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Effects of luteolin on retinal oxidative stress and inflammation in diabetes

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Abstract

Luteolin, a plant flavonoid, has a wide range of therapeutic effects. The aim of this study is to examine the effect of luteolin on diabetes-induced oxidative stress and inflammation in the retina of rats. A diabetic rat model was induced by intraperitoneally given streptozotocin 60 mg/kg and the rats were orally administration of luteolin 25, 50 and 100 mg/kg for 6 weeks. The results showed that luteolin administration prevented diabetes-induced decrease in the antioxidant capacity, and increase in malondialdehyde (MDA), 8-hydroxy-2’-deoxyguanosine (8-OHdG) and nitrotyrosine. Luteolin also inhibited diabetes-induced elevation in the levels of IL-1β, VEGF and NF-κB. However, in the high dose group (100 mg/kg), retinal glutathione (GSH) levels were restored close to normal levels. The effects of luteolin were achieved without amelioration of the severity of hyperglycemia. These data suggest that luteolin can be effective for protection of diabetes induced retinal neurodegeneration by inhibiting the levels of inflammatory markers and oxidative stress.
1. Introduction

Diabetic retinopathy (DR) is the leading cause of acquired blindness in working adults around the world.\(^1\) The molecular mechanism by which diabetes damages the retinal microvasculature is thought to be multifactorial, including hyperglycemia-induced polyol pathway activity, increased nonenzymatic glycation and advanced glycation end products, oxidative stress, protein kinase C (PKC) activity.\(^2\)\(^3\) However, the exact mechanism is still elusive. Diabetes increases oxidative stress in tissues of both humans and animals, and increased oxidative stress play a role in the development of diabetic complications.\(^4\) It has been provided strong evidence in animal models that oxidative stress and inflammatory processes are strongly implicated in development of the vascular lesions characteristic of early stages of this retinopathy.\(^5\) However, the mechanism by which oxidative stress can contribute to the development of diabetic retinopathy remains to be elucidated.

Recent studies have proved the pathological involvement of the immune system in diabetic retinopathy.\(^6\) There is a close association between oxidative stress and inflammation in diabetes and we hypothesize that an increase in oxidative stress-derived inflammation is a major mechanism in the pathogenesis and progression of diabetic retina.\(^7\) Many of the molecular and physiologic abnormalities that have been found to develop in the retina in diabetes are consistent with inflammation. Moreover, a number of anti-inflammatory therapies have been found to significantly inhibit development of different aspects of DR in animal models.\(^8\) The retinal capillaries become nonperfused and ischemic, and the number of platelet-fibrin thrombi increases in diabetes.\(^9\) The levels of pro-inflammatory cytokines are
increased in the retina and vitreous in diabetes. There have shown that intravitreal injection of IL-1β to the normal rats increases retinal capillary cell apoptosis and histopathology, and these IL-1β-induced changes in the retinal capillaries of normal rats are similar to those observed in diabetes. Further, VEGF is implicated in the development of diabetic retinopathy. Increased levels of cytokines (VEGF and IL-1β) in diabetic retinas activates NF-κB, which further influences inflammatory stage leading to increased apoptosis of endothelium cells, pericytes and glial cells.

Luteolin, a 3′,4′5,7-tetrahydroxy flavone, has been reported to possess diverse anti-oxidative and anti-inflammatory activities. It significantly decreases lipid peroxidation, inhibits the LPS-stimulated oxidative stress, increases intracellular antioxidant, GSH, regulates enzymatic antioxidants, and scavenges hyperglycemia-induced ROS. In addition, luteolin is shown to inhibit the pro-inflammatory transcriptional factor, NF-κB, and inhibit VEGF expression and ROS-induced retinal neovascularization. However, the beneficial effect of luteolin on diabetic retinopathy remains to be explored.

In the present study we have investigated the effect of administration of luteolin on oxidative stress and inflammatory markers in the retina of diabetes. The total antioxidant capacity, and the levels of GSH, MDA, 8-OHdG, nitrotyrosine, IL-1β, NF-κB and VEGF were quantified in the retina of diabetic rats that was perfused with or without luteolin for 6 weeks, and for comparison, in the retina of the normal control rats. The results presented show that luteolin administration for 6 weeks prevents diabetes-induced increase in retinal oxidative stress and inhibits the levels of pro-inflammatory markers.
2. Material and methods

2.1 Chemicals and reagents

Luteolin, the purity was ≥98% as determined by HPLC, and streptozotocin (STZ) was purchased from Sigma–Aldrich, St. Louis, USA. The assay kits for tissue malondialdehyde (MDA, batch no. 20140523), GSH (batch no.20140611), nitrotyrosine (batch, no20140509), 8-hydroxy-2’-deoxyguanosine (8-OhdG, batch no.20140413) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2 Animals

Male rats of the Sprague-Dawley strain weighting (200-220g) were procured from Sino-British SIPPR/BK Lab Animal Ltd (Shanghai, China). The experimental rats were maintained in a controlled environment (12:12 ±1-h light/dark cycle; temperature, 22 ± 3°C; relative humidity 55%). Rats were allowed to acclimatize to the laboratory for at least 7 days under climate-controlled conditions. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals, and animal handling followed the dictates of the National Animal Welfare Law of China.

2.3 Experimental design

Diabetes was induced in rats by the intraperitoneal (i.p.) injection of freshly prepared STZ at a dose of 60 mg/kg of 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. Age-matched normal rats served as
control. Diabetic rats were randomly divided into 4 groups containing
eight rats per group as follows:

Group 1: Diabetic control rats treated with saline solution for 6 weeks
Group 2: Diabetic rats treated with Luteolin (25 mg/kg/d) for 6 weeks
Group 3: Diabetic rats treated with Luteolin (50 mg/kg/d) for 6 weeks
Group 4: Diabetic rats treated with Luteolin (100 mg/kg/d) for 6 weeks

Diluted the dissolved compound into suitable concentration, then the
animals were administered with the saline or drugs intragastrically. After
6 weeks of diabetes, the rats were euthanized via pentobarbital overdose
(60 mg/kg), the eyes were removed, and retina was isolated and frozen
immediately in liquid nitrogen for biochemical measurements.

2.4 GSH, Nitrotyrosine, MDA and 8-OHdG measurement

Partial retina tissues were taken and rapidly broken to pieces in ice-cold
normal saline, and the tissue homogenate (10%, w/v) was prepared.
Retina GSH, nitrotyrosine, MDA and 8-OHdG activities were determined
according to the manufacturer’s instructions. We performed all
measurements induplicate. The tissue sample concentration was
calculated from a standard curve and corrected for protein concentration.

2.5 Quantification of mRNA expression

The gene expression levels of IL-1β, VEGF and p65 subunit of NF-κB
in the retina tissue were determined by real-time quantitative
reverse-transcription polymerase chain reaction with the use of ABI 7700 and specific primers as reported previously.\textsuperscript{19-21} Specific PCR primer pairs for the target genes were:

- **GADPH** forward 5’-CCATGGGAGA AGGCTGCGG-3’
  reverse 5’-CAAAGTTGTCATGGATGACC-3’
- **NF-κB** forward 5’-CATTGAGGTGTATTTCACGG-3’
  reverse 5’-GGCAAGTGGCCATTGTGTTC-3’
- **VEGF** forward 5’-ACCTCCACCACGCAAGT-3’
  reverse 5’-TTGGTCTGCATTCACTCTG-3’
- **IL-1β** forward 5’-TTGGGATCCACACTCTCCAG-3’
  reverse 5’-AGAAGCTGTGCGCAGCTACCT-3’

2.5 **Western blot analysis**

Retina tissue samples were processed for Western blot analysis. Equal amount (50μg) of protein was subjected to 12% SDS-PAGE gel, and transferred onto a PVDF membrane in a semi-dry system (Bio-Rad, USA). The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween20, and incubated with specific antibodies against IL-1β, VEGF and p65 subunit of NF-κB (1:1000 dilution)(Cell Signaling Technology, Inc., Danvers, MA). The signals were developed using Super-Signal West Pico chemiluminescent substrate (Pierce, Rockford, USA) and visualized with Quantity One software 4.6.2.

3. **Statistical analysis**
The statistical analysis was performed using the SPSS software package (Version 21). For inter-group comparison, student t-test was used to compare initial value or absolute (net) differences. For multiple comparison, the one-way analysis of variance (ANOVA) was used followed by Tukey’s test. All of the results were expressed as the means ± SEM from the results of eight rats in each group. P values less than 0.05 were considered as significant.

4. Result

4.1 Blood glucose and body weight
In Figure1A, blood glucose levels in the diabetic group (342.95 ± 11.63 mg/dl) were significantly higher compared with those of the control group (103.89 ± 6.84 mg/dl) (P< 0.05) at the end of 6 weeks period. However, these were significantly lower in luteolin-treated rats (50 and 100 mg/kg body weight group), being 234.13 ± 8.82 mg/dl and 196.22 ± 16.56 mg/dl, respectively, compared to untreated diabetic rats (p<0.05 and p<0.001, respectively).

The base line weight of the rats at the beginning of the study was similar in all groups. Body weight in diabetic rats was found to be decreased by 46.62% as compared to that in normal rats. However, the weight gains found in the 25, 50 and 100 mg/kg luteolin-treated rats (51.45%, 35.50% and 10.58 % gains in bodyweight, respectively) were significantly higher (p < 0.05) (Fig.1B) compared to untreated diabetic rats.

4.2 Antioxidant parameters
Retinal GSH levels were significantly lower in diabetic rats as compared to normal rats (p < 0.01). However, in three luteolin-treated groups,
retinal GSH levels were significantly higher than diabetic group (p < 0.01) (Fig.2A). The 100mg/kg group was closest to that in the normal control group, a very significant increase in the GSH level was detected in the model control group (5 times) as compared with the normal control group. Six weeks of diabetes increased nitrotyrosine levels in the retina by about 60% compared to the values obtained from normal control rats (Fig.2B). Luteolin supplementation in diabetic rats prevented increase in retinal nitrotyrosine levels, the values in the luteolin-treated diabetic rats were significantly lower compared to diabetic rats without luteolin (P < 0.05).

As shown in Fig.2C, in the model group, MDA levels (6.75 ± 2.14) were significantly increased, and this effect was gradually recovered by luteolin; however, the luteolin group presented the lowest MDA levels compared with the other dose treatments in 100mg/kg group. 8-OHdG levels were elevated by over 56% (Fig.2D) in diabetes compared to the age-matched normal control rats.

4.3 Inflammatory parameters

At mRNA level, the markedly increased transcripts of IL-1β, VEGF and NF-κB were detected in retina from the diabetes model, whereas luteolin administration significantly reduced the mRNA expression. IL-1β value in diabetic rat retinas was found to be more than 3-fold higher than the untreated normal retinas (p < 0.001) as well as VEGF (Fig.3A, Fig.3B). And NF-κB was elevated by over 2 fold in the retina obtained from diabetic rats (Fig.3C). By the meantime, western blot showed that IL-1β values in luteolin-treated rats were significantly lower than
untreated diabetics (p < 0.05). Differences were significant between 25 mg/kg and 100 mg/kg treated groups (p < 0.05). Supplementation with luteolin prevented diabetes induced increase in VEGF; the values obtained from normal control and diabetes + luteolin rats were not significantly different from each other. The NF-κB levels in the retinas from luteolin-treated rats were significantly lower than untreated diabetic retinas (p < 0.01, Fig. 4B).

5. Discussion

Flavonoids are a group of naturally occurring compounds that are widely distributed as secondary metabolites in the plant kingdom. It has been demonstrated that these compounds have interesting clinical properties, such as anti-inflammatory, antiallergic, antiviral, antibacterial, anticancer and antioxidant activities. Luteolin is found in vegetables, fruits and Chinese traditional herbs. It can prevent oxidant injury and cell apoptosis by several mechanisms. Furthermore, luteolin has been proven to be a potent anti-diabetic agent with an antioxidant and anti-inflammatory profile. This is the first reported showing that luteolin has beneficial effects on retinal metabolic abnormalities, including oxidative stress and inflammation, which are considered to be important in the development of retinopathy in diabetes. The present study showed potential neuroprotective effects of luteolin via its antioxidant and anti-inflammatory mechanisms. Oxidative stress is the keystone in multiple lines of evidence converging on the origin and development of ocular disorders. It can
cause profound damage to the retina through dysregulation of intracellular physiology leading to neurodegenerative disorders.\textsuperscript{27} Strong evidence accumulated over the past 25 years of research indicates correlations between the DR patient and oxidative stress.\textsuperscript{28} In addition, clinical evidence highlights the role of oxidative stress as previously indicated. The antioxidant capacity of luteolin has been considered to be mediated via its beneficial effects on the antioxidant defense system. Here we provide data showing that the administration of luteolin can prevent diabetes-induced decrease in the total antioxidant capacity of the retina. This suggests that luteolin has a potential to inhibit overall oxidative damage experienced by the retina in diabetes.

GSH is an ubiquitous tripeptide that functions as an important intracellular radical scavenger. It is important in antioxidant defense, nutrient metabolism, and regulation of cellular events, including gene expression, apoptosis and cytokine production.\textsuperscript{29} Decreased GSH levels are observed in the retina in diabetes, and diabetes caused a significant decrease in GSH in their sciatic nerves, indicative of oxidative stress.\textsuperscript{30} Here, we provide data demonstrating that luteolin administration has partial beneficial effect on diabetes-induced decrease in retinal GSH. The GSH levels in luteolin-treated diabetic rats remained lower than those in the normal control rats, but were significantly higher than diabetic rats.

8-OHdG is one of the most abundant oxidatively modified lesions in DNA. It has been implicated in the pathogenesis of diabetic retinopathy. The inhibition of increased retinal capillary cell apoptosis
and the development of diabetic retinopathy by lipoic acid are
considered to be mediated via inhibition of increased retinal 8-OHdG
levels. Administration of luteolin decreases diabetes-induced
increase in retinal 8-OHdG levels. Inhibition of diabetes-induced
elevated retinal 8-OHdG levels by luteolin suggests that luteolin could
inhibit the development of diabetic retinopathy, in part, via inhibiting
accumulation of oxidized DNA in the retina.
Oxidative stress is considered to regulate diabetes-induced retinal
nitrotyrosine levels. Our data clearly show that luteolin administration
inhibits increased nitrotyrosine levels in the retina. In support, recent
studies by RC Thuraisingham et al have shown that diabetes
increased nitrotyrosine staining in kidneys from patients with diabetic
nephropathy.
The process of lipid peroxidation is one of oxidative conversion of
polyunsaturated fatty acids to products known as MDA, which is
usually measured as thiobarbituric acid reactive substances (TBARS),
or to lipid peroxides, which is the most studied, biologically relevant,
free radical reaction. Lipid peroxidation of cellular structures, a
consequence of free radical activity, is thought to play an important
role in aging, atherosclerosis and late complications of diabetes.
According to the results, after 6 weeks’ administration of 25, 50,
100mg/kg of luteolin, the level of MDA was all very significantly
decreased. And the decreasing levels in the 100mg/kg group are the
closest to the normal levels. Here we provide data showing that the
administration of luteolin can prevent diabetes-induced decrease in the
total antioxidant capacity of the retina. This suggests that luteolin has
a potential to inhibit overall oxidative damage experienced by the retina in diabetes. Inflammation is common events driving the development of various retinal microvascular changes in patients with hypertension, diabetes, and other metabolic disorders. There are exciting results showing that the importance of inflammation in retinal pericytes apoptosis and in the metabolic memory phenomenon, and pinpoints the importance of the duration of the reversal in its outcome. In the pathogenesis of diabetic retinopathy, diabetes has been shown to upregulate various pro-inflammatory mediators in the retina, including IL-1β, VEGF, NF-κB and localized inflammatory processes is considered to play a role in the development of diabetic retinopathy. IL-1β induces the expression of various genes whose promoters are regulated through complex interactions with NF-kB. NF-kB is considered as a key transcriptional regulator of several genes involved in immune and inflammatory responses, and luteolin is a potent blocker of NF-kB activation. In the pathogenesis of diabetic retinopathy activation of NF-kB is reported to active a developing pro-apoptotic program in retinal pericytes, and accelerated apoptosis can predict the development of retinopathy in diabetes. Here we provide clear evidence that luteolin administration inhibits the activation of NF-kB, accumulation of 8-OHdG and nitrotyrosine in the retina in diabetes. This raises a possibility that luteolin can inhibit apoptosis of retinal capillary cells, a predictor of the development of diabetic retinopathy. The therapies that inhibit diabetes-induced VEGF accumulation in the
retina are shown to inhibit diabetic retinopathy. It is considered to play a pivotal role in the increased permeability and angiogenesis seen in diabetic retinopathy. Here we demonstrate that luteolin can prevent diabetes-induced increase in VEGF levels in the retina. In support, luteolin has been reported to inhibit diabetes-induced increased VEGF in and also is postulated to exert its neuroprotective effects and prevent alcohol-induced liver damage via regulating peroxynitrite levels.

Here, we determined mRNA and protein levels of IL-1β, VEGF and NF-κB using pericytes isolated from the retina. However, luteolin-treated retinas showed significantly lower levels of cytokines compared to diabetic retinas. Luteolin has been widely studied for its strong anti-inflammatory properties in rats. The present study was carried out at three different doses of luteolin (25, 50 and 100 mg/kg body weight). All doses have shown potential neuroprotective effects. However, 100 mg/kg dose level showed better response on anti-oxidant and anti-inflammatory parameters, though effects were comparable with 25 mg/kg body weight treated group on the rest of the parameters, viz., IL-1β, VEGF and NF-κB expressions, retinal edema and apoptosis. Further, 50 mg/kg body weight can be considered for further investigations, as lower dose is liable to carry on less potential adverse events, though toxicity studies were not carried in the present study.

It is well established that both experimental diabetes in rats and diabetes mellitus in humans are accompanied by increased apoptosis of retinal neural cells. The retina contains a robust antioxidant and
inflammation defense system with molecules and endogenous enzymes. Oxidative stress and inflammation play a key role in retinal vascular dysfunction during diabetes. Luteolin can protect the retina of diabetes rat. The potential mechanisms of protection may decrease retinal cell apoptosis by anti-inflammation and anti-oxidative.

6. Conclusions

Luteolin has beneficial effects in experimental studies of the diseases that are characterized by increased oxidative stress and inflammatory reactions supporting its clinical use. Our studies are the first to show that luteolin can inhibit diabetes-induced retinal abnormalities that are postulated in the development of diabetic retinopathy. Thus, luteolin appears to be a useful adjunct therapy to possibly inhibit the development/progression of retinopathy, the sight threatening complication faced by diabetic patients.

Acknowledgements

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References


Figure legend

Figure 1. Effect of luteolin on body weight (A) and blood glucose levels (B) obtained from the experimental groups of rats. (Norm = Normal, Diab = Diabetes, n=8 per group, values are presented as mean ± SEM, #p<0.05 ###p<0.001 compared with Normal, and *p<0.05 **p<0.01 ***p<0.001 compared with Diabetes, one-way ANOVA)
Figure 2. Effect of luteolin on diabetes-induced oxidative stress in retina.

(A) Retinal GSH expression level was detected by kit in the deproteinizing retinal homogenate. Luteolin significantly decreased the oxidative stress in retina, such as nitrotyrosine (B), MDA (C) and 8-OHdG (D). (Norm = Normal, Diab = Diabetes, n=8 per group, values are presented as mean ± SEM, *p<0.05, **p<0.01, ***p<0.001 compared with Normal, and *p<0.05, **p<0.01, ***p<0.001 compared with Diabetes, student t-test)
Figure 3. Luteolin-dependent regulation of inflammation related genes in retina. In retina tissue homogenates of rats, luteolin suppresses inflammation related genes. Graphs show RT-PCR measurements of three differentially regulated genes, they are NF-κB (A), VEGF (B) and IL-1β (C). (Norm = Normal, Diab = Diabetes, n=3 per group, values are presented as mean ± SEM, ##p<0.05 ###p<0.001 compared with Normal, and *p<0.05 **p<0.01 compared with Diabetes, student t-test)
Figure 4. Luteolin decreases the inflammation related proteins retina tissue of rats after diabetes induced by STZ. WB analysis was used in retina tissue to quantify the expression of NF-κB (A), VEGF (B) and IL-1β (C). (Norm = Normal, Diab = Diabetes, n=3 per group, values are presented as mean ± SEM, ##p<0.05 ###p<0.001 compared with Normal, and *p<0.05 **p<0.01 compared with Diabetes, student t-test.)
Figure 1

A

Blood glucose mg/dL

Time (days)

B

Body weight (g)

Norm  Diab  25mg/kg  50mg/kg  100mg/kg  Luteolin

initial  final

30x22mm (300 x 300 DPI)
Figure 4

A

<table>
<thead>
<tr>
<th>Protein</th>
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<td>IL-1β</td>
<td>31KDa</td>
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<tr>
<td>GAPDH</td>
<td>36KDa</td>
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</tbody>
</table>

B

![Bar graph showing NF-κB expression levels in different groups.](image)

C

![Bar graph showing VEGF/GAPDH ratio in different groups.](image)

D

![Bar graph showing IL-1β/GAPDH ratio in different groups.](image)