Pharmacognostic Evaluation of Curcumin on Diabetic Retinopathy in Alloxan-induced Diabetes through NF-KB and Brn3a Related Mechanism

Debasish Pradhan1, Toffa Dasmohapatra2, Gitanjali Tripathy3

INTRODUCTION

Diabetic retinopathy, also known as diabetic eye disease, is when damage occurs to the retina due to diabetes. It can eventually lead to blindness. It affects up to 80 percent of people who have had diabetes for 20 years or more. At least 90% of new cases could be reduced if there were proper treatment and monitoring of the eyes. The longer a person has diabetes, the higher his or her chances of developing diabetic retinopathy. It is also the leading cause of blindness for people aged 20 to 64 years.1,2,10 Microvascular lesions such as microaneurysms, increased vascular permeability caused by the breakdown of the blood-retinal barrier (BRB), and capillary dropout are thought to be key causes of diabetic retinopathy.11,12,13 The sole purpose of retinal circulation is to support the metabolic demands of the inner retinal neurons and glial; these cells may also be damaged by the diabetic state. Retinal ganglion cells (RGC) are the sole output neurons from the eyes, assuming the critical role of transmitting visual signals to the higher visual center at the brain cortex before signal processing.

Curcumin, a pharmacological operator which is utilized as a part of Asian conventional medication because of its mitigating and hostile to oxidation highlights. Curcumin, a characteristic item that has been comprehensively examined by specialists from both natural and compound purpose for view. Curcumin having very nearly a two centuries old consistent history, starting from 1815, when curcumin was initially confined from turmeric.15 In the wake of finding of its threatening to development affect was initially confined from turmeric.16 In the wake of finding of its threatening to development affect curcumin has most cherished subject for all branches of science including natural, inorganic and physical science. It is a α,β-unsaturated-β diketone (Figure 2) and central bioactive section of Curcuma longa has a place with family Zingiberaeceae (Figure 1) which gives grouped tumor counteractive action operator and against inflammatory properties including assurance from metabolic dis-
Hence it is used to treat diabetic retinopathy which hurts each and every genuine cell of retina, vascular cells and shading epithelial cells and makes driving purpose behind visual inadequacy around the world. Almost all patients with type I diabetes and more than 60% with type II diabetes have some level of retinopathy after 20 yrs. In the present review, we have researched the impact of curcumin on oxidative stress and inflammation in the retina of diabetes initiated by alloxan in rats for 12 weeks by comparing with control rats. The results of this research demonstrated that curcumin obtained from Curcuma longa reduces oxidant capacity of retina during oxidative stress condition inside the body by increasing Brn3a transcription factor expression and represses the level of inflammatory protein by diminishing activity of CaMKII and NF-kB transcription factor-intervened retinal vascular damage in patients with diabetic retinopathy. Ca2+/calmodulin dependant protein kinase II (CaMKII) is a multifunctional serine/threonine protein kinase that coordinates destruction of neuronal cells including retinal ganglion cells and releases inflammatory mediators. Along these lines inhibitor of CaMKII and NF-kB transcription factor, curcumin is strongly neuroprotective for neuron-crushing ailments, for instance, diabetic retinopathy.

As shown (Figure 3) Oxidative stress shows up when there is a genuine irregularity between era of ROS and its clearance by cell antioxidant defences. Diabetic-prompts oxidative stress take after by enactment of Brn3a in the retina are early occasions in pathogenesis of diabetic retinopathy. Anti-oxidant properties of curcumin diminish era of ROS by activating of Brn3a factor in the retina.

**MATERIALS**

Chemicals were reagent-review quality and were obtained from Sigma Aldrich, Neheru Nagar, Mumbai.

**Extraction of Curcumin from Curcuma longa and Detection**

Depending on its origin and the soil conditions where it is grown, turmeric contains 2%–9% curcuminoids. The word “curcuminoid” indicates a group of compounds such as curcumin, demethoxycurcumin and bis-demethoxycurcumin and cyclic curcumin. Out of these, curcumin is the major component. Hence Solvent extraction followed by column chromatography has been followed by us for separating Curcumin from turmeric. Ethanol has been used as a most preferred solvent for extracting curcumin in temperature range of 60 to 80-degree celcius. Curcumin was separated from curcumin mix (a mixture of Curcumin, demethoxycurcumin and bis-demethoxycurcumin) by column chromatography by adsorbing mixture on silica gel using solvent like ethanol to yield three different fractions, the curcumin fraction was further purified on silica gel using ethanol as eluent. For detection of curcumin in the UV region using a common detection of wavelength in range 260nm was used.

**Animals**

In this experiment study, male spargue –Dawley rats (8 weeks old) weighing 245g were kept under a 12h dark cycle, the room temperature was maintained in the range of 23-degree celcius of 50%-70% humidity. The experiments were approved by the Institutional Animal Care and Use Subcommittee at our university. Food and water were available. Rats
were fasted for 16h and randomly divided into 2 groups. First groups of Rats was administered a 40mg dose of Alloxan/kg intraperitonially, while second group of rats was administered only citrate buffer (0.1 mol/L, pH 4.5) according to references. Blood glucose levels were monitored 72 h later after Alloxan or vehicle injection, at regular intervals of every week throughout the study and immediately prior to euthanasia, blood samples were obtained by tail prick, and blood glucose concentration measured using a blood glucose meter. Only rats with fasting glucose concentrations (≥300 mg/dl) were included in the DM group. The first group of rats became diabetic as their blood glucose level was more than ≥300mg/dl. Insulin was administered to diabetic rats to permit moderate weight pick up while keeping up hyperglycaemia. 3-4 days in the wake of actuating diabetes, rats were isolated into two groups Diabetic rats and Diabetic rats with curcumin at a dosage of 100mg/kg/day. Each group had 3 rats and whole rat colony got crisp powdered eating routine weekly. The rats were measured two times each week and their nourishment utilization were measured once every week. A total 6 rats were developed into diabetic and been divided into 2 groups and fed with 50:50 mix of normal saline and dimethyl sulfoxide (DMSO, placebo, N = 6) or curcumin in 50% DMSO (N = 3) at 5 mg/kg. Rats in the sham group received standard husbandry care, gavage fed with PBS, but were not treated with Alloxan. At the end of 12 weeks rats were euthanized and retinas harvested for histological and molecular study. After 12 weeks diabetic rats were dark adapted for 1hr and they were anesthetized with an intraperitoneal infusion of 1% pentobarbital sodium(45mg/kg). The pupils were maximally dilated, and the cornea was topically anesthetized.

**In vivo method for anti-inflammatory property of curcumin after extraction**

After anaesthesia of rats, Evans blue(100mg/kg) was administered by means of rat tail vein. Animals were continued a temperature controlled warming cushion for 2hr, after which they were yielded by 1% pentobarbital over measurement >45mg/kg. The eyes were promptly enucleated and settled with 2% paraformaldehyde in PBS (Phosphate buffer saline) for 2hrs. Retinas were then analysed, and level sums were mounted on glass slides and envisioned under a confocal magnifying lens.

**Selection of Tissue Markers**

Rat Endothelial Cell Antigen Protein (RECA) is important biomarkers in vascular biology. Collagen IV (Col-IV) is an important marker of basement membrane competence in blood vessels and the relationship between Col-IV and diabetes has been extensively studied. Important markers for RGC damage include oxidative stress, Thy-1 (a surface glycoprotein of the immunoglobulin superfamily specifically expressed in RGC), and the transcription factor Brn3a (a transcription factor specifically expressed in cells of the developing mammalian nervous system).

**In vitro method for anti-inflammatory property of Curcumin**

**Western Blotting**

The protein focus in the supernatant was measured and protein (50ug) acquired from each retinal specimen was subjected to SDS-PAGE and electro-pheretically exchanged onto a nitrocellulose membrane. The layer was hindered in 5% non-fat dried milk arrangement and hatched overnight with incompletely purified rat hostile to VEGF monoclonal antibody, rabbit against ICAM-1 mAb and rabbit antiphospho INOS mAb against rat CaMKII and NF-kB antibody. Detection of beta actin expression with a mAb was utilized as an inner control to confirm proportionate aggregate protein stacking.

**Immunofluorescence Staining**

After euthanasia, the retina was harvested and immersed in neutral buffered formalin containing 4% formaldehyde for a period of 6 h, embedded in liquid nitrogen. Sections of 8 μm thickness were cut using a freezing microtome. For immunofluorescence, the tissues were cryoprotected in sucrose, frozen and sectioned at 8 μm in a cryostat. Slides were incubated successively with blocking solution. The tissue sections were incubated with primary antibody to Rat Endothelial Cell antigen (RECA), Collagen IV antibody (Col-IV), Thy-1 cell surface antigen, Brain-specific homeobox domain protein 3A(Brn3a), Carbonic Anhydrase II(CA-II). After the hybridization of secondary antibodies, and DAPI staining for the cell nucleus, the sections were observed at the fluorescence microscope (LeCucumin DM 6000 Laser Station). Semi quantitative analysis was performed to evaluate the intensity of RECA, Col-IV, Thy-1, Brn3a, and CA-II staining using Image pro plus software.

**Retina Ganglion Cell Tissue Culture In vitro**

The retinal ganglion tissue of sham group and DM placebo group (untreated) (n = 9) were cultured in vitro, stimulation with different concentration of curcumin and the length of neurite outgrowth was measured. Each retinal tissue specimen was divided into two sections. After PBS rinsing, the freshly dissected retina ganglion fragment was placed on a coverslip, to which a 14 μl drop of growth-factor-reduced Matrigel™ had been added and kept in liquid formulating a cold 35-mm plastic culture dish on ice. The growth factor reduced Matrigel™ was polymerized (5-min incubation at 37°C) and 2 mL of serum-free RPMI-1640 added. Tissue fragments from the control and diabetic group were treated at various concentrations of curcumin for 3 days. Ganglion cultures were maintained at 37°C in a humidified atmosphere with 5% CO2. Photographs of neurite growth at 6 days were captured using a Nikon DXM 1200 digital still camera attached to LeCucumin Laborlux microscope and ACT-1 software. Digital images were analysed using Image-Pro Plus software to determine the longest neurite length per specimen. Mean maximal neurite length was calculated for the control and diabetic group by averaging the longest neurite length from each individual specimen.

**Statistical Analysis**

Results were expressed as means ± standard deviation. One-way ANOVA followed by Bonferron multiple comparisons’ test was used to evaluate whether differences between groups were significant. All calculations were performed using SPSS statistical software. Probability values of less than 5% were considered significant.

**RESULTS**

Curcumin changes morphology of retina in DM rats

Comparing placebo treated diabetic to control animals, numerous Morphological changes were observed in inner nuclear layer (INL), outer nuclear layer (ONL), retinal ganglion cells (RGCs), and the intensity and number of bipolar cells in the INL and ONL. RGC were considerably reduced in diabetic group as compared with those of the controls. The thickness of the basal membrane in diabetic group was significantly decreased (76.18 ± 5.2 um vs. 67.12 ± 4.8 um). In the Curcumin group, the thickness of basal membrane was 73.53 ± 4.1 um (P < 0.05). The morphological structure of the retinal specimens was qualitatively better in the curcumin treated group (Figure 4).
Curcumin inhibits retinal vascular leakage induced by diabetes

Evans blue was utilized as a part of retinal level adds up to assess the impact of curcumin on retinal vein leakage. In control retinas, Evans blue fluorescence was situated inside blood vessel (Figure 5.1). But in alloxan treated rats (Figure 5.2) leakage of colour from vessels and bigger vessels were noted. This spillage was likewise not seen in alloxan treated rats which were controlled by curcumin (Figure 5.3). Evans blue level were lifted in the retinas of alloxan regarded diabetic rats when contrasted with control rats with decreased vascular leakage. This rise was essentially diminished in alloxan treated rats with administration of curcumin.12

Curcumin improve blood vessels density

Retinal blood vessels are clearly defined in retinal pigment epithelium (Figure 6). Collagen IV expression was less in the diabetic retina, consistent with thickening of the micro-vessel basement membrane. Baseline membrane thickening was less in the curcumin treated diabetic group compared to placebo-treated diabetic animals. Curcumin treated animals also had greater expression of RECA and microvessel density (Table 1, Figure 6a and b)

Curcumin increases Retinal Ganglionic Cells in DM rats

Thy-1 and Brn3a expression in diabetic retinas were significantly decreased in the inner nuclear layer, outer nuclear layer ONL), retinal ganglion cells compared to sham control retinas during oxidative stress condition inside the body. Diabetic rats treated with curcumin had greater expression of Thy-1 and Brn3 relative to placebo treated diabetic
Table 1: Effects of curcumin on Col IV and RECA.

<table>
<thead>
<tr>
<th>Groups</th>
<th>RECA</th>
<th>Col IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1423 ± 85</td>
<td>1490 ± 123</td>
</tr>
<tr>
<td>DM+Placebo</td>
<td>1202 ± 97</td>
<td>1220 ± 190</td>
</tr>
<tr>
<td>DM+Cur</td>
<td>1345 ± 84*</td>
<td>1485 ± 105*</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

RECA, Col IV were used to used check retinal micro vessels. Values are the mean values from N=3 animals per group.*P<0.05,**P<0.01 compared with the placebo group.

Table 2: The effects of Curcumin on RGCs Thy-1 and Brn3a and CA II expression in Diabetic retina.

<table>
<thead>
<tr>
<th>Groups</th>
<th>CA-II</th>
<th>Thy-1</th>
<th>Brn3a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1720 ± 101</td>
<td>2090 ± 143</td>
<td>1923 ± 98</td>
</tr>
<tr>
<td>DM+Placebo</td>
<td>1490 ± 192</td>
<td>1756 ± 87</td>
<td>1365 ± 65</td>
</tr>
<tr>
<td>DM+Cur</td>
<td>1560 ± 121*</td>
<td>1870 ± 110**</td>
<td>1509 ± 126**</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
<td>0&lt;01</td>
</tr>
</tbody>
</table>

Thy-1 and Brn3a were used to detect the RGC, CA-II was used to detect the muller cells. Values are the mean values from N=9 animal group.*P<0.05,**P<0.01 compared with the placebo group.
Curcumin suppresses phosphorylation NF-κB p65 through CaMKII pathway in diabetic retina

Phosphorylation of p65 subunit of NF-κB plays an important role regulating expression and encode pro-inflammatory cytokines and adhesion molecules through activation of CaMKII dependent pathway in diabetic retina. But administration of curcumin (100 mg/kg/day) decreased phosphorylation p65 subunit of NF-κB by inhibiting enzyme CaMKII activity (Figure 8 a and b) which were significantly elevated in retinas of Alloxan treated diabetic rats as compared to controls.

Curcumin reduce VEGF, iNOS and ICAM-1 expressions in the diabetic retina

After decreasing expression of encoder NF-κB for pro inflammatory cytokines, effect of curcumin on the expression levels of VEGF, iNOS, ICAM-1 cytokines were measured. These inflammatory cytokines expression were significantly reduced by administration of curcumin as shown Figure 9 a and b.

**Effects of Curcumin on RGC Neurite Outgrowth from Retina in Vitro during oxidative stress condition**

Neurite outgrowth was measured in cultured RGC from diabetic and normal rats. Paired comparisons were made between retina derived from normal control and DM rats at the treatment of 0, 10, 100 and 1000 nmol/mL curcumin at the 72h time point, all retina treated with curcumin had signif Curcuminificantly longer average neurite length when compared to DM group. (Table 5, Figure 10 a and b)

**DISCUSSION**

This is the main report demonstrating that curcumin, a polyphenol has gainful impact on retinal metabolic anomalies including oxidative stress
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Table 5: The neurites sprout from retina after stimulation with curcumin in normal rats and diabetic rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sham</th>
<th>10nM</th>
<th>100nM</th>
<th>1000nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>170 ± 3.5 **</td>
<td>255 ± 1.5 **</td>
<td>272 ± 2.4 **</td>
<td>311 ± 3.4 **</td>
</tr>
<tr>
<td>DM</td>
<td>102 ± 4.8</td>
<td>129 ± 2.3</td>
<td>154 ± 4.1</td>
<td>178 ± 3.8</td>
</tr>
<tr>
<td>P Value</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Paired comparisons were made between retina derived from normal control and DM rats at the treatment of 0, 10, 100 and 1000 nmol/mL CURCUMIN at the 48h time point. Values are the mean values (±standard deviation) from N = 12 animals per group. ** P < 0.01 compared with the DM group.

and inflammation, which is thought to be essential in the improvement of retinopathy in diabetes. We provided the proof that administration of curcumin with vasoactive properties to a rodent model of diabetes can successfully stifle the activity of transcription factor NF-kB through restraint of CaMKII. We likewise demonstrated that this activity downregulates the incendiary cytokines VEGF, INOS and ICAM-1. These changes are joined by diminished vascular penetrability of veins in the diabetic retina. In these studies, we additionally found that curcumin eases the blood retinal barrier(BRB) spillage observed in alloxan treated rats. Inhibition of vascular injuries by curcumin was independent of progress in blood glucose level. In the present study, we also provided evidence that curcumin may be of benefit in the Diabetic
The neurites sprout from retina after stimulation with 27,8,9,15,19,20,32 concentration and the development of proliferative diabetic retinopathy. Interestingly, our results demonstrate that Col IV, RECA expression in retinal microvessel. These perceptions show that high glucose initiates diminish retinal ganglionic cells that thus enact CaMKII a NF-kb, Brn3a and consequently raise cytokine expression and ensuring retinal vascular spillage in diabetes. This model is bolstered by our perceptions that high glucose-actuated articulation of cytokines (INOS, ICAM-1 and VEGF) was blocked by both NF-kb and CaMKII inhibitor i.e Curcumin.REFERENCES


CONCLUSION

CaMKII reacts to increment in Ca2+, resulting from incitement of NMDA receptors. The elevation of VEGF expression and BRB breakdown in alloxan prompted diabetic rats is hindered by NMDA receptor antagonist.17 Our work has demonstrated that curcumin can lift Retinal Ganglionic Cells (RGC) substance to near normal levels in diabetic rats by modulating Thy-1 and Brn3a expression. Curcumin may also be useful in the management of DR by modulating both RECA and Col-IV expression in retinal microvessel. These perceptions show that high glucose initiates diminish retinal ganglionic cells that thus enact CaMKII a NF-kb, Brn3a and consequently raise cytokine expression and ensuring retinal vascular spillage in diabetes. This model is bolstered by our perceptions that high glucose-actuated articulation of cytokines (INOS, ICAM-1 and VEGF) was blocked by both NF-kb and CaMKII inhibitor i.e Curcumin.17,18,19,20

REFERENCES

SUMMARY


Graphical Abstract

Diabetic retinopathy: microvascular complication of diabetes and involves an abnormal pathology of retinal ganglionic cells where activation of enzyme CaMKII and NF-κB expression occur.

Diabetic induced oxidative stress followed by deactivation of Brn3a expression in the retinal ganglionic cells are also pathogenesis of Diabetic retinopathy.

As an anti-oxidant, curcumin raised Retinal Ganglionic cells by increasing Brn3a expression and by inhibiting CaMKII / NF-κB expression.

Curcumin can be used as a therapy against diabetic retinopathy. against diabetic retinopathy.

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