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Fisetin as a caloric restriction mimetic protects rat brain against aging induced oxidative stress, apoptosis and neurodegeneration

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ABSTRACT

Aim: In the present study, attempts have been made to evaluate the potential role of fisetin, a caloric restriction mimetic (CRM), for neuroprotection in D-galactose (D-gal) induced accelerated and natural aging models of rat.
Main methods: Fisetin was supplemented (15 mg/kg b.w., orally) to young, D-gal induced aged (D-gal 500 mg/kg b.w subcutaneously) and naturally aged rats for 6 weeks. Standard protocols were employed to measure pro-oxidants, antioxidants and mitochondrial membrane potential in brain tissues. Gene expression analysis with reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to assess the expression of autophagy, neuronal, aging as well as inflammatory marker genes. We have also evaluated apoptotic cell death and synaptosomal membrane-bound ion transporter activities in brain tissues.
Key findings: Our data demonstrated that fisetin significantly decreased the level of pro-oxidants and increased the level of antioxidants. Furthermore, fisetin also ameliorated mitochondrial membrane depolarization, apoptotic cell death and impairments in the activities of synaptosomal membrane-bound ion transporters in aging rat brain. RT-PCR data revealed that fisetin up-regulated the expression of autophagy genes (Atg-3 and Beclin-1), sirtuin-1 and neuronal markers (NSE and Ngb), and down-regulated the expression of inflammatory (IL-1β and TNF-α) and Sirt-2 genes respectively in aging brain.
Significance: The present study suggests that fisetin supplementation may provide neuroprotection against aging-induced oxidative stress, apoptotic cell death, neuro-inflammation, and neurodegeneration in rat brain.

1. Introduction

Aging, a natural biological process, is manifested by gradual accumulation of oxidized biomolecules and damaged cell organelles leading to progressive loss of structural and functional integrity, and increased risk of mortality [1]. Brain is the most affected organ during aging due to the presence of high lipid content and higher oxygen demand, which are generally assumed to be contributing factors for increased production of highly reactive free radicals [2]. Moreover, brain aging is often associated with a chronic and low grade neurodegeneration and neuroinflammation [3]. However, the impact of aging, to a degree where functions are impaired, varies markedly across individuals, and the underlying biological mechanism for such variability remains poorly understood. Additionally, the development of strategy to slow down brain aging or improve healthy brain aging is elusive.

Caloric restriction (CR) is the most effective intervention known till date that has the potential to robustly increase life span in many species [4]. CR has been employed to maintain overall health and longevity, regulating deterioration of biological functions, and reducing the risk of many age-related diseases [5]. Although CR may have several beneficial effects, its implementation in humans is still a subject of controversy [6].

Caloric restriction mimetics (CRMs), synthetic or natural compounds that mimic metabolic, hormonal, and physiological effects of CR, are being explored for their anti-aging effects. Similar to CR, CRMs also activate stress response pathways, produce CR-like effects on longevity and reduce the risk of aging-induced diseases [7,8]. CRMs modulate several cellular signaling pathways and have also been identified and investigated as sirtuin-activating compounds (STACs), AMPK activators, mTOR inhibitors, and autophagy inducers in various experimental models [9].

Autophagy, a highly conserved cellular cleaning process, is vital for maintaining health and longevity of the organism, that is also known to progressively decline with advancing of age [10]. Thus, the malfunctioning autophagy process plays an important role in age-related pathophysiology and opens different windows of vulnerability towards aging-induced neurological diseases. CR and CRMs stimulate autophagy by favouring the deacetylation of cellular proteins [11].

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Fisetin (3,3,4,7-tetrahydroxyflavone), a flavonoid found in fruits and vegetables, exert anti-oxidant, anti-inflammatory, antitumor effects, neuroprotective and other health benefits [12–14]. These health improving effects of fisetin may be due to its CRM nature and ability to induce sirtuins [15]. Fisetin is not directly involved in modulation of mTOR activity, however, is reported to up-regulate the expression of SIRT1 and activate SIRT1 mediated deacetylation, which further decreases mTOR function and thereby induces autophagy process [16]. In the present study, an attempt has been made to explore the potential role of fisetin as a possible CRM for neuroprotection of aging brain against oxidative damage, apoptosis, neuro-inflammation and neurodegeneration in D-galactose induced accelerated aging and natural aging models of rat.

2. Materials and methods

2.1. Chemicals and reagents

2,4-dinitrophenylhydrazine (DNPH), dithiobis nitro benzoic acid (DTNB), JC-1 dye, propidium iodide, D-galactose and Tri reagent were procured from Sigma Aldrich, St. Louis, MO, USA. Annexin V-FITC apoptosis detection kit was procured from BD Biosciences, USA. Fisetin was purchased from TCI chemical (India) Pvt. Ltd. cDNA synthesis and polymerase chain reaction kits were purchased from New England BioLabs, MA, USA. All other chemicals were of analytical grade available from Merck, Germany and SRL, India.

2.2. Animal model and study protocol

Male Wistar rats (body weight 150–370 g) were used in the study. Rats were bred and maintained in animal house of our department. The rats were individually housed in temperature-controlled (temperature 20–25 °C and relative humidity 55 ± 15%) conditions with a 12-h light/dark cycle, with free access to drinking water and nutrient rich pellets. The study has been divided into three different sub-categories *viz.,* biochemical study, *in vitro* study and molecular study. For each study, rats (n = 6) were randomly divided into following six groups:

Group 1: Young control: Rats (4 months old, b.w. = 150 g ± 20 g) were administered with vehicle (0.9% physiological saline with 0.5% dimethyl sulfoxide).

Group 2: Young fisetin: Rats (4 month old, b.w. = 150 g ± 20 g) were administered fisetin (15 mg/kg b.w., orally, dissolved in 10% DMSO) orally once daily for 6 weeks [17].

Group 3: D-Gal: Rats (4 months old, b.w. = 150 g ± 20 g) were administered with D-Gal (500 mg/kg b.w., subcutaneous) once daily for 6 weeks to induce aging [18].

Group 4: D-gal + fisetin: Rats (4 month old, b.w. = 150 g ± 20 g) were administered with D-galactose (500 mg/kg b.w., subcutaneous) and fisetin (15 mg/kg b.w., orally) once daily for 6 weeks.

Group 5: Old control: Naturally aged rats (24 month old, b.w. = 350 g ± 20 g) were administered with same volume of vehicle.

Group 6: Old fisetin: Rats (24 month old, b.w. = 350 g ± 20 g) were administered with fisetin (15 mg/kg b.w., orally) once daily for 6 weeks.

All the protocols and procedures were followed as suggested by Ethical Committee of University of Allahabad, Allahabad, India.

2.3. Preparation of brain tissue homogenate

At the end of treatment schedule for 6 weeks, the rat brains were isolated after decapitation, washed in ice-cold saline, weighed and homogenized in 10% ice-cold (0–4 °C) medium containing 50 mM Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl) at pH 7.4 and 300 mM sucrose, using homogenizer (Potter Elvehjem glass-teflon homogenizer) following the standard protocol [19]. The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C to remove cell debris and nuclei. The resulting supernatant was stored at − 80 °C for further experimentations.

2.4. Analysis of oxidative stress

Reactive oxygen species (ROS) production in brain homogenates of all the experimental groups was measured using 2,7-dichlorohydrofluorescein diacetate (DCFH-DA) dye following our earlier reported protocol [20]. ROS generation in treated groups is expressed as percent change of control.

Moreover, the level of lipid hydroperoxidation (LHP) was measured according to the FOX-2 method [21]. Briefly, brain homogenates (50 µL) were added to 950 µL of assay buffer (containing 100 µM xylenol orange, 250 µM ammonium ferrous sulfate, 90% methanol, 4 mM butylated hydroxytoluene and 25 mM H_2SO_4) and incubated at room temperature for 30 min. Then, the absorbance was read at 560 nm after removal of any flocculated material by centrifugation. The signal was read against a H_2O_2 standard curve. The data of LHP are expressed as µM.

The protein carbonyl (PCO) contents were analysed by DNPH method as described earlier [22]. The values of PCO are given as nmol/mg protein.

Advanced oxidation protein products (AOPP) level was analysed by the method of Witko-Sarsat et al. [23]. In brief, the brain homogenate was diluted with phosphate buffered saline in a ratio of 1:5 and 25 µL of potassium iodide was added. The sample was incubated for 2 min followed by the addition of 50 µL of acetic acid. The absorbance of the reaction mixture was immediately read on a UV–Vis Spectrophotometer at 340 nm and the concentration of AOPP expressed as µM/mg protein.

The level of intracellular calcium ion $[Ca^{2+}]i$ was determined fluorometrically in primary cultured neuronal cells isolated from rat brain of all the experimental groups using Quin-2 AM fluorescent dye following the method described earlier by us [24]. The data of $[Ca^{2+}]i$ are presented in nM.

Furthermore, accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), was determined by a colorimetric assay using Greiss reagent (0.1% N-(1-napththyl) ethylene diamine dihydrochloride, 1% sulphanilamide and 5% phosphoric acid) following the earlier described protocol [25]. The values of NO are given in µM/g protein.

2.5. Estimation of antioxidant levels

Total thiol (T-SH) was measured according to standard method [26]. Briefly, aliquots of 250 µL of tissue homogenates were mixed in 5 mL test tubes with 750 µL of 0.2 M Tris buffer at pH 8.2, and 50 µL of 0.0 l M DTNB. The mixture was brought to 5 mL with 3.95 mL of absolute methanol. A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. The test tubes were stoppered with rubber caps, color was developed for 15 min and the reaction mixtures were centrifuged at 3000 × *g* for 15 min at room temperature. The absorbance of the supernatants was read in a spectrophotometer at 412 nm and concentration of T-SH was expressed as nmol/mg protein using molar extinction coefficient of 13,100 $M^{-1} cm^{-1}$.

Superoxide dismutase (SOD) was measured following the reported method [27] using NADH as a substrate and the activity is expressed as units / milligram protein.

Catalase activity in brain homogenate of entire experimental groups was measured by the H_2O_2 degradation assay following the reported protocol [28] and the data are expressed as µM H_2O_2 decomposed/min/mg protein.

2.6. Primary culture of neuronal cells

Rat brain from all the experimental groups was used for isolation