Experimental Eye Research 125 (2014) 193–202

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Retinal neuroprotective effects of quercetin in streptozotocin-induced diabetic rats

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ARTICLE INFO

Article history:
Received 25 February 2014
Accepted in revised form 11 June 2014
Available online 18 June 2014

Keywords:
quercetin
aquaporin-4
apoptosis
neuroinflammation
GFAP
bioflavonoid

ABSTRACT

The aim of the present study was to evaluate the effects of Quercetin (Qctn), a plant based flavonol, on retinal oxidative stress, neuroinflammation and apoptosis in streptozotocin-induced diabetic rats. Qctn treatment (25- and 50 mg/kg body weight) was given orally for six months in diabetic rats. Retinal glutathione (GSH) and antioxidant enzymes [superoxide dismutase (SOD) and catalase (CAT)] were estimated using commercially available assays, and inflammatory cytokines levels [tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β)] were estimated by ELISA method. Immunofluorescence and western blot studies were performed for nuclear factor kappa B (NF-kB), caspase-3, glial fibrillary acidic protein (GFAP) and aquaporin-4 (AQP4) expressions. Structural changes were evaluated by light microscopy. In the present study, retinal GSH levels and antioxidant enzyme (SOD and CAT) activities were significantly decreased in diabetic group as compared to normal group. However, in Qctn-treated rats, retinal GSH levels were restored close to normal levels and positive modulation of antioxidant enzyme activities was observed. Diabetic retinas showed significantly increased expression of pro-inflammatory cytokines (TNF-α and IL-1β) as compared to that in normal retinas, while Qctn-treated retinas showed significantly lower levels of cytokines as compared to diabetic retinas. Light microscopy showed significantly increased number of ganglion cell death and decreased retinal thickness in diabetic group compared to those in normal retina; however, protective effect of Qctn was seen. Increased apoptosis in diabetic retina is proposed to be mediated by overexpression of NF-kB and caspase-3. However, Qctn showed inhibitory effects on NF-kB and caspase-3 expression. Microglia showed upregulated GFAP expression, and inflammation of Müller cells resulted in edema in their endfeet and around perivascular space in nerve fiber layer in diabetic retina, as observed through AQP4 expression. However, Qctn treatments inhibited diabetes-induced increases in GFAP and AQP4 expression. Based on these findings, it can be concluded that bioflavonoids, such as Qctn can be effective for protection of diabetes induced retinal neurodegeneration and oxidative stress.

1. Introduction

Diabetic retinopathy (DR) is a disorder of retinal microvasculature. In recent studies, neruoinflammation has been considered to be an early event in the pathogenesis of DR (Cabrera DeBuc and Somfai, 2010; Simó and Hernández, 2012; Hernández and Simó, 2012; Stem and Gardner, 2013; Lasta et al., 2013; Zhang et al., 2013). Hyperglycemia is the main culprit for both neural and vascular inflammation in DR. Further, impaired retinal electrophysiology and neurodegeneration have also been recorded in diabetic patients (Kizawa et al., 2006).

Under normal metabolic processes, reactive oxygen species (ROS) are continually produced, which are quenched by various endogenous antioxidant enzymes [Superoxide dismutase (SOD) and Catalase (CAT)] to maintain normal cellular homeostasis. During long standing hyperglycemic conditions, excessive production of ROS and reduced neutralizing mechanisms [e.g., decreased

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http://dx.doi.org/10.1016/j.exer.2014.06.009
0014-4835/

glutathione (GSH) levels] lead to various pathological conditions like DR. This imbalance between increased production of ROS and their ineffective neutralization leads to a phenomena known as oxidative stress (Baynes and Thorpe, 1999; Brownlee, 2001). Chronic oxidative stress leads to oxidative damage to biological macromolecules such as DNA, lipids, proteins, carbohydrates, and membrane lipid peroxidation (Brownlee, 2001). The resulted oxidation of cellular components leads to generation of important inflammatory cytokines [tumor necrosis factor-α (TNF-α) and interleukin-1β(IL-Iβ)]. Both TNF-α and IL-1β induce the expression of various genes and promoters of these genes are primarily regulated through complex interactions with nuclear factor-kappa β (NF-kB) subunit (Kowluru and Odenbach, 2004; Demircan et al., 2006; Huang et al., 2011). NF-kB, a redox sensitive transcription factor and a key regulator of antioxidant enzymes, can initiate transcription of many genes involved in apoptosis. It has been found to be a direct or indirect activator of caspase-3 expression in retinal cells (Kowluru et al., 2004). Further, NF-kB and Caspase-3 are highly associated with apoptosis of capillary pericytes, ganglion cells and neuroglial cells in diabetic retinas.

On the other hand, it has been shown that Müller cells play an important role in retinal water/fluid homeostasis via aquaporin water channels (Newman, 1996; Newman and Reichenbach, 1996), regulation of neurotransmitter release and degradation (Matsui et al., 1999), and production of various bio-factors such as vascular endothelial growth factor (Eichler et al., 2000). Further, Müller cells are highly responsive to oxidative stress and inflammation in diabetic retinas, as reflected from upregulation of glial fibrillary acidic protein (GFAP) (Lieth et al., 1998; Barber et al., 2000; Bringmann et al., 2006). Aquaporin-4 (AQP4) is the most abundant osmotically driven transmembrane water channel, which is essential in normal retinal fluid and water homeostasis (Nagelhus et al., 1998, 1999). However, during reactive Müller cell gliosis as a result of diabetes, there is redistribution of AQP4 expression. Altered AQP4 expression leads to retinal edema (Bringmann et al., 2005). Therefore, AQP4 has been suggested as a novel therapeutic target for the treatment of DR (Nagelhus et al., 1998; Da and Verkman, 2004; Bringmann et al., 2005).

Quercetin (Qctn; 3,3′,4′,5,7-pentahydroxyflavone, Fig. 1) is a common flavonol found in vegetables and fruits, and a major bioflavonoid in human diet. Qctn is abundantly found in red wine, onions, green tea, apples, berries, *Ginkgo biloba*, and in St. John's wort. It has been found to possess strong antioxidant (Anjaneyulu and Chopra, 2004; Gitika et al., 2006), anti-inflammatory (Cho et al., 2003; Lee et al., 2013), anti-angiogenic (Zhuang et al., 2011), neuroprotective (Sharma et al., 2007; Nakayama et al., 2011; Pandey et al., 2012) and anti-apoptotic (Ishikawa et al., 2000; Choi et al., 2005) properties. Qctn has been found to be efficacious in cataract prevention (Cornish et al., 2002), oxidative damage in RPE cell (*in-vivo* and *in-vitro*) (Cao et al., 2010), and in inhibition of choroidal and retinal angiogenesis (Chen et al., 2008). Our earlier studies on green tea and *Moringa oleifera* (both of which contain Qctn) suggested that potential role of Qctn may be individually evaluated in diabetic retina (Kumar et al., 2012; Gupta et al., 2013).

As neurodegeneration has been directly implicated in the pathogenesis of DR, in this study, we evaluated the effects of Qctn on various neuroinflammatory markers and cytokines, and apoptotic markers on retinal oxidative stress, neuroinflammation and apoptosis in streptozotocin-induced diabetic rats.

2. Materials and methods

2.1. Study design

Wistar albino rats (200–220 g) were used for the present study. Diabetes was induced with a single intraperitoneal injection of streptozotocin (45 mg/kg body weight). Forty eight hours post STZ injection blood glucose level was measured and rats showing a blood glucose level above 300 mg/dl were considered as diabetic and selected for the study. Diabetic rats were divided into three groups: group I received no treatment, group II received oral Qctn @ 25 mg/kg body weight and group III received oral Qctn @ 50 mg/kg body weight by oral gavage soon after establishment of diabetes (48hr after administration of STZ) and continued for 24 weeks. The rats were monitored throughout the study for body weight and blood glucose. One or two units of insulin (subcutaneous) was administered thrice a week or as desired. The blood glucose and body weight of all rats were regularly monitored. Diabetic rats and age-matched normal rats were sacrificed after twenty four weeks of diabetes, and the retinas were immediately removed and homogenized in ice cold phosphate buffer (pH 7.4) for biochemical estimation and retinas fixed in 4% paraformaldehyde for histological and immunohistochemical studies, as described before (Gupta et al., 2011, 2013, 2014; Kumar et al., 2013). Treatment of animals conformed to ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and institutional Animal Ethics Committee guidelines.

2.2. Antioxidant and inflammatory parameters

Estimations of antioxidant parameters such as GSH, SOD and CAT were performed using commercially available kits from Cayman Chemicals Ltd (Ann Arbor, USA), as per manufacturer's instructions. Enzyme activities (IU/mg protein) were measured in retinal homogenate for SOD and CAT.

TNF-α and IL-1β levels in retinas were estimated using commercially available enzyme-linked immunosorbent assay (ELISA) kit from Diaclone, France and Ray Biotech, Inc., USA respectively as per the manufacturer's instructions.

2.3. Light microscopy and morphometric analysis

Cryostat sections (14 μm) were stained with hematoxylin and eosin (H&E) for ganglion cell counting and histopathology. Ganglion cells were counted in central and peripheral retina (both peripheral sides, nasal and temporal), and average ganglion cell number was counted per 100 μm length of the retina in a total six retinas from each group.

The thickness of the retina, and that of the outer and inner nuclear layer was measured in the central and peripheral retina (both peripheral sides, nasal and temporal). The respective measurements were then averaged to get the values. All measurements were performed under a light microscope with attached digital camera (Leica DM6000 B microscope).

2.4. Immunohistochemistry

Cryostat retinal sections (14 μm) were rehydrated in phosphate buffered saline (PBS) for 20 min, blocked with 10% normal goat serum in PBS for 1 h, and incubated overnight in a moist chamber with the primary antibody diluted in PBS containing 3% normal goat serum and 0.5% TritonX-100. For rabbit polyclonal NF-κB detection (1:1000 dilution, Abcam Plc., UK), the sections underwent heat-induced antigen retrieval with a microwave oven (three 5-min cycles in 10 mM Tris–EDTA buffer [pH 9.0] at 650 W). Next, the sections were incubated with the biotinylated secondary antibody and reacted with the avidinbiotin peroxidase complex. The reaction product was visualized by incubating the sections in a solution containing 3, 3-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide. The slides were lightly counterstained with hematoxylin. Finally, the sections were rinsed with distilled water, cleared, dehydrated in ethanol, mounted and cover slipped.