Gentiopicroside attenuates diabetic retinopathy by inhibiting inflammation, oxidative stress, and NF-κB activation in rat model

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Abstract
Diabetic retinopathy, an inflammatory condition, is one of the devastating complication associated with diabetes that can lead to irreversible blindness. Gentiopicroside (GP), a secoiridoid glycoside, exhibits anti-inflammatory and antioxidant activity. The investigation was carried out to explore whether GP could attenuate diabetic retinopathy in diabetic rats. Diabetes was induced by injecting streptozotocin (STZ) (65 mg/kg) intraperitoneally in 8-weeks-old male rats (200–240 g). The treatment group received GP (20, 40, 80 mg/kg) orally for a duration of 10 weeks in diabetic rats (n = 10), and the diabetic group animals received phosphate buffer solution (n = 20). Effect of GP on cell viability study was performed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Oxidative stress markers, inflammatory mediators, and angiogenic factors were quantified in the retinal tissues of diabetic animals. All data were analyzed by one-way analysis of variance (ANOVA) at P < 0.05. Cytoprotective effect of GP was observed in MTT assay. GP effectively downregulated inflammatory cytokine, nuclear factor κB (NF-κB), tumor necrosis factor-α (TNF-α), interleukin 1 beta (IL-1β), and intercellular adhesion molecules-1 (ICAM-1), and upregulated antioxidant markers glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) in the retina of diabetic rats. GP equilibrated the disturbed angiogenic factors in the diabetic retinal tissues. Results clearly indicated defensive role of GP in the treatment of diabetic retinopathy by inhibition of NF-κB and oxidative stress.

Keywords
diabetic retinopathy, gentiopicroside, inflammatory mediators, streptozotocin

Introduction
Diabetes, a disease caused by lack of insulin, had affected 8.8% adults worldwide, and has been predicted to affect around 10% adult population till 2045.1 Diabetes results in a serious cascade of life-threatening conditions including neuropathy, retinopathy, nephropathy, and macroangiopathy.2 Diabetic retinopathy (DR) is one of the most common and serious complications that is known to affect individuals between age groups 30 and 70 years old, after 15 years of diabetes onset.3 DR is a major contributor to blindness in people of working age. Among the early signs of DR, low-grade neuroinflammation is considered as an important indicator.4

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The neurovascular network of the retina is primarily affected during DR, which further cascades to neuroinflammation, neurodegeneration, leakage of plasma, and loss of blood-retinal barrier (BRB) integrity. DR eventually progresses to edema in the retina, neovascularization, loss of vision, and eventually blindness. Limitations in the treatment options available for DR, and their associated adverse effects, the issue of exploring newer and safer molecules for DR treatment, need to be strongly addressed.

A major factor underlying the pathogenesis of DR is a low-grade inflammation. Several genes are over-expressed in retinas of diabetic individuals. During DR, nuclear factor κB (NF-κB) triggers the activity of genes involved in pro-apoptosis, which further upregulates inflammatory mediators, such as cytokines, inducible nitric oxide synthase (iNOS), and intercellular adhesion molecules-1 (ICAM-1). These mediators eventually modulate permeability of BRB coupled with leukostasis. Increased glucose levels, during diabetes, leads to elevated levels of intracellular reactive oxygen species (iROS) and further an oxidative environment, which induces toxicity in the mural, endothelial, and neural cells. Due to this strong oxidative situation, inflammatory responses are stimulated. Unevenness between the antioxidant mechanisms and free radical production results into deposition of oxidative species. The accumulation of these oxidized or nitrosylated species presents persistent retinal damage that cannot be reverted even after normalizing glucose levels and aggravates to disease progression.

Gentiopicroside (GP), a secoiridoid glycoside known for its wide range of pharmacological activities, is obtained from plants belonging to gentian species that are used extensively as medicinal herbs. GP has been investigated to exhibit pharmacological effect such as hepatoprotective, anti-inflammatory, antioxidant, and smooth muscle relaxing effect. GP has also been documented to reduce tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) levels. The mechanism of inhibition of NF-κB by GP is through inhibition of p65 expression.

In consideration of the anti-inflammatory and antioxidant effect exhibited by GP, the investigation was directed toward exploring the protective effect of GP in DR by inhibition of NF-κB. In addition, the effect of GP on various oxidative determinants, that could worsen the diabetic retina, was assessed. The study was carried out on the retinal tissues collected from streptozotocin (STZ)-induced diabetic rat model. Cell viability in the presence of GP was studied in rat Muller cell line induced with high glucose level (HG-induced-rMC1). Among the inflammatory markers, levels of NF-κB, TNF-a, interleukin 1 beta (IL-1b), and ICAM-1 were estimated in the retinal tissues of diabetic and GP treated rats. Variability of histone deacetylases (HDAC) and histone acetyltransferases (HAT) expressions were analyzed in retina of diabetic animals and in those treated with GP. Expression of oxidative stress markers such as iROS, glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) was estimated in the retinal tissues of diabetic and GP-treated animals. Glial fibrillary acid protein (GFAP) levels are slightly elevated in the diabetic retinal tissues and are also assessed after GP treatment. Expressions of proangiogenic and antiangiogenic factors, vascular endothelial growth factor (VEGF) and pigment epithelium-derived factor (PEDF), respectively, were quantified in retinal tissues before and after GP treatment.

**Materials and methods**

**Diabetic animal model**

Eighty-four infection-free male rats (200–250 g), without any eye disease, of age 8 weeks, were procured from the central animal house of the Taipei Medical University. The animals were handled according to the guidelines provided by the Central Committee for Ethical Use of Animals for Research (TMU-F2018/041R/Z). The research protocol was prepared to reduce the number of animals used, and utmost care was taken to reduce the suffering of animal subjects during research. The rats were housed in clean cages and hygienic environment and exposed to 12 h light/dark cycle, coupled with free access to water and standard chow diet. They were further divided randomly into seven groups, each group having 12 animals. Diabetes was induced in rats by injecting STZ (60 mg/kg in 0.1 M sodium citrate buffer, pH = 4.2) intraperitoneally. Rats exhibiting serum-glucose level above 16.7 mmol/L, analyzed after 5 days, were considered as diabetic, and further used for the study. In treatment groups, GP was administered orally as a solution prepared in sterile water for injection, once daily up to 10 weeks.
The animals were divided into following groups:

1. Control group—Non-diabetic and GP untreated.
2. Diabetic group—Injected intraperitoneally with STZ (60 mg/kg) and GP untreated.
3. GP—Non-diabetic and GP treated.
4. GP20—STZ-induced diabetic rats treated with GP 20 mg/kg.
5. GP40—STZ-induced diabetic rats treated with GP 40 mg/kg.
6. GP60—STZ-induced diabetic rats treated with GP 60 mg/kg.
7. GP80—STZ-induced diabetic rats treated with GP 80 mg/kg.

After 10 weeks of the treatment protocol, the rats were weighed, and the respective serum levels were analyzed. After overnight fasting, the rats were euthanized and the eyeballs were surgically removed. The tissues were collected after puncturing one of the eyeball, and the other one was stored in formaldehyde solution (10%) for further investigations. The punctured eyeball was subjected to centrifugation for the separation and collection of the aqueous humor (AqH). Under a surgical microscope, the eyeballs were carefully dissected after the AqH removal.

Cell culture

The viability of rMC1 cells, which were exposed to high glucose content alone and together with GP, was analyzed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The rMC1 cells were subjected to incubation at 37°C, in a mixture of Dulbecco’s modified Eagle medium (DMEM; Gibco Grand Island, USA), and 10% fetal bovine serum (FBS; Hyclone, UT, USA).

Cell viability test

MTT assay was carried out to evaluate the effects of GP on cell survival in normal glucose- and high glucose-induced-rMC1 cells. Group of merged cells (10,000 cells/well), as aggregates, were transferred to plate having 96 wells. A similar procedure was carried out in five wells, to minimize errors. Cells in the individual well were added with 0.2% serum medium and then exposed for 3 h in increasing concentration (0, 10, 20, 50, and 100 µM) of GP. These cells were incubated with high glucose (25 mM) for 48 h and treated later with MTT (5 mg/mL). After 4 h of incubation, the cells were lysed with detergent solution. Furthermore, purple crystalline formazan was dissolved using 200 µL of dimethyl sulfoxide (DMSO), and the corresponding absorbance values were recorded at wavelength of 490 nm. The viability of cells from the control group was considered as 100%, and correspondingly, the GP-treated cell survival percentage was calculated. Each experiment was performed five times.

Determination of HDAC and HAT

The retina of both eyes of the experimental animals from all study groups was processed for the collection of the nuclear extract, according to the manufacturer’s instructions provided with nuclear extraction kit (Active Motif, USA). The activity of HDAC in the diabetic retinal tissues was assessed using an assay kit based on the colorimetric analysis (BioVision Inc., USA), and the readings were recorded at 400 nm using a spectrophotometer. HAT activity in the fraction collected from retina was analyzed using enzyme-linked immunosorbent assay (ELISA), and the recordings were made at 440 nm using a spectrophotometer.

iROS determination

The intracellularly generated iROS and other peroxides readily oxidize non-fluorescent 2′,7′-dichlorodihydrofluorescein diacetate (DCHFD) into dichlorofluorescein (DCF), which is a highly fluorescent compound. An assay kit for detection of iROS was employed and used as per the manufacturer’s instructions (Abcam, MA, USA). The isolated animal retinal cells (8 × 10^6 cells/mL), collected from all experimental groups, were initially diluted to 2 mL using phosphate buffer saline (PBS) (pH 7.4), of which 1-mL cell suspension was added with 100-mL DCHFD (10 mmol/L) and incubated for 3 h at 37°C. Within the homogeneous dispersion of cells, the proportion of fluorescence indicative cells was analyzed at 488 (extinction wavelength) and 535 nm (emission wavelength) with the help fluorescence spectrophotometer (F-2800, Hitachi, Japan).
Protein carbonyl content in the cells was determined in the homogenized retinal tissue extracts, using the respective assay kit (Abcam), following the manufacturer’s guidelines. The principle involved in the protein carbonyl content determination was the generation of protein hydrazone. The generated species was detected using spectrophotometry at an absorbance of 370 nm, considering the absorption coefficient of 22,000/M/cm.

To elucidate lipid peroxidation, the amount of malondialdehyde (MDA) in the homogenized tissues was estimated using lipid peroxidation assay kit (Abcam) according to the instruction provided by the manufacturer. The procedure involved was the addition of 1 mmol/L ethylenediamine tetraacetic acid into 0.5 mL lysate collected from tissue homogenate. The proteins were precipitated after addition of 1 mL cold solution of thiobarbituric acid (15% w/v). The supernatant was again treated with 1 mL thiobarbituric acid solution (0.5% w/v) in a hot water bath for 20 min. The solutions were cooled and the absorbances were recorded at 535 nm. For estimation of thiobarbituric acid reactive substance (TABR), MDA was used as a standard.

**Estimation of oxidative stress markers**

Role of GP in the expression of oxidative stress markers was estimated in diabetic retinal tissues of experimental animals and in rMC1 cells. Using commercially available specific assay kits (Sigma Aldrich, USA) and the instructions provided therewith, levels of GSH, expression of SOD, and CAT activity were quantified in the tissues collected from the retina of diabetic animals and in rMC1 cells.

**Reverse transcription–quantitative polymerase chain reaction (RT-qPCR)**

The homogenized retinal tissues collected from control and diabetic animal groups were subjected to RNA isolation. The separated RNA was dispersed in water pre-treated with diethylylpyrocarbonate, and the total RNA content was analyzed with the help of NanoDrop ND1000 (Thermo Fischer Scientific, USA). After isolation of RNA from the retinal tissues of animals under investigation, mRNA expression of the cytokines responsible for inflammation, namely TNF-α, NF-κB, ICAM-I, and IL-1β, was quantified using RT-qPCR. The total RNA was subjected to reverse transcription into complementary DNA by employing the TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech Co., Ltd., Beijing, China). The forward and reverse primer sequence of β-actin, TNF-α, NF-κB, ICAM-I, and IL-1β is indicated in Table 1.

**Western blot analysis**

The western blotting was carried out to determine the expression of the GFAP, VEGF, and PEDF, using β-actin as an internal control. The retinal tissues collected from experimental animals were homogenized in cold using a mixture of radioimmunoprecipitation assay (RIPA) lysis buffer and phenylmethanesulfonyl fluoride (PMSF). The

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**Table 1. RNA primer sequences used for RT-qPCR.**

| Primer sequence | Forward | Reverse |
|-----------------|---------|---------|
| β-actin         | 5′-AGCCATGTACGTAGCCATCC-3′ | 5′-ACCCTCATAGATGGGCACAG-3′ |
| TNF-α           | 5′-ACACCATGACGACGAAGGC-3′ | 5′-CCGCCACGAGCAAGGAA-3′ |
| NF-κB           | 5′-TGAGGCTGT TTG GTTGAGA-3′ | 5′-TTATGGCTGAGGTCTGTGCTG-3′ |
| ICAM-I          | 5′-GGCCCTAGCTAGTGTA-3′ | 5′-AACCCATTCACCGCTCA-3′ |
| IL-1β           | 5′-AATGGACAGACATTAGCCCAAACA-3′ | 5′-CCCCAGGCAAGAGGAT-3′ |

RT-qPCR: reverse transcription–quantitative polymerase chain reaction; TNF-α: tumor necrosis factor-α; NF-κB: nuclear factor κB; ICAM-I: intercellular adhesion molecules-1; IL-1β: interleukin 1 beta.
resulting homogenate collected was subjected to cooling centrifugation operated at 14,000 rpm for 5 min. The supernatant collected after centrifugation was used for the determination of proteins, using a protein assay kit (BCA, Pierce Biotechnology). Using polyacrylamide gel electrophoresis, 50 mg of protein sample was electrophoresed and loaded on polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with skimmed milk (5%), and subsequently allowed to incubate at 4°C for 12 h with polyclonal antibodies of mouse anti-GFAP (1:10,000), rabbit anti-VEGF (1:1000), rabbit anti-PEDF (1:1000), and rabbit anti-β-actin (1:5000) (Beyotime). The membranes were rinsed and incubated for 2 h at 25°C with a secondary antibody, horseradish peroxidase-conjugate goat immunoglobulin G (1:5000). Bio-Rad image analyzing system, that provides enhanced chemiluminescence, was used to record the optical density of the resulting bands, and Quantity One software was used for quantifying the relative optical density of GFAP, VEGF, and PEDF compared to β-actin.

Statistical interpretation

The data obtained from the methodologies conducted were statistically treated using SPSS Windows software package version 20.0 (IBM SPSS Inc., Chicago, IL, USA). Results were represented as the mean ± standard deviation (SD). Statistical comparisons were made using one-way analysis of variance (ANOVA) and Duncan test. Level of \( P < 0.05 \) was regarded as statistically significant. Least significant difference (LSD) post hoc test was employed for consideration of differences among groups (\( n \approx 3 \)).

Results

Effect of GP on STZ-induced diabetes in rats

All the animals under study, except the control and the GP group, were injected with STZ intraperitoneally for the induction of diabetes. Rats from the STZ-injected groups having serum-glucose levels above 16.6 mol/L were considered as diabetic and, hence, were considered eligible for further investigations. Animal body weight was lowered in the diabetic group that did not receive any treatment. Administration of GP alone in non-diabetic animals produced less impact on the body weight. GP administration improved the reduced body weight of diabetic rats in a dose-dependent manner. The elevated serum-glucose levels were significantly lowered (\( P < 0.01 \)) in diabetic rats treated with GP, compared to the diabetic ones (Figure 1). Results indicated a reduction in the serum-glucose value in diabetic rats, after the administration of GP in a dose-dependent manner.

Effect of GP on cell survival

The rMC1 cells incubated in normal glucose concentration after treatment with variable GP concentrations (0, 10, 20, 50, and 100 µM) for 48 h had no
cytotoxicity. High glucose concentrations exhibited cytotoxicity in rMC1 cells. Incubation of rMC1 cells pre-treated with similarly increasing concentrations of GP followed by incubation in high glucose concentration revealed almost complete cell viability at a concentration above 50 µM (Figure 2). Cell survival at higher glucose concentration was enhanced in a dose-dependent manner, with almost 100% cell surviving at 50 µM.

**Effect of GP on HDAC and HAT levels**

The expression of HDAC in retinas of the STZ-induced diabetic rats was significantly higher than that of the control group. Overexpression of HDAC in the retina of diabetic rats was significantly normalized in retinal tissues of the diabetic rats after administration of GP (80 mg/kg); administration significantly downregulated ($P < 0.05$) the HDAC expression by 38% compared to the enhanced activity observed in the retinal tissues of the diabetic animals (Figure 3(a)). The activity of HAT in the retinal tissues of the diabetic rats was 55.82% lower than in the control group. The HAT expression in the retina of the diabetic rats was enhanced in groups that were on GP therapy. Animals from group GP80 (80 mg/kg) indicated significant normalization (93.25%) of the HAT activity, compared to the control group. GP upregulated the HAT activity by 67%, compared to the untreated diabetic animals (Figure 3(b)).

**Effect of GP on various oxidative stress markers**

Compared to the control group animals, the iROS expression was almost double in retinal tissues of diabetic animals. Diabetic animals, treated with GP (80 mg/kg), revealed a statistically significant reduction in the iROS expression (54.24%), compared to the untreated diabetic group (Figure 4(a)).
The increased MDA and protein carbonyl levels in the retinal extracts of rats were significantly elevated after induction of diabetes, compared to the normal animals. Administration of GP downregulated MDA and protein carbonyl expression by 27.40% and 42.72%, respectively, relative to the diabetic animals (Figure 4(b) and (c)).

Under high glucose condition, the animals depicted a significant reduction in the levels of GSH, SOD, and CAT in the retinal tissues, compared to the corresponding vehicle-treated animals. GP recovered the decreased levels of GSH, SOD, and CAT, in a dose-dependent way. After administration of GP (80 mg/kg) in diabetic animals, retinal
tissue level of GSH, SOD, and CAT was upregulated by 2.08-, 2.19-, and 2.64-fold, respectively, compared to the diabetic animals (Figure 4(d)–(f)).

**Effect of GP on NF-κB, TNF-α, IL-1β, and ICAM-1 expression**

To investigate the regulatory effect of GP in DR, the expressions of NF-κB, TNF-α, IL-1β, and ICAM-1 in the retinal tissues of subject animals were determined by employing RT-qPCR. As indicated in Figure 5(a), western blotting images revealed a significant increase in the expression of NF-κB, TNF-α, IL-1β, and ICAM-1, in the homogenized retinal tissues collected from STZ-induced diabetic rats, compared with the control group animals. Treatment of STZ-induced diabetic rats with gentiopicroside (80 mg/kg) indicated significant lowering of NF-κB, TNF-α, IL-1β, and ICAM-1 expression in the retinal tissue extracts, compared to that of the diabetic animals that were left untreated. Results are presented as mean ± standard deviation (n = 5). *P < 0.05 when compared with the control group; **P < 0.05 when compared with the STZ-induced diabetic group; error bars represent standard deviation.
levels of NF-κB, TNF-α, IL-1β, and ICAM-1, compared to the retinal tissues of the diabetic animals ($P < 0.05$) (Figure 5(b)–(e)). Treatment of DR using GP resulted in 72.87%, 66.69%, 67.01%, and 65.02% reduction of the retinal tissue levels of NF-κB, TNF-α, IL-1β, and ICAM-1, respectively, compared to retinal homogenates of STZ-induced diabetic animals. GP treatment was able to significantly restrict the upregulation of NF-κB, TNF-α, IL-1β, and ICAM-1 expressions that were elevated in diabetic animals confirming DR in the experimental animals ($P < 0.05$).

**Effect of GP on GFAP, VEGF, and PEDF expression**

The western blots exhibiting the expression of GFAP, VEGF, and PEDF (Figure 6(a)) and their respective semiquantitative densitometric determinations are indicated in Figure 6(b)–(d). Western blots indicated significantly elevated levels of GFAP and VEGF in the retinal tissues of the diabetic model animals, compared to that of control group animals ($P < 0.05$). Treatment of diabetic animals with GP revealed significant upregulation in the GFAP and VEGF expression in the retinal extracts, in comparison to the diabetic animals ($P < 0.05$). The PEDF level in the retinal extract of STZ-induced diabetic animals was significantly reduced in comparison to the retina of normal animals ($P < 0.05$). Compared to the retinal PEDF-level reduction, observed in diabetic animals, those on GP therapy revealed marked enhancement in the PEDF expression ($P < 0.05$).

**Discussion**

Investigations reveal that higher blood-glucose concentrations during diabetes result in a cascade
of microvascular and macrovascular implications. Several resulting complications could be avoided and reverted by critically balancing the blood-glucose levels. DR is one such complication that advances and affects the retina of an individual during diabetes. A series of genes are activated that not only result in apoptosis but also trigger the release of various inflammatory and oxidative mediators. DR is associated with the release of inflammatory proteins, such as NF-κB, IL-1β, and TNF-α. The retina in diabetic individuals is more susceptible to oxidative damage that leads further to an elevation in oxygen uptake, higher fatty acid content, and increased oxidation of glucose. The generated oxidative species induce free radical production, that can alter the permeability of BRB, and exhibit irreversible retinal damage on disease progression.

GP, a glycoside having diverse pharmacological effects, is widely present in plants of gentian species. The pharmacological actions exerted by GP include anti-inflammatory, antioxidant, smooth muscle relaxant and hepatoprotective effects. The research was carried out to elucidate the probable effect and its mechanism of GP for the treatment of DR. The present investigation included induction of diabetes by administration of STZ, followed by in-depth examination of parameters responsible for DR. The animal model selected for the investigation was STZ-induced diabetic rat. Animals were divided into control group, diabetic group, and diabetic groups administered with GP. Diabetic induction was verified by recording body weight and serum-glucose levels. Animals induced with diabetes and left untreated with GP indicated a reduction in the body weight and increase in the serum-glucose concentration, compared to the control group animals. Diabetes-induced animals, treated with GP, indicated improvement in the normal body weight and normoglycemic serum levels.

Transcription of several genes known to trigger an inflammatory response is modulated by NF-κB during diabetic complications. Acetylation or deacetylation of NF-κB subunits occurs by HDAC and HAT. Hence, elevated HDAC levels indicate corresponding deacetylation and subsequent activation of NF-κB. Kidney and retinal tissues are widely affected during diabetes due to enhancement activity of HDAC locally. Recent studies have revealed that elevated glucose level during diabetes results into overproduction of superoxide radicals, that further upregulate HDAC activity and downregulate HAT expression. The present investigation indicated a significant reduction in the HDAC activity in the retina of STZ-induced diabetic rat model after administration of GP. In this investigation, GP appears to actively regulate NG-κB, and subsequently modulate HDAC and HAT expression, followed by downregulating the genes involved in inflammatory response.

Reduction in overall antioxidant capacity and increased production of ROS are major contributors to the damage caused during hyperglycemia. High-energy requirements during diabetes and exposure of the retina to light may increase the chances of DR. Therefore, correcting the oxidant and antioxidant equilibrium would be considered as an effective strategy for correcting the damage caused by the DR. Development of high iROS levels during high glucose levels trigger a chain reaction, involving oxidation of intracellular macromolecules such as proteins and lipids. Higher iROS levels may critically impact the cellular functionalities and subsequently induce apoptosis. The present investigation was carried out to quantify the effect of GP on the retinal levels of MDA, GSH, SOD, and carbonyl content, under hyperglycaemic condition. Elevation in the MDA level is linked with damage of lipids due to oxidation, which is eventually because of higher iROS. Damage to the proteins due to increased iROS is marked by increased carbonyl level. Both these
observations are strong indicators of severe oxidative damage caused due to hyperglycemia. Animals with STZ-induced diabetes reflected significantly higher retinal iROS level, in comparison to the normal animals. GP treatment in diabetic animals revealed a significant reduction in the levels of iROS in the retinal tissues. Hyperglycaemia significantly elevated the lipid peroxidation and protein damage, indicated by high retinal MDA levels and carbonyl content, respectively, in the experimental rats. Treatment of STZ-induced hyperglycaemic rats with increasing doses of GP lowered the elevated levels of MDA and protein carbonyl in the retinal tissues. The data thus reveal a strong antioxidant effect of GP in retinal tissues of diabetic rats, in a dose-dependent manner.

SOD and CAT are the enzymes which are known to attenuate peroxides and thereby modulate the antioxidant mechanism in an organism. SODs generate peroxides by dismutation of superoxide radicals. CAT is known to impart protection against reactive hydroxyl radicals and participate in hydrogen peroxide reduction. GSH has been identified as a cosubstrate and a cofactor for several enzymes. In complications, such as diabetes, the oxidative stress has been known to weaken the cellular defense due to reduction of GSH levels. Thus, antioxidants of natural origin may prominently attenuate oxidative stress. The investigation was evidenced with the facts, indicating comparative reduction in the levels of SOD, GSH, and CAT, in tissues collected from retina of the normal and the diabetic animal. After subsequent treatment of diabetic animals with GP, the retinal tissues indicated significant recovery of the SOD, GSH, and CAT levels, indicating the potential of GP to counter the oxidative damage caused during and after hyperglycemia.

One of the most important proinflammatory transcription factors, NF-κB, is known to induce severe inflammatory reactions in DR. Several investigations reveal that elevated blood-glucose levels during diabetes are responsible for oxidative stress in the retina, which is ultimately responsible for cellular inflammation due to flooding of inflammatory mediators. Aggravation of oxidative stress in DR is associated with NF-κB activation. Also, NF-κB triggers activation of several other inflammatory cytokines that induce subsequent retinal damage during hyperglycemia. Thus, it was hypothesized that NF-κB signaling pathway has a profound impact on the inflammation of retina and the progression of DR. Results in the present investigation depicted significantly higher concentrations of NF-κB in the tissues of retina collected from diabetic rats. Administration of GP in animals with hyperglycemia brought a significant decrease in the NF-κB expression in the retinal tissues, almost up to normoglycemic levels, as determined by RT-qPCR. Marked downregulation of NF-κB in the retinal tissues justifies a possible reduction of the inflammatory response and associated damage due to oxidative stress in DR.

Among the other mediators involved, TNF-α is one of the prominent proinflammatory cytokines and is correlated with NF-κB. Studies have revealed that inhibition of TNF-α reduce the NF-κB activity. In a similar way, IL-1β expression rises during retinopathy. During hyperglycemia, the chemoattraction of leukocytes in the vascular walls of the retina is promoted by ICAM-1. White blood cells effectively bind with ICAM-1, followed by adherence to the endothelial linings. In DR, this adherence progresses into localized vascular leakage in the retina. TNF-α regulates the expression of ICAM-1, and further enhances leukostasis and damage to the BRB in DR. RT-qPCR investigations made herewith reveal a significant increase in TNF-α, IL-1β, and ICAM-1 expression in the retinal tissues collected from diabetic animals. Treatment of diabetic rats with GP indicate a remarkable reduction in the elevated levels of TNF-α, IL-1β, and ICAM-1, in the retinal extracts. Reduction of TNF-α, IL-1β, and ICAM-1 levels due to GP administration strongly recommends its potential to reduce the inflammation in the retina and subsequent damage caused during DR.

Furthermore, to investigate the underlying retinoprotection effect of GP in hyperglycemia, western blotting was performed to elucidate the levels of GFAP, VEGF, and PEDF, in the retinal tissues. GFAP is present in a very minor amount in the Muller cells. Muller cells are primarily glial cells present in the retina of vertebrates and are responsible for regulation of neuronal functionality and structural integrity of the retina. The stress-activated Muller cells are known to indicate higher GFAP activity. Elevation of the GFAP levels in the retina of the STZ-induced diabetic animals signified Muller cell activation. Cell loss and loss of normal functionality in DR may be attributed to activation of Muller cells and overexpression of GFAP. The study confirmed marked upregulation
of GFAP in the diabetic retinas, compared to that in the normal animals. Administration of GP in hyperglycaemic rats brought amelioration of Muller cell dysfunction due to high glucose, indicated by downregulation of GFAP activity.

A fine balance between VEGF, a proangiogenic factor, and PEDF, an antiangiogenic factor, is involved in the maintenance of the retinal angiogenesis. Both the factors are actively involved in controlling the vascular leakage and new blood vessel generation. An imbalance of VEGF and PEDF may trigger vascular leakage, disruption, and neovascularization. Reports suggest an inverse co-relationship between VEGF and PEDF expression in DR. Newly developed blood vessels are delicate and can easily break producing hemorrhage, thereby adversely interfering with the normal vision.\textsuperscript{36} As VEGF regulates growth and survival of blood vessels, its overexpression is associated with damage to BRB in DR.\textsuperscript{37} In the present investigation, retinal VEGF was found to be significantly upregulated in diabetic animals, indicating abnormality of the vascular structure of the retina. Concurrently, PEDF expression was downregulated in the retinal tissues of STZ-induced diabetic animals. The reduction in the PEDF expression was indicative of the inhibitory effect on the developing neovascularization in DR. GP effectively inhibited upregulation of VEGF, coupled with enhancement of PEDF expression in the retinal tissues of the diabetic animals. The data provide assurance that GP actively restores the balance of VEGF and PEDF in the retina of rats, that was disturbed after the induction of diabetes.

In conclusion, the investigation demonstrated that STZ-induced diabetes in rats disturbs the structural and functional attributes of the retina. GP imparted retinal protection against free oxygen radicals and oxidative remains of proteins and lipids, which were produced due to hyperglycemia. In vitro, GP induced cytoprotective effects on rMC1 cells. Furthermore, GP attenuated DR by inhibition of the transduction pathway involved in NF-κB signaling. GP actively downregulated expression of TNF-α, IL-1β, and ICAM-1, which are known for progressive damage to the diabetic retina. Administration of GP normalized the balance between VEGF and PEDF, which are known to balance angiogenesis in the retina. The findings shed light on the pharmacological effect of GP to attenuate the high glucose-induced damage of retinal cells and thus provide a research ground for further in-depth research to treat diabetes-associated blindness.

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