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Methodology

Neuroprotective effect of quercetin in murine cortical brain tissue cultures

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SUMMARY

Purpose: Quercetin (QR) is bioflavonoids known for its antioxidants property and its ability to alleviate oxidative stress and promote cellular survival. The aim of this study was to explore the neuroprotective potential of QR against the induced oxidative stress.
Methods: Cortical brain tissue cultures from one week old Wister rats were set up in four groups. Group 1: the control group without any treatments; Group 2: cortical cultures treated with 1 mM H_2O_2 for one hour; Group 3: Cortical cultures pretreated with varying doses of QR for 24 h followed by treatment with 1 mM H_2O_2 for 1 h; Group 4: Cortical cultures treated with the vehicle alone (DMSO). The cortical Tissues from all four groups were homogenized, both the homogenized cortical tissues and conditioned medium was used for the biochemical assay. For histology studies cortical brain tissue were fixed in 10% formalin and stained with H&E.
Results: Pretreatment with 100 μg/ml showed the optimum concentration, which completely ameliorates the effect of induced oxidative stress by H_2O_2. All of the biochemical markers of oxidative stress such as lipid peroxidation, GST enzyme assay, DNA damage and fragmentation were completely reversed with the pretreatments of QR. Histology of the cortical tissues further confirmed the biochemical assays as it showed the pretreatments with QR resulted in the neuronal survival and viability.
Conclusions: This study further reiterated the neuroprotective role of QR against oxidative stress-related neurodegenerative disorders.

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1. Introduction

Brain is one of the main organs susceptible to the damaging effect of oxidative stress by the excessive generation of reactive Oxygen species (ROS). It has a limited capacity to detoxify ROS due to its low glutathione producing capacity of neuronal cells as well as its high metabolic rate, and above all its limited capacity to regenerate and repair as compared to other organs [1].

Quercetin (QR) is a polyphenolic flavonoid widely distributed in plants, with the glycosylated forms includes rutin and quercetrin. In-vitro studies in neuronal cell lines and primary neurons have shown that at low micro molar concentrations, QR antagonise cell toxicity induced by various oxidants (hydrogen peroxide, linoleic acid and hydro peroxide) and other neurotoxic molecules inducing oxidative stress [2–4]. Other study showed that quercetin glycoside (rutin, isoquercitrin) was capable of antagonising the changes in the gene expression induced by 6-hydroxydopamine in PC12 cells [5]. Protection of neuronal cells from Beta amyloid toxicity has been also reported [6]. The antioxidant capacity of QR has been ascribed to the presence of two pharmacophores within the molecule that have the optimal configuration for free radical scavenging, that is, the catechol group in the B ring and the OH group at position 3 [7]. When the QR reacts with a free radical, it donates a proton and becomes a radical itself, but the resulting unpaired electron is delocalized by resonance, making the QR radical too low in energy to be reactive. Three structural groups aid in Quercetin's ability to maintain its stability and act as an antioxidant when reacting with free radicals: The B ring o-dihydroxyl groups, the 4-oxo group in conjugation with the 2,3-alkene, and the 3- and 5-hydroxyl groups. The functional groups can donate electrons to the rings, which increase the number of resonance forms available in addition to those created by the benzene structure [4,8,9].

The neuroprotective role of QR has been suggested in a number of studies but the exact effect of QR on the neuronal morphology and apoptosis was not studied in detail. In this study we looked at the neuroprotective effect of different doses of QR on the neuronal cells under induced oxidative stress.

2. Materials and methods

2.1. Chemicals

QR and all other chemicals were purchased from “Sigma–Aldrich Co., St Louis, MO, USA”. QR was dissolved in Dimethylsulfoxide (DMSO) and used as 100× dilutions.

2.2. Rat brain culture

Male and female I week old Wister Albino rats were used for this study. The rats were kept at a facility of King Saud university research Centre under the strict guidelines provided by the Experimental Animal Laboratory and approved by the animal care and use committee at the College of Applied Medical (Sciences at King Saud university). All procedures dealing with animals were followed in accordance with the standard ethically approved protocol.

Eight brains of the rats were removed immediately after being anesthetized and decapitated in a sterile condition. Cerebral cortex was separated from the front and hind lobe. The left and right cortex was separated carefully by a sharp scalpel and used for this study. The cortexes were washed with sterile saline and then kept in DMEM F12 medium supplemented with glutamine, and 100 μg/ml of streptomycin and penicillin added to it and kept at 37 °C in a humid atmosphere of 5% CO_2.

2.3. Treatment of the brain culture

Each cortex was kept in separate well of 12 well tissue culture plates (Merck Millipore Corporation USA).

They were divided into following treatment groups. Each treatment group consists of 2 brains and each brain was dissected into left and right cortexes.

Group 1: the Control group without any treatment.
Group 2: The cultures treated with DMSO alone
Group 3: The cultures treated with H_2O_2 alone
Group 4: The cultures pretreated with varying doses of QR (50, 100, 150 μg/ml) for 24 h followed by induced oxidative stress by 1 mM H_2O_2.

All the cortical tissues were homogenized in 5 ml ice cold Phosphate Buffered Saline (PBS) after the treatments. One ml aliquots of Conditioned medium and the homogenized tissues were kept at −80 °C for the biochemical assays.

2.4. Lipid peroxidation assay

Lipid peroxidation was calculated using the method of Garcia et al. [10], using the TBARS (thiobarbiturate reactive substances) assay. 1 ml of Brain homogenate was incubated in a metabolic shaker at 37 °C for one hour. 1.5 ml of 20% TCA was added and centrifuged at 600×*g* for 10 min. 1 ml of freshly prepared Thiobarbituric acid (TBA) .67% was added to 1 ml of supernatant, and the reaction mixture was heated in a boiling water bath for 10 min. Absorbance was read at 535 using a reagent blank. Values were expressed as expressed as Mmoles of malonaldehyde formed hour/g of Tissue.

2.5. Glutathione S transferase assay

The activity of Glutathione S-transferase was assayed in a reaction mixture containing 100 mM phosphate buffer, pH 6.5, 1 mM 1-Chloro-2,4-dinitrobenzene (CDNB) and 1 mM reduced GSH. Reaction was initiated by adding 10 μl of Cell lysate and formation of S-(2, 4-dinitrophenyl) glutathione (DNP-GSH) was measured spectrophotometrically as Units per minute per gm of tissue [11].

2.6. DNA fragmentation and quantitation assay

The extent of DNA fragmentation was determined by the method described by Taylor [12]. Brain Tissue homogenate were lysed with equal volume of lysing buffer (5 mM Tris/HCl pH 8, 20 mM EDTA, and .5% triton X-100) and the lysate were centrifuged to separate intact and fragmented chromatin. Both pellet and supernatant were precipitated with 12.5% trichloroacetic acid (TCA). The DNA precipitate was heated to 90 °C for 10 min in 1 ml of 5% TCA, and quantitative analysis was carried out by reaction with diphenylamine at room temperature for overnight. O.D was measured at 600 nm against blank. The percentage of fragmentation was calculated as the ratio of DNA in the supernatant to the total DNA (supernatant plus pellet).

2.7. DNA damage by 8-OH guanosine assay

DNA damage was performed by using the ELISA kit from Cell Biolabs inc USA (Catalogue no ST322). .5 ml of the conditioned medium from the cultures was used for the assay.

2.8. Histology

The cortexes for each treatments group were first fixed in neutral buffered 10% formalin solution. The Block preparation was done in paraffin followed by section cutting in 5–6 μm thick followed by staining with Toluidine blue as well as haematoxylin and Eosin.

2.9. Statistical analysis

Values were expressed as mean ± S.D. The data were represented statistically in the form of numbers, standard deviation (SD), and mean. The contrast among various groups was done using an independent sample T-Test using Microsoft Excel program 2010 for comparing two groups. A

Table 1
The effect of QR on 8-OHG, lipid peroxidation and GST enzyme activity.

Treatments groups	8-oHG assay nM/ml	Lipid peroxidation assay nM/mg protein	GST assay U/mg protein
Control	7.39 ± .001	14.3 ± .205	6.26 ± .001
H_2O_2	14.89 ± .037*	19.3 ± .318*	2.7 ± .636*
50 Q + H_2O_2	7.06 ± .01	16.2 ± 1.016	8.33 ± .423
100 Q + H_2O_2	8.23 ± .04**	12.5 ± 1.07**	9.88 ± .353**
DMSO	7.56 ± .09	15.4 ± 1.4	6.34 ± .256

Values were expressed as mean ± SD, n = 6.
*p < .05; **p < .01.

"probability value" (*p* value) of ≤.05 was referred to be significant. For the statistical analyses, SPSS program (v22.0.0.0) was used.

3. Results

3.1. Effect of QR on DNA damage by 8-OH-dG assay

The Levels of 8-OH dG levels were greatly elevated in the Brain cultures treated with H_2O_2 alone when compared to the control cultures (p < .05). Pretreatment with quercetin prior to H_2O_2 treatment protected the DNA in a dose dependent manner (Table 1).

3.2. Effect of QR on DNA damage

The effect of quercetin on extent of DNA fragmentation was also measured by diphenyl assay. Figure 1 revealed that the extend of DNA fragmentation was significantly increased in the Hydrogen peroxide treated alone brain cultures, while when the brain cultures were pretreated with 50 and

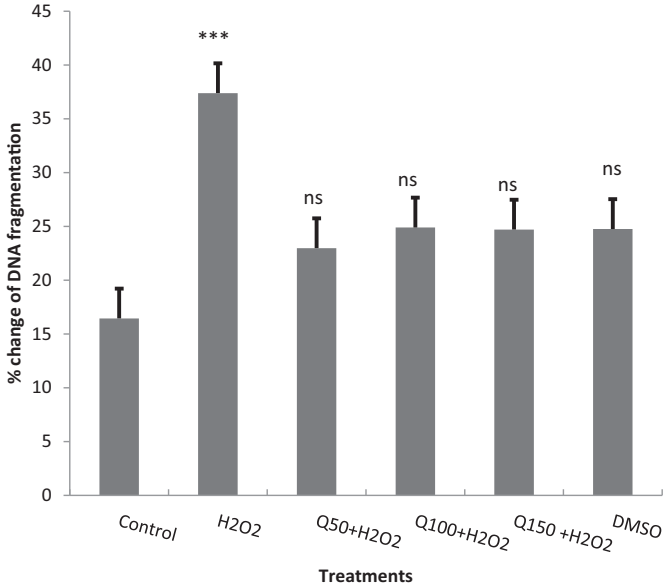

Fig. 1. Percentage change of DNA fragmentation in the control, H_2O_2 treated, and H_2O_2 treatments along with 50, 100 and 150 μg/ml pre-treatments with Quercetin and Treatments with DMSO alone. Data between the groups were compared with an analysis of Variance (ANOVA) and Tukey's multiple comparison tests ***p < .0001 as compared to the control group. The readings were taken from three independent sets of experiments.

100 μ/ml of quercetin and then exposed to H_2O_2 complete protection of genomic DNA strands were observed.

3.3. Effect of QR on GST levels

Exposure of Brain cultures with H_2O_2 resulted in a decreased GST activity, when compared to the control samples ($p < .05$). The pretreatments with QR prior to H_2O_2 treatments however significantly elevated the GST activity in a dose dependent manner (Table 1).

3.4. Effect of QR on lipid peroxidation

The level of lipid peroxidation was significantly increased ($p < .05$) when the brain cultures were treated with H_2O_2 alone when compared to control. The brain cultures pretreated with QR before exposing to H_2O_2 treatment significantly reduced the levels of lipid peroxidation ($p < .01$) again in a dose dependent manner.

3.5. Effect of QR on the histopathology

Small slices of brain tissues were used for histopathological studies.

The control brain cultures normal nucleated healthy neurons with glial cells and pyramidal cells. The brain cultures treated with H_2O_2 however showed distortion of the cellular architecture, with condensed nuclei and disintegrated cytoplasm (Fig. 2).

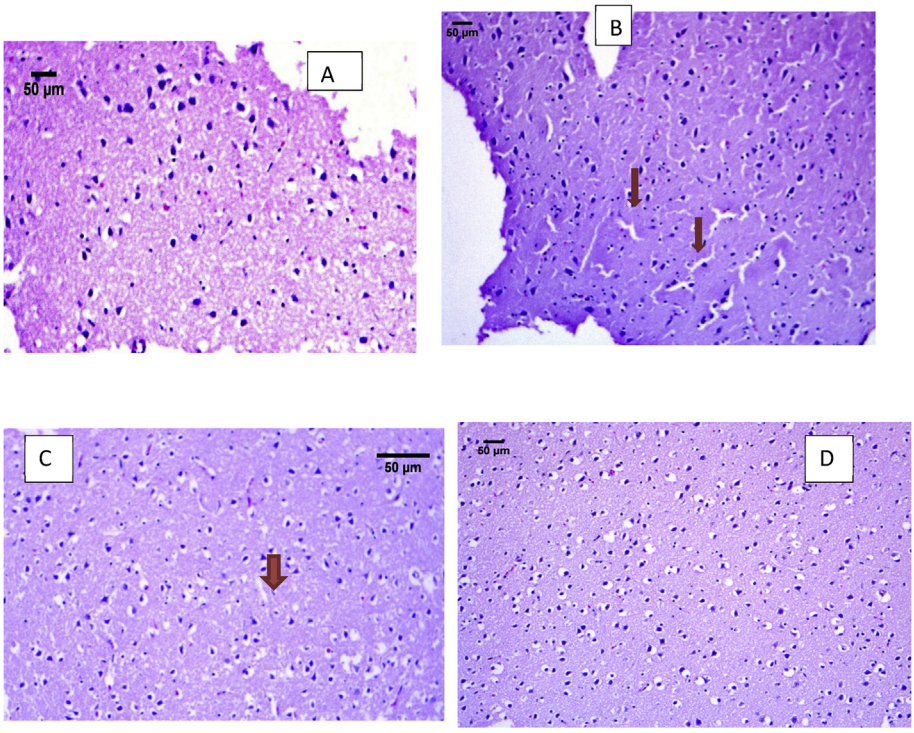

Fig. 2. H&E staining of the rats brain cortical cultures. A: the control cultures. B: H_2O_2 treated alone. C: Cultures pre-treated with 50 μg/ml of QR for 24 h followed by induced OS by 1 mM H_2O_2 for 1 h. D: cultures pre-treated with 100 μg/ml of QR + 1 mM H_2O_2. The red arrows in B and C indicated the cytoplasmic vacuolization.

The neurons cell density was demonstrated by staining with toluidine blue staining. The stain indicated a significant loss of viable healthy neurons with the treatment of H_2O_2. The neurons pre-treated with a 100 μg/ml of QR showed significant ameliorative effect and completely maintains the neuronal health and viability (Fig. 3).

4. Discussion

Oxidative stress is a major contributor to the pathophysiology of many neuronal diseases. Studies have shown that increased Free radical formation with a deficit in antioxidant defense mechanisms could lead to various neurodegenerating disorders. Hydrogen peroxide is cytotoxic and exposure of cultured cells and tissues with it can cause imbalance in energy metabolism and peroxidation of cell membrane lipids and proteins and the loss of membrane integrity and normal function that eventually results in apoptosis and neuronal death [13–15].

Lipid peroxidation is regarded as a vital marker for the toxicity induced by oxidative stress. It has been demonstrated that exogenous treatment with hydrogen peroxide increases the levels of LPO, even at low doses the treatments can cause necrosis and apoptosis of brain cells [16,17] suggesting the cellular injury by the action of the free radicals. In our study treatment of brain cultures with hydrogen peroxide resulted in significant high levels of the LPO when compared to the control. The QR pre-treatments clearly showed to decreased the levels of LPO thus demonstrating the neuroprotective role of QR in activating the antioxidant defense mechanisms by preventing the lipid peroxidation in cellular membrane as supported by a similar study *in-vivo* [18].

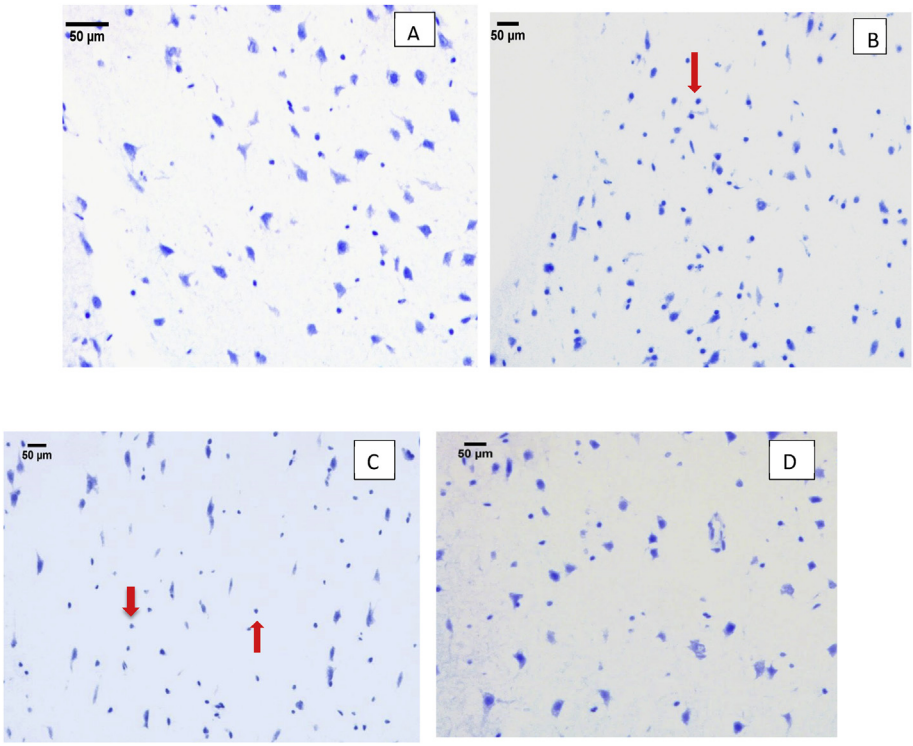

Fig. 3. Toluidine blue staining of the rats brain cortical cultures. A: the control cultures. B: H_2O_2 treated alone. C: Cultures pre-treated with 50 μg/ml of QR for 24 h followed by induced OS by 1 mM H_2O_2 for 1 h. D: cultures pre-treated with 100 μg/ml of QR + 1 mM H_2O_2. Note in B the neuronal cell undergoes necrosis and pyknosis with no surrounding cytoplasm. The arrow in picture C shows only few normal neuron cells which were left after H_2O_2 treatment as compared to picture D where the pre-treatment with 100 μg/ml of QR resulted in ameliorated the OS induced by H_2O_2.

GST is the natural antioxidant enzyme that catalyzes the conjugation of glutathione with various electrophiles and free ionic species produced by different toxins. Under oxidative stress the excessive ROS induces an increase in the GST activity in order to metabolize the toxic products of lipid peroxidation and other molecules [19]. In our study significant decrease in GST activity was observed by H_2O_2 treatments showing the extreme oxidative stress conditions which overwhelm the enzyme. The low activity of GST could be due to modification of the proteins or low glutathione levels. The pretreatments with QR however reduce the oxidative stress induced by H_2O_2 significantly and clearly showed a shift in the ratio of antioxidants verses oxidant, which results in the upregulation of GST activity.

Enhanced oxidative stress induced by H_2O_2 in the brain cells was reflected in the histological sections of the brain stained with both H&E staining and Toluidine blue staining. The tissue sections of the brain cultures derived from control cultures showed normal neurons, glial cells and pyramidal cells arranged in several layers. The treatment with H_2O_2 showed a marked change in the morphology of the glial cells (enlarged) as well as nuclear pyknosis and cytoplasmic vacuolization and disintegration. Toluidine blue is a basic thiazine metachromatic dye and has been used preferentially to investigate the changes in the morphology of the neurons under different experimental or pathological conditions [20–22]. The pretreatment of quercetin with 100 μg/ml showed a remarkable protective effect on the overall morphology and viability of neuronal cells, which was more clearly showed by the toluidine blue staining.

In Conclusion, QR exerted a neuroprotective effect against the induced oxidative stress and neuronal injury by hydrogen peroxide in a dose-dependent manner. The effect of QR on the improvement of neuronal survival and viability was presumably related to its antioxidant capacity and prevention of ROS generation. These results further emphasize the therapeutic potential of QR and the importance of taking QR enriched foods and marketed supplements, in order to prevent the neuronal loss due to physiological ageing and neurodegenerative diseases.

Conflict of interest

No potential conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yclnex.2018.10.002.

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