Pasteur's Institute, Tehran), weighing 250–300 g at the start of the experiment were housed three to four per cage in a temperature-controlled colony room under light/dark cycle. The animals were given free access to water and kept at 80–85% of their free feeding body weight throughout the experiment. All behavioral experiments were carried out between 10 a.m. and 4 p.m. This study was conducted in accordance with the policies stipulated in the Guide for the Care and Use of Laboratory Animals (NIH) and by the Research Council of Iran University of Medical Sciences (Tehran, Iran).

2.2. Experimental procedure

Rats ($n = 37$) were randomly allocated to the following groups: (1) sham operation ($n = 6$); (2) genistein treatment, sham operation ($n = 8$); (3) A β injection (A-beta; $n = 8$); (4) A β injection, genistein treatment ($n = 8$); (5) A β injection and genistein treatment, followed by administration of fulvestrant as an estrogen receptor antagonist ($n = 7$). For stereotaxic surgery, rats were anesthetized with a combination of ketamine (100 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) and then placed in a Stoelting stereotaxic apparatus (incisor bar -3.3 mm, ear bars positioned symmetrically). The scalp was cleaned with iodine solution and incised on the midline, and a burr hole was drilled through the skull and A β_{1-40} was injected at coordinates of -3.5 mm posterior to bregma, 2 mm lateral to sagittal suture, and 2.8 mm below dura, according to the stereotaxic atlas (Paxinos & Watson, 1986). Genistein (Sigma Chemicals, USA) was dissolved in Cremophor and administered orally by gavage at a dose of 10 mg/kg body weight one hour before surgery. The dosage was chosen according to the results of our pilot study and an earlier investigation (Baluchnejadmojarad et al., 2009). Animals in the A β group were bilaterally injected in the dorsal hippocampus with 4 μ l of a solution containing A β_{1-40} (2 nmol/4 μ l; Sigma Chemicals, USA, product no. 79793). The amount of A β (0.5 nM/ μ l dissolved in 0.9% normal saline; pH = 8.0) was chosen based on our earlier experiment, and the solution was prepared according to a previously described protocol (Miguel-Hidalgo, Alvarez, Cacabelos, & Quack, 2002) and then

immediately stored at -70°C until used. Sham-operated rats received 4 μl of 0.9% normal saline instead of $\text{A}\beta$ solution. The ER antagonist fulvestrant (Sigma Chemicals, USA) was injected i.c.v. at a dose of 10 $\mu\text{g}/\text{rat}$ (5 μl) at coordinates of -0.8 mm posterior to bregma, 1.4 mm lateral to bregma, and 4 mm below dura; this was done using a Hamilton microsyringe 30 min before $\text{A}\beta$ injection. Fulvestrant was dissolved in dimethyl sulfoxide (DMSO) and diluted to the required volume with artificial CSF (ACSF) containing the following: 120 mM NaCl, 3 mM KCl, 1.15 mM CaCl_2 , 0.8 mM MgCl_2 , 27 mM NaHCO_3 , and 0.33 mM NaH_2PO_4 ; pH adjusted to 7.2 (Merck Chemical, Germany). Post-operatively, the rats were given special care until spontaneous feeding was restored. Behavioral tests were conducted two weeks after the surgery and were evaluated blind to the treatments by the observer.

2.3. Y-maze task

Spatial recognition memory was assessed by recording spontaneous alternation behavior in a single-session Y-maze on the 14th day post-surgery, as described elsewhere (Rasoolijazi, Joghataie, Roghani, & Nobakht, 2007). The maze was made of black Plexiglas. Each arm was 40 cm long, 30 cm high and 15 cm wide. The arms converged in an equilateral triangular central area that was 15 cm at its longest axis. The procedure was as follows: each rat, naive to the maze, was placed at the end of one arm and was allowed to move freely through the maze during an 8-min session. The series of arm entries were recorded visually. Entry was considered to be complete when the base of the animal's tail was entirely within the arm. Alternation was defined as successive entries into the three arms on overlapping triplet sets. The maximum number of possible spontaneous alternations was determined as the total number of arms entered minus 2, and the percentage was calculated as the ratio of actual to possible alternations $\times 100$.

2.4. Single trial passive avoidance test

The apparatus (40 cm long $\times$ 20 cm wide $\times$ 30 cm high) consisted of an illuminated chamber connected to a dark chamber by a guillotine door. Electric shocks were delivered to the grid floor by an isolated stimulator. On the first and second days of testing, each rat was placed in the apparatus for 15 min to habituate. On the third day, an acquisition trial was performed. Rats were placed individually in the illuminated chamber. After a habituation period (5 min), the guillotine door was lifted, and, after the rat had entered the dark chamber, the door was lowered and an

inescapable scrambled single electric shock (1 mA, 1 s) was delivered. In this trial, the initial latency (IL) of entrance into the dark chamber was recorded, and all rats had ILs greater than 60 s and were included in the study. Twenty-four hours later, each rat was placed in the illuminated chamber for retention trial. The interval between placement in the illuminated chamber and entry into the dark chamber was measured as step-through latency (STL, up to a maximum of 300 s). This test was conducted on 17 to 20 days after surgery.

2.5. RAM task

Spatial learning and memory were tested using a radial maze according to the paradigm described previously (Baluchnejadmojarad & Roghani, 2006). The apparatus consisted of a 50-cm-elevated (above the floor) eight-armed radial maze (RAM) made of black Plexiglas. The maze was placed in a sound-attenuated and dimly lit room. The 60-cm-long, 10-cm-wide, and 15-cm-high arms extended radially from a central octagonal starting platform (35 cm in diameter), and there was a recessed food cup at the end of each arm. In some of the arms, the cup contained a single small food pellet as a reinforcer. A plastic cylinder (30 cm in diameter, 20 cm high) was placed on the central platform, and a rat was placed inside this cylinder 15 s before the test. Following this interval, the rats were allowed to move freely and timing began. The RAM was surrounded by various extra-maze cues; their orientation relative to the maze was kept constant throughout the experiment. The maze was cleaned with diluted ethanol between trials.

Prior to acquisition (i.e., before surgery), the rats were maintained on a restricted feeding schedule designed to keep their body weight at about 85% of the free-feeding level. The rats learned to visit each arm, eat the pellet, and not re-enter the arm that had been visited during the same test. Each entry into each arm with all four paws was scored during a period of 10 min. Behavioral observation was discontinued after 10 min, even if the animal did not finish the task. The number of correct choices or errors was used to assess the performance of the animal in each session. An error was defined as a re-entry into an already visited arm. Rats that made at least seven correct choices in each of three consecutive sessions were used in the subsequent behavioral experiments. Training was performed at 24-h intervals, and rats that fulfilled the above-mentioned criteria within two weeks were included in the study (37 of 45 eligible rats). Retention trials were performed once on the 16th day post-surgery.

2.6. Determination of hippocampal MDA concentration

The rats were anesthetized with ketamine (100 mg/kg) and decapitated. Hippocampi were isolated and blotted dry, and then weighed and prepared as a 5% tissue homogenate in ice-cold 0.9% saline solution. After centrifugation (1000×g, 4 °C, 10 min), the supernatant was aliquoted and stored at –80 °C until assayed. The concentration of malondialdehyde (MDA), used as a marker of lipid peroxidation index, was calculated by measuring thiobarbituric acid reactive substances (TBARS) in the supernatant as described previously (Roghani & Baluchnejadmojarad, 2009). Briefly, trichloroacetic acid and TBARS reagent were added to aliquots of the supernatant, which were subsequently mixed and incubated at 100 °C for 80 min. After cooling on ice, the samples were centrifuged at 1000×g for 10 min, and the absorbance of the supernatant was read at 532 nm. The results of TBARS measurements were expressed as MDA equivalents, using tetraethoxypropane as standard.

2.7. Measurement of hippocampal SOD activity

The supernatant of hippocampal homogenate was obtained as described above. Superoxide dismutase (SOD) activity was measured as previously reported (Roghani & Baluchnejadmojarad, 2009). Briefly, supernatant was incubated with xanthine and xanthine oxidase in potassium phosphate buffer (pH 7.8, 37 °C) for 40 min, and then nitroblue tetrazolium (NBT) was added. Thereafter, blue formazan was monitored spectrophotometrically at 550 nm. The amount of protein that inhibited NBT reduction to 50% maximum was defined as 1 nitrite unit (NU) of SOD activity.

2.8. Assay of hippocampal nitrite concentration

Supernatant nitrite (NO_2^-) content was assayed by the Griess method. The compound NO has a short half-life and is rapidly converted to the stable end products nitrate (NO_3^-) and NO_2^- . In the assay used here, NO_3^- is converted to NO_2^- by cadmium, and this is followed by color development with Griess reagent (sulfanilamide and N-naphthyl ethylenediamine) in acidic medium. The absorbance was determined using a spectrophotometer at 540 nm.

2.9. Protein assay

The protein content of the supernatant was measured by the Bradford method, using bovine serum albumin (Sigma Chemical, St. Louis, MO) as the standard (Bradford, 1976).

2.10. Statistical analysis

All results were expressed as mean $\pm$ S.E.M. The nonparametric Kruskal-Wallis test was used to analyze the behavioral tests, and if a difference was found to be significant, pair-wise comparison was done using the Mann-Whitney U-test. Parametric one-way ANOVA was used to assess the biochemical tests. In all calculations, a difference at $p < 0.05$ was regarded as significant.

3. Results

3.1. Alternation behavior in Y-maze task

Figure 1 illustrates the performance of rats in the Y-maze task, which was studied to assess spatial recognition memory. In this respect, the alternation score was found to be significantly lower for A β -injected rats ($49.5 \pm 5.1\%$) compared to the sham group ($81.1 \pm 5.4\%$) at the end of the study ($p < 0.01$). Moreover, the score was significantly higher for genistein-treated A β -injected rats ($72.6 \pm 6.1\%$) compared to the animals in the A β group ($p < 0.05$). The alternation score was also higher for genistein-treated A β -injected rats that received the estrogen receptor antagonist fulvestrant ($67.4 \pm 6.5\%$) compared to the A β -injected group, but this difference was not significant. In addition, the genistein-treated sham-operated group ($78.4 \pm 5.3\%$) did not differ significantly from the sham-operated group with regard to this parameter.

To avoid a compounding effect of locomotor activity on the performance of the rats in memory evaluation tests, we used the total number of arms entered by rats as an index of such activity. In this respect, although the total number of arm entrances was slightly lower in some groups (data not shown) these differences were not statistically significant when compared with the sham group.

3.2. Passive avoidance test

Figure 2 shows the performance of rats in the passive avoidance paradigm determined as IL and STL. For IL, no significant difference was found between the groups. Regarding STL, the A β -injected rats (22.3 ± 4.2 s; $p < 0.005$), and the A β -injected rats with genistein and fulvestrant treatment (31.2 ± 7.8 s; $p < 0.01$) developed a marked impairment in retention and recall capability compared with the sham-operated (99.7 ± 13.4 s). Genistein treatment did produce a significant improvement in STL (72.4 ± 12.9 s) compared to A β injection ($p < 0.05$), but this difference was significantly abolished in the presence of fulvestrant ($p < 0.05$). Fulvestrant application to genistein-treated A β -injected rats significantly impaired the improved retention and recall capability of genistein-treated A β -injected animals ($p < 0.05$).

Furthermore, the STL values were similar for the sham-operated rats and the sham-operated genistein-treated rats.

3.3. RAM task

Pre-trained A β -injected rats showed a significant deficit in spatial cognition in the eight-arm radial maze task, as indicated by a lower number of correct choices ($p < 0.01$) and a higher number of errors ($p < 0.01$) compared to relevant data for the sham-operated group.

Administration of genistein caused a non-significant increase in the number of correct choices and lowered the number of errors at a ratio of 35.6% and 36.8%, respectively ($p < 0.05$).

Administration of fulvestrant to genistein-treated A β -injected rats did not improve the number of correct choices or errors (Fig. 3).

3.4. Markers of oxidative stress

A β -injected rats exhibited significantly elevated levels of MDA (9.3 ± 0.8 nmol/mg protein; $p < 0.01$) and nitrite (8.8 ± 0.7 nmol/mg protein; $p < 0.005$), and a significant reduction in SOD activity (3.8 ± 0.7 unit/mg protein; $p < 0.005$) in hippocampal tissue (Figures 4–6) compared to the sham-operated group (MDA, 6.1 ± 0.7 nmol/mg protein; nitrite, 5.9 ± 0.6 nmol/mg protein; SOD 7.2 ± 0.6 unit/mg protein). Pretreatment of A β -injected rats with genistein significantly attenuated the increased MDA content (6.7 ± 0.6 ; $p < 0.05$) relative to sham-operated genistein treated rats. However, the levels of nitrite and SOD were not significantly changed by treatment with genistein. Values for these markers were similar for the genistein-treated A β group and the genistein-treated A β group that also received fulvestrant.

4. Discussion

The aim of the present study was to evaluate the beneficial effect of genistein on learning and memory deficits in an intrahippocampal A β -injected rat model of AD, and we also examined the involvement of estrogen receptors and oxidative stress in impairment of cognitive function. The main findings were as follows: (1) compared with the sham group, within 2–3 weeks the A β _{1–40}-injected rats had a lower alternation score in the Y-maze task, impaired retention and recall in the passive avoidance test, and fewer correct choices and more errors in the RAM task; (2) genistein pretreatment significantly improved short-term spatial recognition memory in the Y-maze task and retention and recall aspects of learning and memory in the passive avoidance test; (3) using fulvestrant to block estrogen receptors significantly abolished the beneficial effect of genistein regard to STL in the passive avoidance test; (4) genistein pretreatment attenuated the increased level of MDA caused by A β _{1–40} injection but did not improve the level of nitrite or SOD activity.

In previous studies of rats, it was found that infusion of A β _{1–40} into the cerebral ventricles resulted in impaired learning and memory (Nabeshima & Nitta, 1994; Nitta, Itoh, Hasegawa, & Nabeshima, 1994). In addition, A β _{1–40} infusion decreased choline acetyltransferase activity in the cerebral cortex and hippocampus (Nitta et al., 1994; Yamada, Tanaka, Senzaki, Kameyama, & Nabeshima, 1998) and activated glial cells, seen as increased immunoreactivity for glial fibrillary acidic protein (Nitta, Fukuta, Hasegawa, & Nabeshima, 1997). Furthermore, Itoh and colleagues (1996) observed a marked reduction in nicotine- and/or KCl-induced release of acetylcholine in the hippocampus and cerebral cortex, as well as reduced dopamine release in the striatum following infusion of A β . These data obtained using rats suggest that infusion of A β in the brain can affect different neuronal pathways. Regarding the neurodegenerative effect of A β , some investigators (Miguel-Hidalgo & Cacabelos, 1998; Yamada et al., 1999), have found morphological signs of cell damage after A β injection in the brain. In the current study, we observed impaired learning and memory in rats after bilateral injections of soluble A β _{1–40} into the dorsal hippocampus, which agrees with the results of previous investigations (Nitta et al., 1997; Tanaka et al., 1998). Furthermore, pretreatment of genistein improved, albeit did not prevent, the

development of memory loss in the rats, and this enhancement may be related to the beneficial effect of genistein on some, but not all, of these above mentioned neuronal pathways. Another mechanism that has been related to impaired cognitive function in AD is endogenous estrogen deficiency. Many studies have indicated that estrogen replacement therapy has a beneficial effect on cognitive function. Genistein has a structure similar to that of estrogen and it is a relatively selective estrogen receptor β agonist (An, Tzagarakis-Foster, Scharschmidt, Lomri, & Leitman, 2001). As mentioned, genistein improved the memory of A β -injected rats in our study. However, this positive effect was not observed when we used estrogen receptor antagonist, which suggests that the favorable effect of genistein that we detected may have been partly due to the estrogen-like activity of this isoflavone. In a study of rats, fulvestrant was detected both in the plasma and in brain tissue 2.5 h after subcutaneous administration of this drug, and the maximum concentration in those tissues remained stable for 24 h (Alfinito, Chen, Atherton, Cosmi, & Deecher, 2008). In humans, the peak serum concentration (C_{max}) and area under the curve (AUC) have been found to increase in a dose-dependent manner for up to 28 days after a single i.m. injection of fulvestrant (Robertson & Harrison, 2004).

It has also been shown that A β has the potential to induce oxidative stress, including increased production of hydrogen peroxide and lipid peroxides in neurons (see reviews by (Behl, 1997; Varadarajan, Yatin, Aksenova, & Butterfield, 2000). Oxidative stress plays an important role in the development and progression of AD (Reddy, 2006). A β interacts directly with the mitochondria and induces production of free radicals, mitochondrial dysfunction, and cell death (Reddy, 2006); for these reasons, antioxidants such as α -tocopherol have a protective effect against learning and memory deficits induced by A β (Yamada et al., 1999). The antioxidant quality of genistein might be due to the ability of this compound to decrease oxidant production by mitochondria (Borras, Gambini, Lopez-Grueso, Pallardo, & Vina, 2010). Superoxide dismutase, glutathione peroxidase, and catalase are the three main enzymes involved in cellular protection against damage caused by oxygen-derived free radicals (Crack, Cimdins, Ali, Hertzog, & Iannello, 2006). In the present study, genistein reversed the A β -induced decrease in MDA, but it did not affect nitrite production, nor did it improve SOD activity, suggesting that the beneficial effect of genistein does not occur mainly via its antioxidant capacity. We cannot, however, exclude the possibility that longer pretreatment with genistein might inhibit the induction of

oxidative stress. Notably, in experiments performed by other researchers (King, Broadbent, & Head, 1996), it was found that the plasma level of genistein reached a maximum at 2 h after a single oral dose and declined with a half-life of approximately 9 h. Genistein penetrates the blood-brain barrier (Tsai, 2005) in a dose-dependent manner, and it has been detected in brain tissue (Chang, Churchwell, Delclos, Newbold, & Doerge, 2000), although the amounts found in that tissue are lower than the levels observed in other tissues. The low penetration rate in the blood-brain barrier does not exclude the possibility that even a relatively low concentration of genistein can have a beneficial effect on learning and memory.

In the current study, all behavioral assessments were done on days 14 to 20 post-surgery. The reason for that approach was that, in our preliminary studies, we had not observed any significant changes in learning and memory indices during days 7–13 after A β _{1–40} injection (data not shown), which indicated that injection of A β into the hippocampus causes cumulative cell damage. The single dose of genistein was given to the rats one hour before surgery. Hence, our results illustrate the acute effect of genistein after A β injection and the impact of this compound on behavioral and biochemical factors after a short period of time. Estrogen elicits enhancement of neurite growth and differentiation in the developing brain (Toran-Allerand, 1996a, 1996b) and it regulates the level of growth factors such as NGF and BDNF in the cerebral cortex. Estrogen may also induced activation of ERKs (Singh, Setalo, Guan, Warren, & Toran-Allerand, 1999) which have a key position in intracellular signaling pathways mediating growth factor effects. Activation of ERKs involves their phosphorylation (Robbins et al., 1993), and translocation to the nucleus to regulate transcription factors and induce early and late gene responses (Marshall, 1995). Among different brain regions, hippocampus and some other brain areas have the highest level of ERKs (Ortiz et al., 1995). Borrás and colleagues (2006) showed that genistein acts via interaction with estrogen receptors leading to activation of MAP kinases and nuclear translocation of NF κ B, and overexpression of manganese superoxide dismutase (MnSOD) which acts as antioxidant. Hence, the positive effect of genistein seems to be due to activation of other mechanisms which require time to be activated. In this way, these positive effects may last for a while even after clearance of genistein in the brain tissue. The question that arises is whether the behavioral alterations induced by A β are due solely to physiological dysfunction of neurons, or if

morphological abnormalities are also involved. We are presently addressing this question in our laboratory.

As mentioned, A β can lead to cholinergic dysfunction and cognitive impairment (reviewed by (Kar & Quirion, 2004). Choline acetyltransferase and acetylcholinesterase are important for maintaining a stable level of acetylcholine in the brain. Flavonoids like genistein can moderately inhibit acetylcholinesterase (Orhan, Kartal, Tosun, & Sener, 2007) and in this way delay degradation of the neurotransmitter. Further research is needed to determine possible effects of genistein and fulvestrant on the cholinergic pathway in the rat model of AD used in our study. Genistein also has an anti-angiogenesis effect, and it can inhibit the activity of tyrosine kinase (Akiyama et al., 1987; Siow & Mann, 2010), which is expressed extensively in hippocampus and is involved in the induction of long-term potentiation (LTP). This means that genistein can impair learning and memory by inhibiting LTP, but to have such a harmful impact, it must be present at a very high concentration *in vivo* (Kim et al., 2009). To avoid this negative effect of genistein, we used only a single dose of the compound. We also found that A β -injected genistein-treated rats showed the same level of learning and memory as sham-operated rats in the Y-maze and passive avoidance tests, which suggests that genistein does not inhibit the induction of LTP.

In conclusion, our results suggest that genistein pretreatment via an estrogenic pathway and by inducing attenuation of oxidative stress prevents A $\beta_{(1-40)}$ -induced impairment of short-term spatial recognition memory in a Y-maze and learning and memory in the passive avoidance test.

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Figure Legends

Fig. 1. Alternation behavior displayed in the Y-maze by rats given vehicle (sham; n= 6), sham + genistein (n = 8), hippocampal A β injection (bilateral; 2 nmol/4 μ l; n = 8), or genistein pretreatment (single dose 10 mg/kg; n= 8) followed by fulvestrant (estrogen receptor antagonist; i.c.v. 10 μ g/5 μ l; n = 7). Values are means $\pm$ S.E.M.; * $p < 0.01$ (vs. sham), # $p < 0.05$ (vs. A β).

Fig. 2. Initial latency (IL) and step-through latency (STL) recorded in a single-trial passive avoidance test for rats given vehicle (sham; n = 6), sham + genistein (n = 8), hippocampal A β injection (bilateral; 2 nmol/4 μ l; n = 8), or genistein pretreatment (single dose 10 mg/kg; n = 8) followed by fulvestrant (estrogen receptor antagonist; i.c.v. 10 μ g/5 μ l; n = 7). Values are means $\pm$ S.E.M.; * $p < 0.01$, ** $p < 0.005$ (vs. sham), # $p < 0.05$ (vs. A β), § $p < 0.05$ (vs. A β + genistein).

Fig. 3. The effect of genistein on the spatial cognition deficit induced by A β injection in rats, measured 2 weeks after treatment. Values are means $\pm$ S.E.M. of the number of correct choices or the number of errors. * $p < 0.05$ (vs. A β ; n = 8), ** $p < 0.01$ (vs. sham group) (n = 6-8/each group).

Fig. 4. Malondialdehyde (MDA) concentration in hippocampal homogenate from rats given vehicle; sham, sham + genistein, hippocampal A β injection (bilateral; 2 nmol/4 μ l), or genistein pretreatment (single dose 10 mg/kg) followed by fulvestrant (estrogen receptor antagonist; i.c.v. 10 μ g/5 μ l) (n=6/each group). Values are means $\pm$ S.E.M.; * $p < 0.01$ (vs. sham), # $p < 0.05$ (vs. A β).

Fig. 5. Nitrite content in hippocampal homogenate from rats given vehicle; sham, sham + genistein, hippocampal A β injection (bilateral; 2 nmol/4 μ l), or genistein pretreatment (single dose 10 mg/kg) followed by fulvestrant (estrogen receptor antagonist; i.c.v. 10 μ g/5 μ l) (n=6/each group). Values are means $\pm$ S.E.M., * $p < 0.005$ (vs. sham).

Fig. 6. Superoxide dismutase (SOD) activity in hippocampal homogenate from rats given vehicle; sham, sham + Genistein, hippocampal A β injection (bilateral; 2 nmol/4 μ l), or genistein pretreatment (single dose 10 mg/kg) followed by fulvestrant (estrogen receptor antagonist; i.c.v. 10 μ g/5 μ l) (n=6/each group). Values are means $\pm$ S.E.M., * p < 0.005 (vs. sham).

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References

- Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., et al. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. *J Biol Chem*, 262(12), 5592-5595.
- Alfinito, P. D., Chen, X., Atherton, J., Cosmi, S., & Deecher, D. C. (2008). ICI 182,780 penetrates brain and hypothalamic tissue and has functional effects in the brain after systemic dosing. *Endocrinology*, 149(10), 5219-5226.
- An, J., Tzagarakis-Foster, C., Scharschmidt, T. C., Lomri, N., & Leitman, D. C. (2001). Estrogen receptor beta-selective transcriptional activity and recruitment of coregulators by phytoestrogens. *J Biol Chem*, 276(21), 17808-17814.
- Baluchnejadmojarad, T., & Roghani, M. (2006). Effect of naringenin on intracerebroventricular streptozotocin-induced cognitive deficits in rat: a behavioral analysis. *Pharmacology*, 78(4), 193-197.
- Baluchnejadmojarad, T., Roghani, M., Nadoushan, M. R., & Bagheri, M. (2009). Neuroprotective effect of genistein in 6-hydroxydopamine Hemi-parkinsonian rat model. *Phytother Res*, 23(1), 132-135.
- Bang, O. Y., Hong, H. S., Kim, D. H., Kim, H., Boo, J. H., Huh, K., et al. (2004). Neuroprotective effect of genistein against beta amyloid-induced neurotoxicity. *Neurobiol Dis*, 16(1), 21-28.
- Bastianetto, S., Yao, Z. X., Papadopoulos, V., & Quirion, R. (2006). Neuroprotective effects of green and black teas and their catechin gallate esters against beta-amyloid-induced toxicity. *Eur J Neurosci*, 23(1), 55-64.

- Behl, C. (1997). Amyloid beta-protein toxicity and oxidative stress in Alzheimer's disease. *Cell Tissue Res*, 290(3), 471-480.
- Borrás, C., Gambini, J., Gómez-Cabrera. M.C., Sastre, J., Pallardó, F.V., Mann, G.E. & Viña, J. (2006). Genistein, a soy isoflavone, up-regulates expression of antioxidant genes: involvement of estrogen receptors, ERK1/2, and NFkappaB. *The FASEB journal*, 12, 2136-2138.
- Borras, C., Gambini, J., Lopez-Grueso, R., Pallardo, F. V., & Vina, J. (2010). Direct antioxidant and protective effect of estradiol on isolated mitochondria. *Biochim Biophys Acta*, 1802(1), 205-211.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 72, 248-254.
- Butterfield, D. A., & Lauderback, C. M. (2002). Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid beta-peptide-associated free radical oxidative stress. *Free Radic Biol Med*, 32(11), 1050-1060.
- Chang, H. C., Churchwell, M. I., Delclos, K. B., Newbold, R. R., & Doerge, D. R. (2000). Mass spectrometric determination of Genistein tissue distribution in diet-exposed Sprague-Dawley rats. *J Nutr*, 130(8), 1963-1970.
- Clarkson, T. B., Anthony, M. S., Williams, J. K., Honore, E. K., & Cline, J. M. (1998). The potential of soybean phytoestrogens for postmenopausal hormone replacement therapy. *Proc Soc Exp Biol Med*, 217(3), 365-368.

- Crack, P. J., Cimdins, K., Ali, U., Hertzog, P. J., & Iannello, R. C. (2006). Lack of glutathione peroxidase-1 exacerbates Abeta-mediated neurotoxicity in cortical neurons. *J Neural Transm*, 113(5), 645-657.
- File, S. E., Jarrett, N., Fluck, E., Duffy, R., Casey, K., & Wiseman, H. (2001). Eating soya improves human memory. *Psychopharmacology (Berl)*, 157(4), 430-436.
- Gongadze, N., Antelava, N., Kezeli, T., Okudjava, M. & Pachkoria, K. (2008). The mechanisms of neurodegenerative processes and current pharmacotherapy of Alzheimer's disease. *Georgian Medical News*, 155, 44-48.
- Hebert, L. E., Beckett, L. A., Scherr, P. A., & Evans, D. A. (2001). Annual incidence of Alzheimer disease in the United States projected to the years 2000 through 2050. *Alzheimer Dis Assoc Disord*, 15(4), 169-173.
- Ho, K. P., Li, L., Zhao, L., & Qian, Z. M. (2003). Genistein protects primary cortical neurons from iron-induced lipid peroxidation. *Mol Cell Biochem*, 247(1-2), 219-222.
- Itoh, A., Nitta, A., Nadai, M., Nishimura, K., Hirose, M., Hasegawa, T., & Nabeshima T. (1996). Dysfunction of cholinergic and dopaminergic neuronal systems in β -amyloid protein-infused rats. *Journal of Neurochemistry*, 66, 1113-1117.
- Jellinger, K. A., & Attems, J. (2010). Prevalence of dementia disorders in the oldest-old: an autopsy study. *Acta Neuropathol*, 119(4), 421-433.
- Kar, S., & Quirion, R. (2004). Amyloid beta peptides and central cholinergic neurons: functional interrelationship and relevance to Alzheimer's disease pathology. *Prog Brain Res*, 145, 261-274.

- Kim, D. J., Seok, S. H., Baek, M. W., Lee, H. Y., Na, Y. R., Park, S. H., et al. (2009). Developmental toxicity and brain aromatase induction by high genistein concentrations in zebrafish embryos. *Toxicol Mech Methods*, 19(3), 251-256.
- King, R. A., Broadbent, J. L., & Head, R. J. (1996). Absorption and excretion of the soy isoflavone genistein in rats. *J Nutr*, 126(1), 176-182.
- Klafki, H. W., Staufenbiel, M., Kornhuber, J., & Wiltfang, J. (2006). Therapeutic approaches to Alzheimer's disease. *Brain*, 129(Pt 11), 2840-2855.
- Liang, H. W., Qiu, S. F., Shen, J., Sun, L. N., Wang, J. Y., Bruce, I. C., et al. (2008). Genistein attenuates oxidative stress and neuronal damage following transient global cerebral ischemia in rat hippocampus. *Neurosci Lett*, 438(1), 116-120.
- Liu, L. X., Chen, W. F., Xie, J. X., & Wong, M. S. (2008). Neuroprotective effects of genistein on dopaminergic neurons in the mice model of Parkinson's disease. *Neurosci Res*, 60(2), 156-161.
- Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, 80(2), 179-185.
- Miguel-Hidalgo, J. J., Alvarez, X. A., Cacabelos, R., & Quack, G. (2002). Neuroprotection by memantine against neurodegeneration induced by beta-amyloid(1-40). *Brain Res*, 958(1), 210-221.
- Miguel-Hidalgo, J. J., & Cacabelos, R. (1998). Beta-amyloid(1-40)-induced neurodegeneration in the rat hippocampal neurons of the CA1 subfield. *Acta Neuropathol*, 95(5), 455-465.
- Molteni, A., Brizio-Molteni, L., & Persky, V. (1995). In vitro hormonal effects of soybean isoflavones. *J Nutr*, 125(3 Suppl), 751S-756S.

- Munch, G., Schinzel, R., Loske, C., Wong, A., Durany, N., Li, J. J., et al. (1998). Alzheimer's disease--synergistic effects of glucose deficit, oxidative stress and advanced glycation endproducts. *J Neural Transm*, 105(4-5), 439-461.
- Mura, T., Dartigues, J. F., & Berr, C. (2010). How many dementia cases in France and Europe? Alternative projections and scenarios 2010-2050. *Eur J Neurol*, 17(2), 252-259.
- Nabeshima, T., & Nitta, A. (1994). Memory impairment and neuronal dysfunction induced by beta-amyloid protein in rats. *Tohoku J Exp Med*, 174(3), 241-249.
- Nitta, A., Fukuta, T., Hasegawa, T., & Nabeshima, T. (1997). Continuous infusion of beta-amyloid protein into the rat cerebral ventricle induces learning impairment and neuronal and morphological degeneration. *Jpn J Pharmacol*, 73(1), 51-57.
- Nitta, A., Itoh, A., Hasegawa, T., & Nabeshima, T. (1994). beta-Amyloid protein-induced Alzheimer's disease animal model. *Neurosci Lett*, 170(1), 63-66.
- Orhan, I., Kartal, M., Tosun, F., & Sener, B. (2007). Screening of various phenolic acids and flavonoid derivatives for their anticholinesterase potential. *Z Naturforsch C*, 62(11-12), 829-832.
- Ortiz, J., Harris, H. W., Guitart, X., Terwilliger, R. Z., Haycock, J. W., & Nestler, E. J. (1995). Extracellular signal-regulated protein kinases (ERKs) and ERK kinase (MEK) in brain: regional distribution and regulation by chronic morphine. *J Neurosci*, 15(2), 1285-1297.
- Paxinos, G., & Watson, C. (1986). *The rat brain in stereotaxic coordinates*. (2nd edn.): Academic Press, New York.
- Rasoolijazi, H., Joghataie, M. T., Roghani, M., & Nobakht, M. (2007). The beneficial effect of (-)-epigallocatechin-3-gallate in an experimental model of Alzheimer's disease in rat: a behavioral analysis. *Iran Biomed J*, 11(4), 237-243.

- Reddy, P. H. (2006). Amyloid precursor protein-mediated free radicals and oxidative damage: implications for the development and progression of Alzheimer's disease. *J Neurochem*, 96(1), 1-13.
- Retz, W., Gsell, W., Munch, G., Rosler, M., & Riederer, P. (1998). Free radicals in Alzheimer's disease. *J Neural Transm Suppl*, 54, 221-236.
- Robbins, D. J., Zhen, E., Owaki, H., Vanderbilt, C. A., Ebert, D., Geppert, T. D., et al. (1993). Regulation and properties of extracellular signal-regulated protein kinases 1 and 2 in vitro. *J Biol Chem*, 268(7), 5097-5106.
- Robertson, J. F., & Harrison, M. (2004). Fulvestrant: pharmacokinetics and pharmacology. *Br J Cancer*, 90 Suppl 1, S7-10.
- Roghani, M., & Baluchnejadmojarad, T. (2009). Chronic epigallocatechin-gallate improves aortic reactivity of diabetic rats: underlying mechanisms. *Vascul Pharmacol*, 51(2-3), 84-89.
- Ruiz-Larrea, M. B., Mohan, A. R., Paganga, G., Miller, N. J., Bolwell, G. P., & Rice-Evans, C. A. (1997). Antioxidant activity of phytoestrogenic isoflavones. *Free Radic Res*, 26(1), 63-70.
- Sano, M., Ernesto, C., Thomas, R. G., Klauber, M. R., Schafer, K., Grundman, M., et al. (1997). A controlled trial of selegiline, alpha-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's Disease Cooperative Study. *N Engl J Med*, 336(17), 1216-1222.
- Shah, R. S., Lee, H. G., Xiongwei, Z., Perry, G., Smith, M. A., & Castellani, R. J. (2008). Current approaches in the treatment of Alzheimer's disease. *Biomed Pharmacother*, 62(4), 199-207.

Singh, M., Setalo, G., Jr., Guan, X., Warren, M., & Toran-Allerand, C. D. (1999). Estrogen-induced activation of mitogen-activated protein kinase in cerebral cortical explants: convergence of estrogen and neurotrophin signaling pathways. *J Neurosci*, 19(4), 1179-1188.

Siow, R. C., & Mann, G. E. (2010). Dietary isoflavones and vascular protection: Activation of cellular antioxidant defenses by SERMs or hormesis? *Mol Aspects Med*.

Sonee, M., Sum, T., Wang, C., & Mukherjee, S. K. (2004). The soy isoflavone, genistein, protects human cortical neuronal cells from oxidative stress. *Neurotoxicology*, 25(5), 885-891.

Stuchbury, G., & Munch, G. (2005). Alzheimer's associated inflammation, potential drug targets and future therapies. *J Neural Transm*, 112(3), 429-453.

Tanaka, T., Yamada, K., Senzaki, K., Narimatsu, H., Nishimura, K., Kameyama, T., et al. (1998). NC-1900, an active fragment analog of arginine vasopressin, improves learning and memory deficits induced by beta-amyloid protein in rats. *Eur J Pharmacol*, 352(2-3), 135-142.

Toran-Allerand, C. D. (1996a). The estrogen/neurotrophin connection during neural development: is co-localization of estrogen receptors with the neurotrophins and their receptors biologically relevant? *Dev Neurosci*, 18(1-2), 36-48.

Toran-Allerand, C. D. (1996b). Mechanisms of estrogen action during neural development: mediation by interactions with the neurotrophins and their receptors? *J Steroid Biochem Mol Biol*, 56(1-6 Spec No), 169-178.

Tsai, T. H. (2005). Concurrent measurement of unbound genistein in the blood, brain and bile of anesthetized rats using microdialysis and its pharmacokinetic application. *J Chromatogr A*, 1073(1-2), 317-322.

Valles, S. L., Borrás, C., Gambini, J., Furriol, J., Ortega, A., Sastre, J., et al. (2008).

Oestradiol or genistein rescues neurons from amyloid beta-induced cell death by inhibiting activation of p38. *Aging Cell*, 7(1), 112-118.

Valles, S. L., Dolz-Gaiton, P., Gambini, J., Borrás, C., Lloret, A., Pallardo, F. V., et al.

(2010). Estradiol or genistein prevent Alzheimer's disease-associated inflammation correlating with an increase PPAR gamma expression in cultured astrocytes. *Brain Res*, 1312, 138-144.

Varadarajan, S., Yatin, S., Aksenova, M., & Butterfield, D. A. (2000). Review: Alzheimer's amyloid beta-peptide-associated free radical oxidative stress and neurotoxicity. *J Struct Biol*, 130(2-3), 184-208.

Yamada, K., Tanaka, T., Han, D., Senzaki, K., Kameyama, T., & Nabeshima, T. (1999).

Protective effects of idebenone and alpha-tocopherol on beta-amyloid-(1-42)-induced learning and memory deficits in rats: implication of oxidative stress in beta-amyloid-induced neurotoxicity in vivo. *Eur J Neurosci*, 11(1), 83-90.

Yamada, K., Tanaka, T., Senzaki, K., Kameyama, T., & Nabeshima, T. (1998).

Propentofylline improves learning and memory deficits in rats induced by beta-amyloid protein-(1-40). *Eur J Pharmacol*, 349(1), 15-22.

Yu, H. L., Li, L., Zhang, X. H., Xiang, L., Zhang, J., Feng, J. F., et al. (2009).

Neuroprotective effects of genistein and folic acid on apoptosis of rat cultured cortical neurons induced by beta-amyloid 31-35. *Br J Nutr*, 102(5), 655-662.

Zeng, H., Chen, Q., & Zhao, B. (2004). Genistein ameliorates beta-amyloid peptide (25-35)-induced hippocampal neuronal apoptosis. *Free Radic Biol Med*, 36(2), 180-188.