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Research Article
Estrogen-like Properties of Quercetin Protect Rat Hippocampal Neurons by Estrogen Receptor Alpha

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Abstract

Background: Early change of cells in Alzheimer's Disease (AD) occurs in hippocampal region. Investigation of hippocampal neuronal cells were provide useful information about this disease. Quercetin is a phytoestrogen, which has similar biological activity as estrogen. Since the mechanism of quercetin in prevention of AD development is still unknown, there is a need to investigate whether, quercetin can regulate hippocampal neuronal cells. **Methodology and Results:** Rat hippocampal neurons were isolated from one day old Sprague Dawley rats and treated with estrogen, quercetin and estrogen receptor antagonists. Cell viability was determined by MTT assay, neurite outgrowth was measured by fluorescent microsope and estrogen receptors were determine by Western blot. Quercetin functions like estrogen to increase hippocampal neuronal cell viability. It can also increase neurite outgrowth. Moreover, the effect of quercetin on hippocampal neuronal cells was mediated by estrogen receptor alpha instead of estrogen receptor beta. Furthermore, quercetin increased estrogen receptor alpha expression, but did not affect estrogen receptor beta expression. **Conclusion:** In conclusion, quercetin like estrogen can enhance hippocampal neuronal cell viability and promote neuritogenesis through estrogen receptor alpha.

Key words: Estrogen, quercetin, hippocampus, Alzheimer's disease, estrogen receptor

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder of older adults and leading to death in about 10 years. In 2010, official death certificates recorded 83,494 deaths from AD, making AD the 6th leading cause of death in the United States and the 5th leading cause of death in Americans aged 65 years or older[1]. Alzheimer's disease has a significant economic impact across the globe as seen with more than $600 billion in total patient care costs. Therefore, treatment and prevention of Alzheimer's disease attracts world-wide attention.

The AD is characterized by two pathological hallmarks, extracellular plaques of amyloid-β (Aβ) peptide aggregates and intracellular neurofibrillary tangles. This disease is believed to result from the loss of synapses and the subsequent loss of neuronal cells in the hippocampus and cortex, as synaptic loss is correlated with early events of AD[2,3]. So far, AD remains incurable, therefore postponing or delaying the occurrence and development of AD is particularly important for improving the quality of patient's life and reducing the family burden.

Estrogen is a sex hormone and can protect neurons enhance neuronal survival, inhibit apoptosis and promote synapse generation through its regulation of neuroendocrine homeostasis[4,5]. The synapses and dendritic learning and memory-related hippocampal neurons are especially sensitive to estrogen levels. Estrogen replacement therapy can delay the onset of AD, reducing the incidence rate by about 50%, however, estrogen replacement also carries the risk of endometrial cancer, ovarian cancer, breast cancer and other cardiovascular and cerebrovascular diseases. Phytoestrogens (PEs) are plant-derived substances, which share a similar chemical structure with endogenous estrogen. Phytoestrogens can improve the neuronal cell survival rate, thus reducing the age-related cognitive decline[6].

Quercetin (Que) belongs to the class of flavonoids and is a type of plant estrogen. Flavnoids induce the expression of synaptic proteins, such as synaptotagmin and the post-synaptic density protein-95 in cultured rat cortical neurons[7]. Quercetin enhances the effect of brain-derived neurotrophic factor, stimulating neurite outgrowth, which is an important step in the process of neuroregeneration[8]. Moreover, quercetin can improve the survival of P19 neurons and provent neuronal damage from cerebral ischemia reperfusion injury, amyloid beta protein and heavy metals[9-11]. However, the mechanism of quercetin's neuronal protection still requires investigation. The present study is to evaluate the potential neuropotective effects of quercitrin on intact cultured hippocampal neurons and to examine its possible mechanisms.

MATERIALS AND METHODS

Animals: One day old Sprague Dawley (SD) rats were employed for isolation of hippocampal neuronal cells. They were generated from 3 months old SD rats mated with a female to male ratio of 2:1 and natural birth. Rats were housed in an environment of 12 h cycle of light and dark and free access to water and food. All procedures related to handling and sacrificing the animals were approved by the Institutional Animal Care and Use Committee of Hebei North University.

Hippocampal neuronal cultures: The hippocampal neuronal cells were prepared from one-day-old rats. Hippocampi were dissected and freed of meninges on ice and then digested with 0.25% trypsin-EDTA (0.5% trypsin-EDTA dilution 1:1 in PBS; Gibco, USA). Dissociated cells were plated on 96/6-well plates coated with poly-L-lysine (Sigma, USA) at a density of $4\text{-}10\times10^5$ mm^{-3} neurons and cultured in phenol red-free Neurobasal medium supplemented with B-27 (Gibco, USA).

Cell viability determination: Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay. Neurons were plated on 96-well microplates. At 4 or 5 days in culture, the culture medium was replaced with the media (20 µL) including medium alone or in addition of: 17 β-estradiol (E_2) (0.02 µM; Abcam, USA), genistein (Gen) (2 µM; National Institutes for Food and Drug Control, CN), quercetin (Que) of different concentrations (12.5, 25 or 50 µM; National Institutes for Food and Drug Control, CN) and estrogen receptor antagonist ICI182,780 (0.02 µM, intervention 1 h; Abcam, USA). After cells were further cultured for 24 h, 20 µL MTT stock solution in ultra pure water (5 mg mL^{-1}; Sigma, USA) was then added to the culture medium in each well and incubated for another 4 h at 37°C. The dark blue formazan crystals, formed in intact cells were dissolved with 200 mL of DMSO and Optical Density (OD) at 490 nm was read by a microtiter plate reader (Tecan safire 2 microplate reader; Tecan grop Ltd., Switzerland). The cell Survival Rate (SR) was calculated as:

$$\text{Survival rate} = \frac{\text{OD of treatment groups}}{\text{OD of control groups}} \times 100\%$$

Immunocytochemistry: After 4 or 5 days in culture of hippocampal neuronal cells, the culture medium was replaced

with the following media including medium alone or in addition of: E_2, Gen, Que at different concentrations estrogen receptor antagonist ICI182,780, estrogen receptor α antagonist MPP (2 μM, intervention 1 h, Sarta Cruz, USA) and estrogen receptor β antagonist PHTPP (2 μM; intervention 1 h; Sarta Cruz, USA). Cells were then further cultured for 24 h. Cells were fixed in 4% paraformaldehyde for 20 min at room temperature and permeabilized for 10 min with penetrating fluid (0.5% triton-x dissolved in PBS). Cells were washed three times with PBS and incubated with anti-β tubulin mouse monoclonal antibody (Tuj1, dilution 1:350 in PBS; Beyotime, CN) at room temperature for 1 h[12]. After washing in PBST (0.1% tween 20 dissolved in PBS), cells were incubated for 1 h at room temperature with goat anti-mouse IgG conjugated with Cy3 (dilution 1:500 in PBS; Jackson Immuno-research, US) for the detection of β tubulin. The cells were washed three times with PBST and the cell nuclei were stained with DAPI. Images were taken using fluorescence microscopy and analyzed with image-pro plus. Automatic measurement of dendrites using count/size features.

Western blotting: After 4 or 5 days in culture of hippocampal cells in the 6 wells plates, culture medium were replaced with following media for 24 h including medium alone or in addition of: E_2, Gen and Que at different concentrations (25 and 50 μM). Whole cellular proteins were extracted by protein extraction solution@@. Equal amount of proteins (20 μL) were loaded on each lane in 8% SDS-PAGE gel. The proteins were then resolved by electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked with blocking buffer (5% non-fat dry milk in PBST) for 1 h at room temperature. One part of each membrane was incubated overnight at 4°C with mouse monoclonal antibodies against estrogen receptor beta/alpha, respectively (dilution 1:400 in blocking buffer; novus biologicals, USA) and the other part was incubated with mouse monoclonal antibody against β-actin (dilution 1:500 in blocking buffer; Santa Cruz, USA). The membranes were washed three times with PBST and incubated for 2 h with rabbit anti-mouse antibodies conjugated with horseradish peroxidase (Novus biologicals, USA). Immunoreactivities were detected by an enhanced chemiluminescence kit. Protein contents were estimated from staining intensity of antibody-binding bands in comparison to the corresponding protein molecular weights using Imag J.

Statistical analysis: Data were expressed as Mean±SEM. Statistical significance was assessed by one-way analysis of variance (ANOVA) using GraphPad Software. The data was considered statistically significant if the probability had a value of 0.05 or less.

RESULTS

Neuron cell purity identification: Neuronal cell purity was identified by staining cells with antibody against βIII tubulin (Tuj1) and DAPI for nucleis. Figure 1 shows that, purity of hippocampal neuronal cells were reached 95%, which met the requirements for experiment.

Cell viability: Cell viability was detemined by MTT assay. Table 1 shows that, when hippocampal neuronal cells were treated with E_2 (0.02 μM), Gen (2 μM) and Que (12.5, 25 and 50 μM) for 24 h, the cell viability were significantly improved as comared with that of the control $p<0.05$. The improvement of cell viability with treatment of quercetin showed a dose-dependent manner. However, when hippocampal neuronal cells were incubated with estrogen receptor antagonist-ICI182,780 for 1 h before treatment with medium alone, E_2 (0.02 μM) and Que at different concentrations (12.5, 25 and 50 μM), the cell viability of hippocampal neuronal cells was decreased except the cells treated with 50 μM Que, which showed increased cell viability compared with that of medium alone ($p<0.05$). However, after pre-treatment of 1 h ICI182,780, there were no statistically significant difference between E2, Que (12.5 and 25 μM) and medium.

Neurite outgrowth: Neurite outgrowth were evaluated by fluorescence microscopy and analyzed with image-pro plus (Fig. 2). When hippocampal neuronal cells were treated with E_2 (0.02 μM), Gen (2 μM) and Que (25 μM) for 24 h, there were significant increase in neuritogenesis (Fig. 2). However, after pre-treatment of ICI182,780 for 1 h, neuronal cells treated with E_2 (0.02 μM) and Que (25 μM) showed significant increase in neuritogenesis. Other treatments did not have significant effect on neuritogenesis (Fig. 3).

To further investigate whether, the quercetin-induced neuritogenesis is due to its binding to ERα or ERβ. Specific antagonists to ERα-MPP and to ERβ-PHTPP were employed. Hippocampal neuronal cells were incubated with MPP or PHTPP respectively for 1 h. Cells were then incubated with E_2 (0.02 μM) and Que (25 μM), respectively for 24 h. Figue 4 shows that, E_2 (0.02 μM) and Que (25 μM) significantly increased neurite outgrowth. However, ERα antagonist-MPP eliminated E2 or Que induced neurite outgrowth, but ERβ antagonist-PHTPP did not affect E2 or Que induced neuritogenesis Fig. 5.

ERα/β protein expression: To elucidate whether, Que affected ERα or ERβ expression, Western blot was used to examine protein levels of ERα or ERβ. Figure 6 shows that,

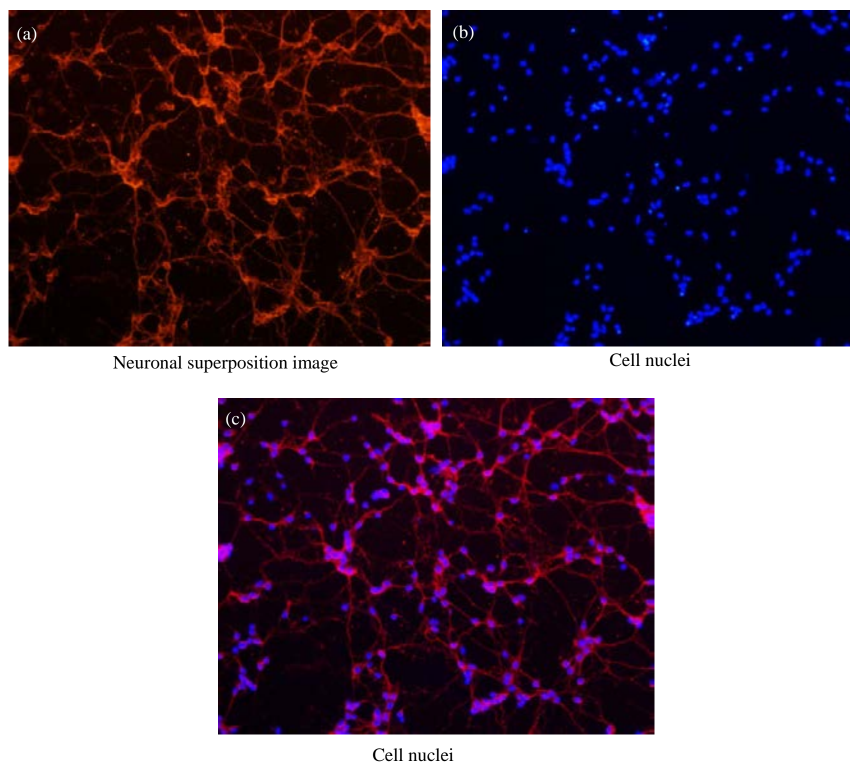

Fig. 1(a-c): Morphology and purity of hippocampal neurons stained with βIII-tubulin for cells and DAPI for nuclei (magnification x200), (a) Neuronal superposition image and (b-c) All cell nuclei

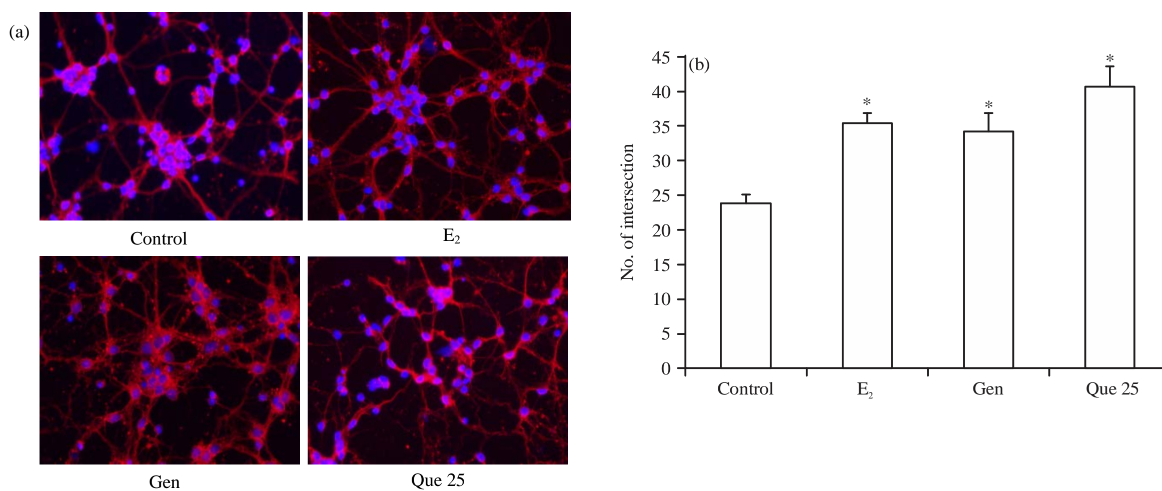

Fig. 2(a-b): Neurite outgrowth of hippocampal neuronal cells treated with E_2 (0.02 μM), Gen (2 μM) and Que (25 μM) for 24 h, (a) Morphology of hippocampal neurons immunostained for βIII-tubulin and DAPI, respectively (x400) and (b) Histogram of neurite outgrowth after different treatments, data are presented as Mean±SEM and *indicates p<0.05 between treatments and control

after the primary cultured rat hippocampal neurons were treated with E_2 (0.02 μM), Gen (2 μM) and Que (25 and 50 μM) for 24 h, there were significant increase in ERα protein but not ERβ protein.

DISCUSSION

In the present study, it has been demonstrated that quercetin can maintance cell viability of estrogen receptor

Table 1: Effect of quercetin on the activity of antagonist estrogen receptor in hippocampal neurons $\bar{X}\pm$SEM (n = 5)

Treatments (μmol L^{-1})	OD	SR (%)
ICI+DMSO	0.255±0.045	
ICI+E_2 0.02	0.249±0.024$^{\Delta}$	103.10
ICI+Que 50	0.323±0.016$^{\blacktriangle}$	126.70
ICI+Que 25	0.275±0.013$^{\Delta}$	107.90
ICI+Que 12.5	0.267±0.010$^{\Delta\Delta}$	104.80
DMSO	0.239±0.039	
E_2 0.02	0.285±0.023*	119.50
Que 50	0.320±0.013**	134.10
Que 25	0.305±0.018**	127.74
Que 12.5	0.289±0.007*	121.13
Gen 2	0.279±0.013*	116.70

Hippocampal cells treated with E_2 (0.02 μM), Gen (2 μM) and Que (12.5, 25 and 50 μM) for 24 h, Cell viability was determined by MTT. Data are represented as Mean±SEM from five experiments, *,**significant difference between treatments and control of p<0.05 and p<0.01, respectively, $^{\blacktriangle}$represents p<0.05 between ICI182,780+Que (50 μM) and ICI182,780+DMSO, $^{\Delta,\Delta\Delta}$ indicate p<0.05 and p<0.01 between antagonistic group and non-antagonistic group

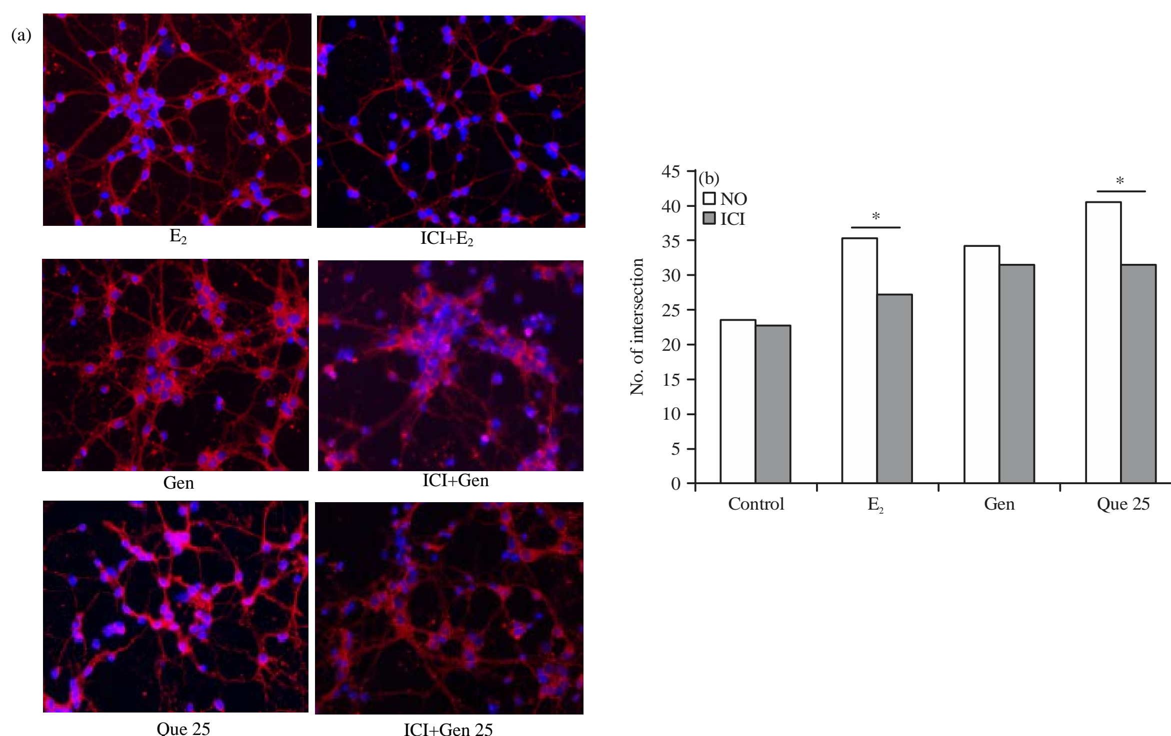

Fig. 3(a-b): Neurite outgrowth of hippocampal neuronal cells treated with E_2 (0.02 μM), Gen (2 μM) and Que (25 μM) for 24 h (left column of panel a) or incubated with ICI182,780 for 1 h and treated with the same treatments (right column of panel a), (a) Morphology of hippocampal neurons immunostained for βIII-tubulin and DAPI, respectively (x400) and (b) Histogram of neurite outgrowth after different treatments, data are presented as Mean±SEM and *indicates p<0.05 between treatments and control

positive neuronal cells in a certain dose range in estrogen depletion culture medium, suggesting its estrogen-like bio-activity[13]. Several studies have shown that quercetin attenuates $A\beta_{25-35}$-induced neurotoxicity in a comparable manner[10]. Quercetin also provides neuroprotection to hippocampal neurons during exposure to hypobaric hypoxia through anti-oxidative and anti-apoptotic mechanisms and possesses promising therapeutic potential to ameliorate hypoxia-induced memory dysfunction[14]. However, the effect of quercetin on intact hippocampus neurons have not been studied comprehensively.

This study from the MTT assay revealed that quercitrin could enhance the cell viability of hippocampal neurons in primary culture. This biological activity of quercitrin was in a

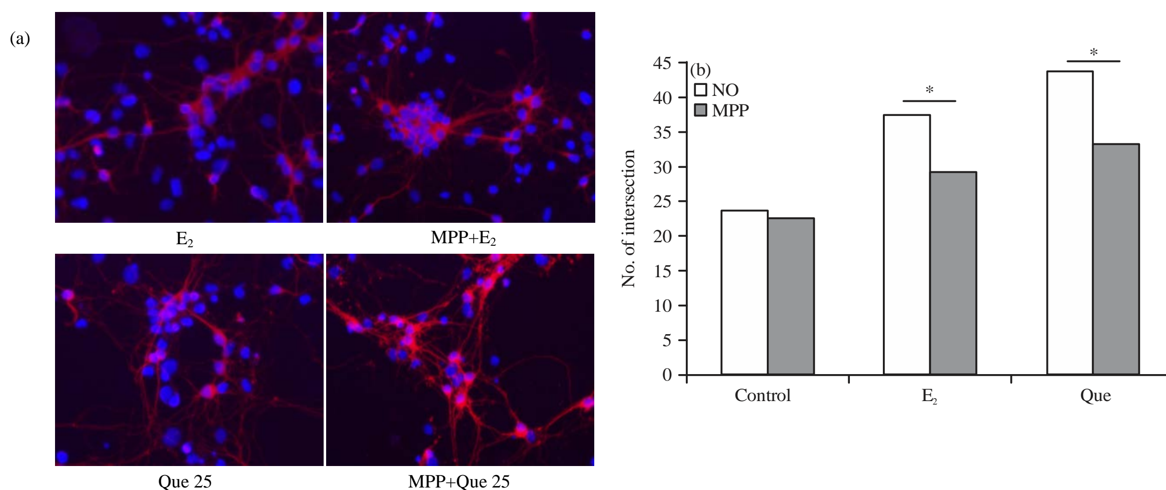

Fig. 4(a-b): Neurite outgrowth of hippocampal neuronal cells treated with E_2 (0.02 μM) and Que (25 μM) for 24 h (left column of panel a) or incubated with ERα antagonist-MPP for 1 h and treated with the same treatments (right column of panel a), (a) Morphology of hippocampal neurons immunostained for βIII-tubulin and DAPI, respectively (x400) and (b) Histogram of neurite outgrowth after different treatments, data are presented as Mean±SEM and *indicates $p<0.05$ between treatments and control

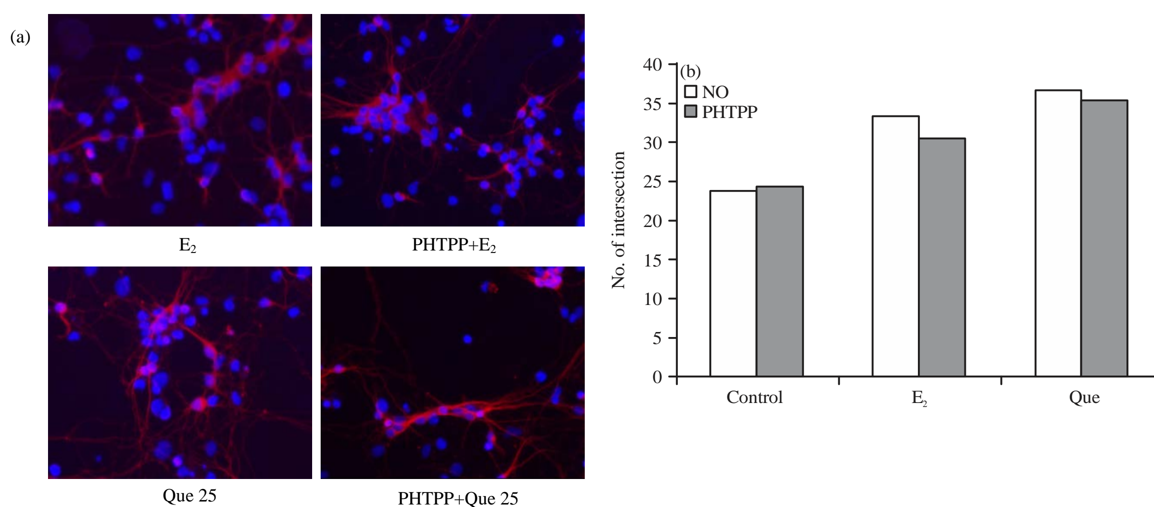

Fig. 5(a-b): Neurite outgrowth of hippocampal neuronal cells treated with E_2 (0.02 μM) and Que (25 μM) for 24 h (left column of panel a) or incubated with ERβ antagonist-PHTPP for 1 h and treated with the same treatments (right column of panel a), (a) Morphology of hippocampal neurons immunostained for βIII-tubulin and DAPI, respectively (x400) and (b) Histogram of neurite outgrowth after different treatments. data are presented as Mean±SEM

dose-dependent manner with highest viability in Que (50 μM) gradually to lowest viability in Que (12.5 μM). This effect is similar to 17 β-estradiol in neuroprotective effects on rat hippocampal neurons.

Learning and memory systems of the human brain is complex. Synaptic plasticity forms the basis of learning and memory formation. Estrogen's function is mainly on learning and memory by affecting the realization of synaptic plasticity[15]. The number of synapses in rat hippocampal CA1 region after ovariectomy significantly reduced exogenous estrogen, which can reverse the changes[16]. Previous studies demonstrated that estradiol can increase the density of synapses by estrogen receptor[17]. This study suggested that similar to 17 β-estradiol and genistein, quercetin can increase the number of synapses in hippocampal neurons.

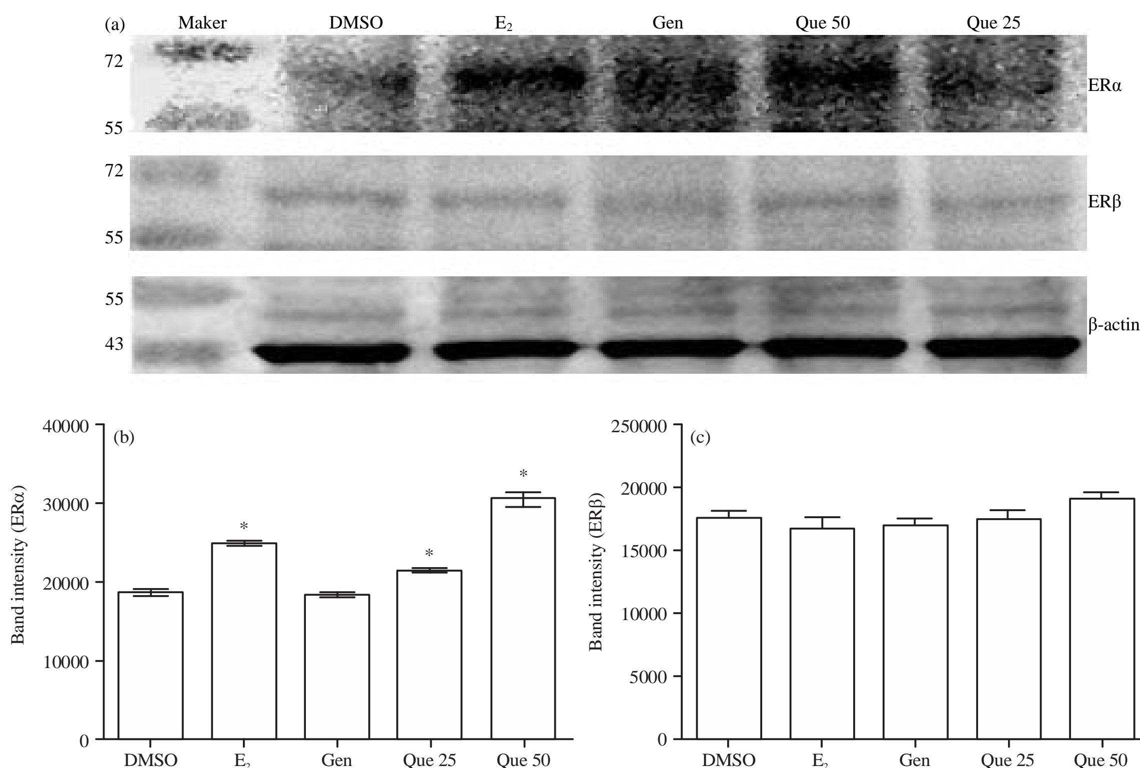

Fig. 6(a-c): Protein abundances of ERα and ERβ in hippocampal neuronal cells treated with E_2 (0.02 μM), Gen (2 μM) and Que (25 and 50 μM) for 24 h, (a) Typical Western blotting of ERα and ERβ, (b) Shows the histogram of ERα and (c) ERβ bands density analyzed by Image J, data are presented as Mean±SEM and *indicates $p<0.05$ between treatments and control

Estrogen-like effects of phytoestrogens are often mediated through the estrogen receptor[18]. The classical estrogen receptors, ERα and ERβ are expressed in the hippocampus[19] and the expression of ERα was higher than that of ERβ[20,21]. Phytoestrogens have different binding capacity with ERα and ERβ. The binding capacity of genistein with ERβ is 22 times higher than that with ERα and the neuroprotective effect of genistein is through the activation of ERβ[22]. Studies have shown that quercetin can bind ERα to regulate proliferation of MCF-7 cells[13]. Neuroprotective effects of quercitrin and 17 β-estradiol may be mediated through estrogen receptor-dependent mechanisms. This hypothesis was tested by pre-incubating cultured neurons with ICI 182780, a specific estrogen receptor antagonist. When incubated with ICI182,780 for 1 h, only highest concentration of Que (25 μM) could improve the viability of neurons and increase neuritogenesis. The protective effects of Gen (2 μM) was iliminated. The 17 β-estradiol promotes neuronal viability via ERβ but after estrogen receptor antagonism, neuritogenesis was not increased[23,24]. The protective effect of quercetin on hippocampal neurons require the participation of the estrogen receptor, but do not rely solely on the estrogen receptors, as high concentrations of quercetin may continue to play a protective role through other pathways.

When ERα was antagonized by MPP, the effect of quercetin and 17 β-estradiol on neuritogenesis was reduced. But, when ERβ was antagonized by PHTPP, 17 β-estradiol and quercetin remained to promote hippocampus synapse formation. Western blot results showed that quercetin can significantly increase the expression of estrogen receptor alpha and does not increase the expression of estrogen receptor beta protein. So, quercetin may exert estrogen-like activity through estrogen receptor alpha on synaptic plasticity of hippocampal neurons.

CONCLUSION

Firstly it was illustrated that the potential neuroprotective effect of quercetin on the intact hippocampus neurons. Moreover, quercetin exerts its neuroprotective activity

through estrogen receptors, mainly ERα. Furthermore, neuroprotective activity of higher concentration of quercitrin might not be related to estrogen receptor mechanism. Although, the exact mechanisms of quercetin-induced neuroprotection and its potential therapeutic applications in neurodegenerative disorders are still unclear, our findings in this study provide a hope for the prevention of AD.

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