To Study Protective Effects of Hesperetin on Retinal Neurovascular Degeneration in Streptozotocin-Induced Type-I Diabetic Rats

**Introduction**

Diabetes is a chronic disease characterized by hyperglycemic state. Uncontrolled hyperglycemia has been the major culprit for various secondary diabetic complications. Diabetic retinopathy (DR) is one of the most debilitating disorder of the microvasculature of the retina as a result of uncontrolled hyperglycemic state. Depending on the severity of the disorder it has been divided into two stages: (1) Early stage or non-proliferative DR, which is characterized by leaky vessels; (2) Late stage or proliferative DR, where proliferation of retinal vessels occurs due to activation of growth factors (Frank, 2004).

Various biochemical mechanisms have been implicated in the pathogenesis of hyperglycemia induced vascular damage, but increased de novo synthesis of diacylglycerol (DAG), is considered a direct endogenous activator of protein kinase C – β (PKC- β) (Donnelly et al., 2004). Therefore, PKC-β is primarily involved in mediating a variety of unwanted functional and structural abnormalities in vascular tissues. VEGF-induced vasopermeability *in vivo* and *in vitro* requires PKC activity. However, actual mechanism by which PKC regulates barrier properties is not fully understood (Aiello, et al., 1997; Geraldes and King, 2010; Ayo et al., 1991; Geraldes et al., 2009). In DR, PKC mediated alterations in vascular permeability, blood flow as well as both the formation and response to angiogenic growth factors contribute to retinal leakage, ischemia, and neovascularisation (Frank, 2004). PKC activation also contributes to loss of capillary pericytes and thickening of vascular basement membrane (BM) are early feature of DR (Kim et al., 2010a). Therefore, both PKC-β and VEGF are considered very important parameters in diabetes induced microangiopathy (Kuiper et al., 2007; Pfeiffer and Schatz, 1995; Aiello et al., 1994).

Hyperglycemia results in the production of free radicals, which are actually associated in the pathologies such as cancer, atherosclerosis, diabetic complications, heart diseases etc. When insufficient neutralisation of free radicals occurs due to an
imbalance between Pro-oxidants and Anti-oxidants in diabetes mellitus (DM) leading to oxidation of cellular lipids, proteins and nucleic acids (Guler et al., 2000; Kowluru and Koppolu, 2002a; Kowluru and Kowluru, 2007). As a result oxidative stress exerted on the retina result in the activation of NF-κB (Romeo et al., 2002; Kowluru et al., 2003), an inflammatory cascade, and acts as a trigger for the release of pro-inflammatory cytokines (IL-1β, TNF-α) (Gupta et al., 2011; Zhang et al., 2011; Gupta et al., 2012). Multiple pro-inflammatory cytokines, chemokines and adhesion molecules activate endothelium to increase expression of adhesion molecules (E-Selectin, ICAM-1) (Masuzawa , et al., 2006; Zernecke and Weber, 2010). Further, leukostasis in retinal capillaries leads to capillary occlusion causing non-perfusion and ischemia. Alternatively, Cytokines (IL-1β, TNF-α) are involved in the activation of several apoptosis regulatory genes including caspase (Behl et al., 2008). Moreover, Cytokine induced NF-kB activation in retinal pericytes plays a significant role in the hyperglycaemia induced pericyte death as observed in diabetic retinopathy. Pericyte loss is a critical stage in diabetic retina leading to increased vascular permeability and formation of microaneurysms (Romeo et al., 2002). That is how; retinal inflammation and oxidative pathways are strongly implicated in the pathogenesis of DR.

Over the past many decades, bioflavanoids are the most common group of plant polyphenols that give color and flavor to fruits and vegetables. The common structural feature of the flavonoids is the flavone nucleus (2-phenyl chromone or 2-phenyl benzopyrone), characterized by a C6-C3-C6 carbon skeleton with the C6 component being aromatic in nature (Figure 2). This basic skeleton may contain numerous substituent groups: (1) hydroxyl groups, generally present at the 4, 5 and 7 positions; (2) sugars, generally linked with the hydroxyl group positioned at 7; and (3) methyl and isopentyl units. Hydroxyl groups and sugars impart hydrophilicity, while methyl groups and isopentyl units impart lipophilicity to the flavonoids (Majumdar and Srirangam, 2010) (Fig.1). In plants, flavonoids occur as the glycosides
or occasionally as the aglycones. Initially, they were known as ‘Vitamin P’.

Flavonoids have gained wide importance in therapeutics as a result of their beneficial properties. Bioflavanoids possess strong anti-oxidant (Gupta et al., 2011), anti-angiogenic (Kumar et al., 2012) or anti-inflammatory properties (Kumar et al., 2013). They have also shown beneficial effects on reducing fluid retention and strengthening capillary walls (Kumar et al., 2012). Therefore, bioflavanoids have been considered effective in prevention or treatment of various ocular disorder/diseases like DR, macular degeneration, glaucoma and cataract.

Hesperetin (Hsp) is a plant based active flavanone found in citrus fruits (Fig. 2). Hsp has been shown to be a potential anti-oxidant (Choi, 2008; Hwang and Yen, 2008; Cho, 2006), anti-inflammatory (Hirata et al., 2005), neuroprotective agent (Choi and Ahn, 2008) and also found to decreases vascular permeability (Paysant et al., 2008). Importantly, as described in earlier reports (Srirangam and Majumdar, 2010), some of the pharmacological activities of Hsp can be very useful in the prevention and treatment of eye disorders such as DR, diabetic macular edema and cataract.

To the best of our knowledge no scientific study have been conducted earlier which shows direct effect of Hsp on retinal neurovascular degeneration and, correlate them
with angiogenesis and neuroinflammatory markers after chronic diabetes. Therefore,

Figure 2. Chemical Structure of Hesperetin (2,3-dihydro-5,7-dihydroxy-2-(3-hydroxy-methoxyphenyl)-4H-1-benzopyran-4-one) and mass spectroscopy chromatogram of product ion scan of hesperetin molecule (MH+, 303.1, Obtained from Cayman chemical Company, USA).

Figure 3. Picture from our experimental work demonstrating use of fundus camera for taking fundus images in rats.
in the present study we investigated the effect of oral treatment with Hsp (Purity ≥ 98 %, Cayman Chemicals, USA) on hyperglycemia induced retinal oxidative stress, neuroinflammation, vasculopathy and apoptosis with supported structural and ultrastructural findings.

Figure 4. Fundus photographs from different study groups. (A). Normal rat fundus not showing any vascular dysfunction, (B). Diabetic rat fundus showing hyperpermeable optic nerve head (arrow) and dilated retinal vessels, (C and D). Hsp-treated (Hsp-100 and 200 groups) rat fundus not showing any vascular dysfunction.

**Materials & Methods**

**Study Design**

Diabetes was induced in Wistar albino rats (200 to 250 g) with streptozotocin (STZ, 45 mg/kg body weight). Blood glucose was measured prior to the induction of diabetes and 48 hours post STZ/vehicle injection in all groups. The rats showing a blood glucose concentration greater than 300 mg/dl were considered diabetic. The present work was accomplished using three studies. Study I- Age-matched normal rats served as control. Diabetic rats were divided into three groups of 18 rats each: the rats
in group 1 received normal diet without Hsp, group 2 received oral Hsp at a dose of 100 mg/kg BW and group 3 received Hsp at a dose of 200 mg/kg BW by oral gavage soon after establishment of diabetes (48hr after administration of STZ). Study II - Age-matched normal rats served as control. Diabetic rats were divided into 3 groups of 16 rats each: the rats in group 1 received normal diet without Hsp, group 2 received oral Hsp at a dose of 100mg/kg BW and group 3 received oral Hsp at a dose of 200mg/kg BW by oral gavage soon after establishment of diabetes (48hr after administration of STZ). Study III - the rats in group 1 received normal diet without Hsp and group 2 received oral Hsp at a dose of 100mg/kg BW. After 24 weeks of diabetes, the rats were euthanized by an overdose of pentobarbital, the eyes removed, and the retinæ were isolated and homogenised in phosphate buffer (50 mM, with added Protease inhibitor). Four retinæ from each group were processed for Transmission Electron Microscopy. Treatment of the animals conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research, and prior approval was taken from Institutional Animal Ethics Committee.

Figure 5. Effect of Hsp on retinal arteriolar and venular diameter after 24 weeks of diabetes. Values are mean ± SD, n=12. *p<0.001 compared with normal arterioles and venules; #p < 0.05 compared with Diab.+ Hsp-100 and Diab.+ Hsp-200 arteriols. $= Differences are insignificant between Diab.+ Hsp-100 and Diab.+ Hsp-200 groups.

Glycemic parameters
Blood glucose was estimated with the help of Accu-Chek® Active Glucose Test Strips using an Accu-Chek® meter (Roche Diagnostics India Pvt. Ltd). Accu-chek active device was calibrated before every use to check accuracy and sensitivity. Glycosylated hemoglobin (HbA₁c) was estimated at 24 weeks by ion exchange resin kit (Biosystems S.A.Costa Brava 30, Barcelona, Spain).

**Fundus photography and Vessel Diameter**

Animals were trained before start of the study so that they become accustomed to the fundus photography procedure. KOWA Handheld Digital Retinal Camera (Genesis – Df, Kowa Company Ltd., Japan, Tokyo) was used to photograph rat fundi. Photographs were taken using conscious rats. This avoids complications of anesthesia such as clouding of the ocular media and the poor tolerance of rats to anesthesia. The vibrissae were trimmed to prevent them from obscuring the photograph. Eyes were dilated with a drop of 1 % tropicamide (Sunways India Pvt. Ltd.). Fundus photography was done regularly till 24 weeks to monitor the fundus changes (Fig.3).

![Figure 6. Fundus fluorescein angiograms from different study groups. (A). Normal Group fundus fluorescein angiogram not showing any vascular leakage, (B). Diabetic Group fundus fluorescein angiogram showing vascular leakage as evident in the form of hyper-fluorescent spots (arrows), (C and](image-url)
D). Hsp-treated (Hsp-100 and 200 groups) fundus fluorescein angiogram not showing any vascular leakage.

Final fundus photographs were used for estimating arteriolar and venular diameter. Arteriolar and venular diameters were estimated as per methodology (Vucetic et al., 2004). Diameter measurements were obtained at three different locations along each vessel near optic disk within circles drawn (25, 50 and 100 µm diameters from optic nerve head), with an average of three measurements reported. Before diameter estimation, the retinal photographs from all groups were randomized and 3 independent observers did the estimations. V2K software (Towa Optics, Japan) was used for analyzing and garbing the fundus photographs.

**Fluorescein angiography**

For retinal angiography the same general fundus photography procedure was opted except that barrier filter is used for fluorescein angiography, and the illumination & strobe of the camera were adjusted for angiography. Rats were intraperitoneally injected with 20% sodium fluorescein Injection USP (Samarth Life Sciences Pvt. Ltd., India) at a dose of 0.012 ml per 5-6 gm body weight. Soon after injecting the dye angiograms were captured without any delay at regular intervals.

**Estimation of antioxidant parameters**

Estimation of anti-oxidant parameter such as Glutathione (GSH), Superoxide dismutase (SOD) and Catalase (CAT) were performed using commercially available
kits from Cayman Chemicals Ltd. All estimations were done as per manufacturer’s instructions.

Figure 8. (A). Effect of Hsp on retinal TNF-α levels in normal, diabetes and diabetes + Hsp groups after 24 weeks of diabetes. Values are mean ± SD, n=6. *p<0.001 diabetic vs. normal; #p<0.05 compared Hsp-100; @p<0.001 compared with Hsp-200; $p<0.05 compared with Hsp-200. (B). Effect of Hsp on retinal IL-1β levels in normal, diabetes and diabetes + Hsp groups after 24 weeks of diabetes. Values are presented as mean ± SD, n=6. *p<0.001 diabetic vs. normal; #p<0.05 compared Hsp-100; @p<0.001 compared with Hsp-200; $p<0.05 compared with Hsp-200.

Inflammatory parameters

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

Angiogenesis parameters

VEGF and PKC-β levels in retinae were estimated using commercially available enzyme-linked immunosorbent assay (ELISA) kit from RayBiotech, Inc, USA and USCN Life Science, Wuhan, China, respectively as per the manufacturer’s instructions. Protein estimation in each sample was done by Lowry’s method (Lowry et al., 1951).

Light Microscopy (LM) & 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 one hour, and incubated overnight in a moist chamber with the primary antibody diluted in PBS containing 3% normal goat serum and 0.5% Triton X-100. For rabbit polyclonal anti-Caspase-3 (1:1000), the sections underwent heat-induced antigen retrieval with a microwave oven (three 5-minute cycles in 10 mM Tris-EDTA buffer [pH 9] at 650 W). Further,
the sections were incubated with the biotinylated secondary antibody and reacted with the avidin-biotinylated peroxidase complex. The reaction product was visualized by incubation in development solution (containing 3, 3-diaminobenzidine (DAB) and hydrogen peroxide). The slides were faintly counterstained with hematoxylin in case of caspase-3. Finally, the sections were rinsed with distilled water, cleared, mounted and cover slipped.

Cryostat cut sections (4 µm) were stained with Haemotoxylin & Eosin (H&E) Stain 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 were reported per 100 µm length in total six retinas from each group.

Figure 9. (A). VEGF levels in Hsp-treated rats after 24 weeks of diabetes. Values are presented as mean ± SD, n=6. *p < 0.001 compared with normal; #p < 0.001 compared with Diab.+Hsp-100 and Diab.+Hsp-200 groups; $p <0.05 compared with Diab.+Hsp-200 groups. (B). PKC-β levels in Hsp-treated rats after 24 weeks of diabetes. Values are presented as mean ± SD, n=6. *p <0.001 compared with normal; #p < 0.001 compared with Diab.+Hsp-100 and Diab.+Hsp-200 groups; $p < 0.05 compared with Diab.+Hsp-200 groups.

**Glila Fibrillary Acidic Protein (GFAP), Aquaporin-4 (AQP4), Collagen IV and Fibronectin Immunofluorescence**

Fourteen µm cryosections were incubated in 10% normal goat serum for 1 h. The sections were then incubated in polyclonal rabbit anti-rat AQP4 antibody (Abcam Plc., UK, 1:1000), GFAP antibody (Abcam Plc., UK,1:1000), collagen IV (1:500 dilution, Santacruz) and fibronectin (1:1000 dilution, Santacruz) overnight at 4 °C, followed by anti-rabbit secondary antibody labelled with FITC (Molecular Probes) or Rhodamine to identify the localization of GFAP, AQP4, Collagen IV and Fibronectin in the retinal sections. Slides were mounted in anti-fade medium containing DAPI
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(Vectashield-DAPI; Vector Laboratories) to counterstain the nuclei, and images were obtained with microscope (Leica fluorescent microscope).

Figure 10. Caspase-3 Expression in normal, diabetic and Hsp-treated retina. A. Normal Retina shows normal pattern of Caspase-3 expression. Counterstained with hematoxylin, B. Diabetic retina showing increased expression of caspase-3 in astrocytes (NFL, arrow heads) and along Müller cell fibres (IPL, arrows), and INL, C and D. Hsp-treated Retina shows relatively lesser expression caspase-3 in NFL, IPL and INL. Counterstained with hematoxylin. NFL-Nerve Fibre Layer, INL- Inner Nuclear Layer, IPL-Inner Plexiform Layer, ONL- Outer Nuclear Layer, PRL - Photoreceptor Layer.

RT-PCR Analysis

cDNA was synthesised using a kit (QuantiTect Reverse Transcription Kit, Valencia, CA, USA) as per manufacturer’s protocol and stored at −20°C until use. Real-time PCR was performed with a sequence detection system (ABI Step One) and a Quantititect Sybr Green PCR Kit (Qiagen, Valencia, CA, USA). Primers (Table 5) were designed using NCBI Primer Blast software. cDNA was diluted to 8 ng/μl. The
PCR reaction mixture contained 10 μl of Sybr PCR master mix, 8 μl RNAse-free water, 1 μl (18μM) of forward and reverse primers, and 1 μl cDNA constituting a total volume of reaction mixture to 20 μl. Reactions were performed in triplicate at 95°C for 15 min; then 95°C for 15 s, 58°C for 30 s and 30 s at 72°C for 40 cycles, followed by a melt cycle consisting of stepwise increase in temperature from 72 to 99°C. The fold change values were calculated using Livak Method and values are normalized in response to GAPDH.

Figure 11. GFAP expression in normal, diabetic and treated retina. It is prominent in Müller cell inner processes (arrows) in diabetic retina (D-F), but insignificant in Hsp-100(G-I) and Hsp-200 treated retina (J-L). Normal retina is GFAP negative (A-C).
**Flat Mount Immunolabeling**

Immunolabeling of tight junction proteins was performed on whole mounted retinas prepared and processed as described by Barber et al. 1998 with some modifications. Eyes fixed in formalin for two hours were washed in PBS, and the retina was isolated, mounted nerve fibre layer side up on glass slides. The whole mounts were dehydrated through graded alcohols, defatted in xylene overnight at 4°C, then rehydrated, permeabilized with 0.3% Triton X-100 for 15 min, and treated with proteinase K (20 µg/ml) for 30 min at room temperature. Endogenous peroxidase was quenched with 3% hydrogen peroxide in methanol water solution for 10 min at room temperature. The preparations were blocked with 5% BSA in PBS and incubated for 2 h at room temperature. The flat mount sections were incubated overnight with the primary antibodies. The primary antibodies were polyclonal rabbit anti-Occludin (1:500; Invitrogen), mouse monoclonal anti-Claudin-5 (1:1000; Invitrogen), and rabbit polyclonal anti-ZO-1 (1:500; Invitrogen).

After incubation with primary antibody, retinal flat mounts were washed and incubated with secondary antibody (tagged with FITC or Rhodamine conjugates). Finally, washed and mounted with an anti-fade medium (Vectashield) and sealed with white nail polish. The preparations were observed under the fluorescence microscope, and multiple fields were photographed (Asnaghi et al., 2003).

**Western blotting analysis**

The retinae were isolated and homogenized in ice-cold RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, and 1% Nonidet P-40, with a protease inhibitor cock- tail. The protein concentrations of the samples were measured using the bradford's reagent. Each protein sample (25 µg) was separated by 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for Collagen-IV and fibronectin, and then transferred onto nitrocellulose membranes. Immunoblotting was performed using rabbit anti-rat Collagen IV (1:1000) and rabbit anti-rat Fibronectin (1:1000) as the primary antibodies. Further, the membranes were incubated with the biotinylated secondary antibody and reacted with the avidin-biotinylated peroxidase complex. The protein bands were visualized by incubation in development solution (containing 3,3-diaminobenzidinetetrahydrochloride (DAB) and hydrogen peroxide).
Hesperetin

Figure 12. Representative fluorescence micrographs showing AQP4 immunoreactivity in different study groups. (A-C), AQP4 immunoreactivity is moderately present in normal Müller cell endfeet; (D-F) In diabetic retina, AQP4 is strongly expressed in Müller cell endfeet (arrows) and in perivascular space (star); (G-I) Hsp-100 treated retina showing lesser immunoreactivity compared to that in diabetic retina and less staining in Müller cell endfeet; (J-L) Hsp-200 treated retina also showing lesser immunoreactivity compared to that in diabetic retina and less staining in Müller cell endfeet. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar: 25 µm (shown in Figure A, common to all micrographs).

Transmission Electron Microscopy

Eyes were enucleated from respective groups. Soon after enucleating cornea and lens were removed. The posterior segment carefully freed from vitreous adhering to retina gently with a blunt forceps and retina isolated. Further, retinal tissues were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) for 6 h at 4 °C. After fixation, the retina was circumscribed around 2mm around the optic
Hesperetin

nerve head and further trimmed into 1 mm² pieces. After fixation in 1% osmium
tetraoxide, tissue is dehydrated, and embedded in araldite CY 212. Semithin section
(500 nm thick) were stained with 0.5% toluidine blue and examined under a light
microscope. The LM images thus obtained were evaluated to study LM changes in
retina of respective groups. After selecting the areas of interest, the blocks (70nm)
were cut on an ultra-microtome and placed on the grids. Final staining was done with
uranyl acetate and lead citrate, and viewed under TECNAI G20 transmission electron
microscope (FEI Company, Netherlands). Digital Micrograph™ software was used
for analysing the ultramicrographs.

Quantification of Photoreceptor Nuclei - Photoreceptor damage was quantified by
counting the number of Photoreceptor nuclei per 25 µm Square grid using Digital
MicrographTM Software. Quantification was done in central retina, nasal peripheral retina and temporal peripheral retina in a total of six retinae per group.

**Measurement of Basement Membrane Thickness** - We evaluated only the capillaries from the outer plexiform layer. Electron micrographs of six randomly selected capillaries from mid-retina per animal and a total of 4 rat retinae were analysed per experimental group. Only cross-sectioned capillaries were considered. BM thickness was measured as per method described by Siperstein, et al., 1966 method. A grid equally divided into 24 clock hours was superimposed on a vessel. The BM thickness was measured at point of intersection by each spoke. We have also included two points having minimal BM thickness.

**Statistical analysis**

All data are expressed as mean ± SD. The groups were compared by one-way ANOVA with suitable tukey post hoc multiple comparison test. Data were considered statistically significant at p-value < 0.05.

**Results**

**Blood Glucose and Body Weight (Study-I)**

Blood glucose levels in the diabetic group (498.50 ± 18.51 mg/dl) were significantly higher than in the normal rats (103.50 ± 13.86 mg/dl) (p<0.001) at the end of 24 weeks period. In Hsp-100 and Hsp-200 treated rats the blood glucose levels (458.27 ± 33.95 and 390.57±37.07 mg/dl) were significantly lower than in the diabetic group

Table 1. Effects of Hsp on body weight and glycemic parameters

<table>
<thead>
<tr>
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<th>Diabetic</th>
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<th>Hsp-200</th>
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<tr>
<td><strong>Body Weight (gms)</strong></td>
<td>450.00±18.79</td>
<td>293.60± 4.86*#</td>
<td>381.09±23.48NS</td>
<td>379.42±25.47</td>
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<tr>
<td><strong>Blood Glucose (mg/dl)</strong></td>
<td>103.50 ± 13.86</td>
<td>498.50 ± 18.51*#</td>
<td>458.27 ± 33.95NS</td>
<td>390.57±37.07</td>
</tr>
<tr>
<td><strong>%HBA1C</strong></td>
<td>3.81±0.45</td>
<td>8.60±0.64*@</td>
<td>6.68 ± 0.48NS</td>
<td>6.82 ± 0.70</td>
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Values are Mean ± S.D. *P<0.001 (Normal Vs Diabetic); *P<0.001 (Diabetic Vs Hsp-100 and Hsp-200); @P<0.05 (Diabetic Vs Hsp-100 and Hsp-200). Differences in body weight and blood glucose were analysed by Kruskal wallis test. One way ANOVA was used for %HBA1C. NS = Difference between Hsp-100 and Hsp-200 were insignificant.
Table 2. Effects of Hsp on angiogenic parameters.

<table>
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<th>Hsp-200</th>
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<tr>
<td>VEGF (pg/mg protein)</td>
<td>8.35±1.02</td>
<td>19.80±1.31*#</td>
<td>14.95±1.05$</td>
<td>12.58±1.22</td>
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<tr>
<td>PKC-beta (pg/mg protein)</td>
<td>31.90±3.68</td>
<td>144.12±14.09*#</td>
<td>92.16±10.18$</td>
<td>74.00 ±11.22</td>
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Values are Mean ± S.D, n=6. *P<0.001 (Diabetic Vs Normal); #P<0.001 (Diabetic Vs Hsp-100 and Hsp-200); $P<0.05 (Hsp-100 Vs Hsp-200). Differences were analyzed by one way ANOVA followed by post hoc tukey test.

(p<0.001), though remained higher than normal (p<0.001). Similarly, HbA1C levels in diabetic rats (8.60± 0.64) were significantly higher than the normal rats (3.81± 0.45) (p<0.001). In Hsp-treated (Hsp-100 and Hsp-200) rats the HbA1C levels (6.68 ± 0.48 and 6.82 ± 0.70) were significantly lower (p<0.05) than in the diabetic group.

Body weight in normal group was found to be increased by 51.71% as compared to diabetic group with a weight gain of 22.34%. Rats in Hsp-100 and Hsp-200 group gained 45.54 and 45.35%, respectively. Diabetic rats showed all three cardinal signs i.e. Polyuria, polyphagia and polydipsia (Table-1).

**Blood Glucose and Body Weight (Study-II)**

Blood glucose levels in the diabetic group (541.50 ± 52.09 mg/dl) were significantly higher than in the normal rats (100.67 ± 5.01 mg/dl) (p<0.001) at the end of 24 week period. In Hsp-treated (Hsp-100 and Hsp-200) rats the blood glucose levels (464.85 ± 37.53 and 452.92 mg/dl) were significantly lower than in the diabetic group (p<0.05) (Table-3).

Body weight in normal group was found to be increased by 54.32% as compared to diabetic group with a weight gain of 20.86 %. Rats in Hsp-treated (Hsp-100 and Hsp-200) group gained 35.63% and 38.92%.

**Fundus Photographs and Microvasculature Diameter**

Fundus photographs from diabetic group showed hyperpermeable optic nerve head and region around it as compared to normal group retinae (Fig. 4A and B). On the other hand, Hsp-treated group retinae do not show any such vascular dysfunction.
(Fig.4C). Retinal blood vessels (arterioles and venules) in diabetic group were estimated to be dilated than normal group (p<0.001). However, Hsp-treated rats showed significantly lesser dilated vessels (arterioles and venules) as compared to diabetic group (p<0.05) (Fig. 5A and B).

**Fluorescein Angiography**

Normal rat angiograms showed no dysfunction of either kind at the end of six months (Fig.6A). Diabetic rat angiograms showed vascular leakages in the form of hyperfluorescent dots in the retinae (Fig. 6B) and also around the optic nerve head (Fig.6C). Hsp-treated rat retinal angiograms showed lesser degree of vascular dysfunction compared to untreated rats (Fig.6D).
Table 3. Effects of Hsp on body weight and blood glucose.

<table>
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<tr>
<td>Body Weight (gms)</td>
<td>445.17±19.94</td>
<td>273.75±8.16</td>
<td>326.23±14.50</td>
<td>343.08±18.25</td>
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<tr>
<td>Blood Glucose (mg/dl)</td>
<td>100.67±5.01</td>
<td>541.50±52.09</td>
<td>464.85±37.53</td>
<td>452.92±49.73</td>
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Values are Mean ± S.D, * P < 0.001 (Normal Vs Diabetic); # P < 0.001 (Diabetic Vs Hsp-100 and Hsp-200); @ P < 0.05 (Diabetic Vs Hsp-100 and Hsp-200). Differences in body weight and blood glucose were analysed by Kruskal wallis test. One way ANOVA was used for %HBA1C. NS = Difference between Hsp-100 and Hsp-200 were insignificant.

Table 4. Effects of Hsp on inflammatory and anti-oxidant parameters.

<table>
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<td>TNF-α (pg/mg protein)</td>
<td>18.71±2.58</td>
<td>42.29±4.83</td>
<td>28.77±4.76</td>
<td>23.85±2.64</td>
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<td>IL-1β (pg/mg protein)</td>
<td>37.32±7.14</td>
<td>93.51±7.34</td>
<td>70.52±6.47</td>
<td>62.79±6.13</td>
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<tr>
<td>GSH (nM/mg protein)</td>
<td>17.81±0.77</td>
<td>3.76±0.62</td>
<td>7.17±0.67</td>
<td>9.20±0.98</td>
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<tr>
<td>SOD (IU/mg protein)</td>
<td>7.76±0.99</td>
<td>2.17±0.17</td>
<td>4.51±0.47</td>
<td>5.76±0.74</td>
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<tr>
<td>CAT (IU/mg protein)</td>
<td>10.59±1.35</td>
<td>3.01±0.41</td>
<td>6.77±0.76</td>
<td>9.25±0.54</td>
</tr>
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Values are Mean ± S.D, n=6. * P < 0.001 (Diabetic Vs Normal); # P < 0.05 (Diabetic Vs Hsp-100); @ P < 0.001 (Diabetic Vs Hsp-200); $ P < 0.05 (Hsp-100 Vs Hsp-200). Differences were analyzed by one way ANOVA followed by post hoc tukey test.

Antioxidant parameters

Retinal GSH levels were almost four fold lower in diabetic rats as compared to normal rats (p<0.001). However, in Hsp-treated (Hsp-100 and Hsp-200) rats, retinal GSH levels were significantly higher than diabetic group (p<0.05 and p<0.05,
The antioxidant enzymes SOD and CAT showed more than three fold decrease in activity in diabetic retinas as compared to normal retinae (p<0.001). Both SOD and CAT enzymatic activities were restored close to normal in Hsp-treated (Hsp-100 and Hsp-200) diabetic rats (p<0.05 and p<0.05, respectively) (Fig. 7B) (Table-4).

**Inflammatory parameters**

TNF-α estimations in untreated diabetic retinas showed more than 2 fold higher levels than in the normal retinae (p<0.001). The TNF-α levels in the retinae from Hsp-treated (Hsp-100 and Hsp-200) rats were significantly lower than untreated diabetic retinae (p<0.05 and p<0.05, respectively) (Fig. 8A).

Figure 16. Representative immunofluorescence micrographs of Collagen-IV, (A-C). Normal retina showing lesser normal pattern of collagen in inner retina, (D-F). Diabetic retina showing strong expression of collagen-IV in inner retina and in outer retina between intracellular spaces of outer nuclear layer, (G-I). Hsp-treated retina showing reduced expression of collagen as compared to diabetic retina.
Mean IL-1β value in normal rat retinas was found to be more than 2 fold lower than the untreated diabetic retinas (p<0.001). Mean IL-1β values in Hsp-treated (Hsp-100 and Hsp-200) rats were significantly lower than untreated diabetics (p<0.05 and p<0.05, respectively) (Fig. 8B) (Table-3).

**Angiogenic Parameters**

VEGF estimations in untreated diabetic retinas showed more than 2 folds higher levels than in the normal retinas (p<0.001). The VEGF levels in the retinas from Hsp-treated rats were more than 1.5 fold lower than the untreated diabetic retinas (p<0.001), but remained significantly higher than normal retinas (p < 0.05) (Fig. 9A).

Similarly, PKC-β levels in normal rat retinas were found to be more than 4 folds lower than the untreated diabetic retinas (p<0.001). PKC-β values in Hsp-treated rats were significantly lower than the untreated diabetics (p<0.001), but remained significantly higher than normal retinas (p<0.001) (Fig. 9B) (Table-2).
Figure 18. (A). Retinal proteins were extracted using RIPA buffer and separated by SDS-PAGE. (B). showing relative expression of collagen-IV and fibronectin in normal, diabetic and Hsp-treated group. Normal vs Diabetic, *p< 0.001; Diabetic vs Hsp, #p< 0.05.

**Immunohistochemistry**

Generalised pattern of caspase-3 expression was seen in normal rat retina (Fig. 10A). On the other hand, intense expression of caspase-3 was seen in nerve fibre layer (astrocytes) and Müller cells in case of diabetic retina (Fig. 10B). However, Hsp-treated (Hsp-100 and Hsp-200) retina showed relatively lesser expression of caspase-3 than diabetic retina (Fig.10C and D).

**GFAP and AQP4 Immunofluorescence**

Positive GFAP expression was seen in diabetic retina (Fig.11A and B); however insignificant GFAP expression was seen in treated retina (Fig. 11C and D). Normal retina served as negative control for GFAP expression.
Hesperetin

Figure 19. Photomicrographs of rat retina in normal and other experimental groups. A. Retinal architecture in normal group, B. Diabetic retina showing degenerated outer nuclear layer (ONL) and photoreceptor layer (PRL). Inner retina is still attached by many Müller cell processes as seen in the form of strands (arrows), extensive degenerated photoreceptor layer might be a complication of retinal detachment (this is a rare observation), C. Magnified view of selected region from Fig. B, showing clearly visible Müller cell strands (arrows), D. Hsp-100 treated retina showing intact retinal layers without any signs of diabetes induced structural changes, E. Hsp-200 treated showing normal retinal layer NFL, nerve fibre layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; PRL, photoreceptor layer; PEL, pigment epithelial layer.

In diabetic retina, AQP4 was strongly expressed in Müller cell endfeet and in perivascular space (Fig. 12E and F) as compared to normal retina showing moderate immunoreactivity in Müller cell endfeet (Fig. 12C and D). Hsp-100 (Fig. 12H and I) and Hsp-200 (Fig. 12K and L) retina showed lesser immunoreactivity compared to diabetic retina and less immunofluorescence in Müller cell endfeet.
**Figure 20.** Effect of Hsp on retinal ganglion cell apoptosis after 24 weeks of diabetes. Ganglion cell counting was done per 100µm of retina in normal, diabetes and diabetes + Hsp groups. Values are mean ± SD, n=6. (*p < 0.05) diabetic vs. normal; (#p < 0.05) diabetic vs. Hsp-treated (Hsp-100 and Hsp-200) diabetics.

**RT-PCR Analysis**

Occludin gene expression has been significantly reduced in diabetic retinae. However, Occludin gene expression was significantly increased in Hsp-treated group (Fig.13). Similarly, in case of Claudin-5 and ZO-1 genes expression have been significantly reduced in diabetic retina. However, Claudin-5 and ZO-1 genes expression were significantly increased in Hsp-treated retina (Fig. 14).

**Flat Mount Immunolabelling**

Uniform expression of tight junction proteins was observed in flat mount retinal sections of normal rats compared to diabetic rats showed reduced expression of tight junction proteins. However, Hsp-treated retinae showed similar expression pattern of tight junction proteins as compared to normal retinae (Fig.15).
Figure 21. Representative LM images of different study groups. (A). Normal retina showing retinal layers without any obvious damage (B). Diabetic retina showing edematous Müller cell endfeet (arrow), increases in intercellular spaces in INL and ONL due to apoptosis and degenerated PRL (arrow) (C and D). Hsp-treated (Hsp-100 and Hsp-200) retina showing lesser edematous changes in GCL and INL. The ONL and PRL appear intact. GCL-ganglion cell layer, IPL- inner plexiform layer, INL-inner nuclear layer, ONL-Outer nuclear layer, PRC-photoreceptor layer. Scale bar -50 μm.

Collagen IV and Fibronectin Immunofluorescence

Diabetic rats showed strong expression of Collagen IV in inner retina as compared to normal rats. However, there was relatively lesser expression of Collagen IV expression in Hsp-treated retina (Fig. 16).

Similarly, normal retinae showed normal pattern of fibronectin expression in AND around blood vessels in NFL and OPL. However, diabetic retinae showed over-expression of fibronectin in blood vessels of NFL and OPL. On the other side, Hsp-treated retinae showed lower expression of fibronectin in NFL and OPL (Fig. 17).
Figure 22. Effect of Hsp on photoreceptor degeneration, A. Normal photoreceptor nuclei structure, B. Diabetic retina showing degenerated and pyknotic photoreceptor nuclei, C-D. Hsp-100 and Hsp-200 treated retina showing well characterized photoreceptor nuclei, except few pyknotic nuclei (star; in fig.C).

Figure 23. Photoreceptor nuclei were counted per 25µm square area at mid and periphery retina regions. Values are mean ± SD, n=6. (p < 0.001) diabetic vs. normal; (p < 0.05) diabetic vs. Hsp-treated (Hsp-100 and Hsp-200) diabetic.
**Western blot**

Collagen-IV band was localized at 170 kDa and fibronectin band was localized at 220 kDa. We found that protein expression of collagen–IV and fibronectin were significantly increased in diabetic retinae. However, Hsp inhibited protein expression of collagen-IV and fibronectin to a significant extent (Fig. 18).

**Light and Electron microscopic Studies**

Diabetic retina showed degenerated outer nuclear layer and photoreceptor layer as a result of separation from retinal pigment epithelium layer (Fig. 19B). Inner detached retina is attached by many Müller cell processes as seen in the form of strands (Fig. 19B and C). Hsp-treated (Hsp-100 and Hsp-200) retina showing intact retinal layers without any signs of diabetes induced structural changes (Fig. 19D and E). The retinal detachment and extensive photoreceptor degeneration was rare in experimental rat and other models, and only detected once out of twelve diabetic retinae studied in the present study (six from each diabetic and Hsp-treated diabetic) after six months of diabetes.

Average ganglion cell counts in diabetic retinae were found to be significantly less than normal retinae. However, Hsp-treatment helped in rescuing apoptotic ganglion cell death as compared to diabetic retinae (Fig. 20).

LM image of toluidine stained normal retina showed well organized structural morphology (Fig. 21A). On the other hand, LM image of diabetic retina has shown edematous Müller cell endfeet, increase inter-cellular spaces in inner nuclear and outer nuclear layers. Further, photoreceptor layer was also shown to be degenerated and damaged (Fig. 21B). However, Hsp-treated retina showed comparatively lesser degenerated changes in ganglion cell layer and inner nuclear layer. Outer nuclear layer and photoreceptor layer appeared to be normal in structure (Fig. 21C and D).

Further, electron microscopic ultrastructure showed degenerated and pyknotic photoreceptor nuclei; and degenerated rod outer segment in diabetic retina (Fig. 22B, Fig.23, 24B, C and H). On the other hand, Hsp-treated (Hsp-100 and Hsp-200) retina showed well defined photoreceptor nuclei and rod outer segment (Fig. 22C, Fig.23, 24D-F and I-J).
Electron microscopic observations of normal rat retinas clearly showed thin BM as compared to diabetic group. However, treatment with Hsp in diabetic rats prevented thickening of BM as compared to diabetic rats (Fig. 25 and 26).

**Discussion**

Diabetic complications such as DR are a result of multiple metabolic, vascular and inflammatory defects. Uncontrolled hyperglycemia is the key metabolic abnormality in DM. The early signs of DR in experimental diabetic models include vascular inflammatory response due to oxidative stress results in over-expression of pro-inflammatory cytokines (TNF-α, IL-1β etc), and consequent over-expression apoptotic markers. As a result pericyte death retinal capillary leads to ischemia, thereafter causing compromised vascular and neural functions.

Uncontrolled hyperglycemia induced retinal metabolic abnormalities lead to increased oxidative stress, which contribute to development of diabetic complications such as DR (Gurler et al., 2000; Kowluru and Koppolu, 2002a; Kowluru and Kowluru, 2007). Retina has a highly efficient antioxidant defence mechanism comprising of free radical scavengers such as α-tocopherol, glutathione, and ascorbic acid and antioxidant enzymes such as glutathione peroxidase, SOD and CAT (Armstrong et al., 1981; Castorina et al., 1992; Kowluru, et al., 2001). The diabetic rats in our study shows decreased levels of GSH and subnormal activity of SOD & CAT as compared to normal rats (Fig. 7A and B). These changes in antioxidant parameters are in accordance with earlier studies (Obrosova, et al., 2006; Gupta et al., 2011). Hsp-
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treated retinae shows restoration of antioxidant defense system of retina back to normal, thereby preventing retinal inflammatory changes (Fig. 7A and B). Hsp being a flavanone offers powerful anti-oxidant activity which modulates the enzymatic activity effectively (Aranganathan and Nalini, 2009; Aranganathan et al., 2009; Kim et al., 2004). Similarly, in our study positive modulation of GSH and antioxidant enzymes was observed.

Pro-inflammatory cytokines (TNF-α & IL-1β) are the key mediator in neurovascular inflammation and upregulated in DR. IL-1β is very well studied in Blood Retinal Barrier Breakdown (Bamforth et al., 1997) and induction of retinal capillary cell apoptosis in diabetic retina (Kowluru and Odenbach, 2004a; Kowluru and Odenbach, 2004b). Further, contribution of TNF-α to the pathogenesis DR is supported by a number of reports (Joussen et al., 2002; Joussen et al., 2003) and significantly higher levels were found in the plasma of diabetic patients (Foss et al., 1992; Zorena et al., 2007). Similarly, in the present study significantly higher levels of pro-inflammatory cytokines (TNF-α & IL-1β) were found in the retinae of diabetic rats versus normal rat retinae (Fig. 3A and B). However, Hsp-treated diabetic retinae showed significantly lower levels of TNF-α & IL-1β as compared to diabetic retinae (Fig. 8A and B). Similarly, various studies have shown that dietary flavanoids including Hsp inhibits IL-1β (Kowluru and Odenbach, 2004b; Sharma et al., 2007) & TNF-α (Xangorari et al., 2001; Vafeiadou et al., 2009) mediated retinal inflammation and apoptosis.

VEGF, also known as vascular permeability factor, is a cytokine with strong angiogenic and mitogenic actions as a result plays major role in retinal vascular leakage and neovascularisation during late stage of retinopathy. Ocular VEGF levels are strongly correlated with neovascularisation in patients with diabetes (Aiello et al., 1994), and also intravitreal administration of anti-VEGF proteins has neutralized the angiogenic effects of VEGF in experimental models (Aiello et al., 1995). VEGF interacts with VEGF-R1 and VEGF-R2 receptors results in phosphoinositol hydrolysis and release of DAG, which again leads to activation of PKC-β (Xia et al., 1996). PKC-β is important mediator in VEGF signaling pathway. Various
experimental studies have shown that angiogenic response, as a result of retinal

Figure 25. Retinal capillary endothelial BM thickness in different groups. (A) Capillary from normal group, showing a thin BM (90.45 nm, arrowheads), (B) Capillary from diabetic group, showing a thick BM (194.32 nm), (C and D) Retinal capillary from Hsp-treated (Hsp-100 and 200 groups) group, showing a relatively thin BM (140.12 and136.18 nm). l, lumen of capillary; n, nucleus of endothelial cell; RBC, Red blood cells. Scale Bar-500 nm.

ischemia, is increased in mice over-expressing the PKC- β isoform and also mitogenic action of VEGF has been found to be increased in retinal endothelial cells over expressing the PKC- β isoform (Suzama et al., 2002). Further, others have also shown that raised VEGF and PKC- β levels in retinas of patients with DR (Aiello et al., 1994; Kim et al., 2010a). Similarly, in the present study there are increased levels of VEGF and PKC- β are estimated in diabetic retinæ compared to normal retinæ. However, Hsp treatment prevented increase in the expression of angiogenic parameters (VEGF and PKC- β) suggesting it as potential anti-angiogenic candidate (Fig. 6A and B). Further, various earlier studies have also shown its potential anti-
angiogenic effects (Choi et al., 2006). Apart from this, dietary polyphenolic compounds are very well studied for their vasoprotective effects and have shown to downregulate expression of VEGF (Nicholson et al., 2010; Mojzis et al., 2008; Kumar et al., 2012).

In the present study, fluorescein angiography was performed by injecting intraperitoneal sodium fluorescein and obtained angiograms showed normal arterial-venous cycle. Subtle retinal vascular abnormalities, retinal capillary angiography and vascular leakage can only be detected using this procedure. The retina from diabetic group shows vascular leakage as evident from fluorescein angiograms and a significant greater grading score was calculated as compared to diabetic retinæ (Fig. 6). Similarly, various experimental studies have shown breakdown of blood retinal barrier as a results of activation of growth factors (VEGF & PKC-β) due to hyperglycemia (Grant et al., 2010; Kim et al., 2010a, 2010b; Wang et al., 2010). Fundus photographs shows significant dilatation of arterioles and venules, and hyper-pigmentation, due to increased vascular permeability, around the optic nerve head as compared to normal retinæ (Fig.4). So, these are certain classic features of early stage DR that have seen in diabetic retinæ in the present study. Other experimental studies have also shown similar findings in diabetic retinæ (Maharjan et al., 2011, Kim et al., 2010a, 2010b). However, Hsp-treated retinæ shows normal fundus photographs and significantly less dilatation of arterioles and venules as compared to diabetic retinæ. Fluorescein angiograms of the Hsp-treated retinæ detected no clearly defined vascular leakage and significantly lower grading score was calculated as compared to diabetic retinæ. Therefore, it can be said that Hsp treatment has offered a protective effect on diabetes induced vasculopathy as evident from results of the present study. The vasculo-protective effect of Hsp can be postulated due to its strong anti-angiogenic effects via inhibiting VEGF & PKC-β pathways. Similarly, in earlier studies Hsp has found to prevent or decrease vascular permeability or leakage (Paysant et al., 2008). Moreover, vascular protective effect of flavonoids has been very well studied in animal studies as well as in patients clinically (Stoclet et al., 2004).
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Figure 26. Effect of Hsp on retinal capillary BM thickness after 24 weeks of diabetes. Values are mean ± SD, n=6. *p≤0.001 compared with normal; #p < 0.001 compared with Diab.+Hsp-100 and Diab.+Hsp-200 groups. $=differences are insignificant between Diab.+Hsp-100 and Diab.+Hsp-200 groups.

BRB is composed of arrangement of tight junction proteins in between endothelial cells forming a tight barrier to restrict the normal movement of large molecular weight compounds across the barrier. The principal proteins found specifically in retinal endothelial tight junctions are occludin and the claudin-5. Both, occludin and claudin-5, are transmembrane proteins that are linked to the actin cytoskeleton via interaction with the cytosolic protein ZO-1. ZO-1 is a membrane-associated TJ adaptor protein that links occludin and the claudin-5 to the cytoskeleton (Paris et al., 2008). Hyperglycemia results in over-expression of MMP-9 activity in retinal endothelial cells both in-vivo and in-vitro, leading to proteolytic degradation of tight junction proteins (Giebel et al. 2005). Similarly, in the present study, we have found low expression of tight junction proteins (Occludin, Claudin-5 and ZO-1) in diabetic retinal vasculature as compared to normal retinal vessels. However, hesperetin treatment was found to be very useful in preserving tight junction structures.

Hyperglycemia acts as a trigger that causes activation of various apoptotic proteins involved in apoptotic cell death, including members of the caspase family (Allen et
al., 2005). Earlier studies showed that caspases play important role in the initiation and execution of apoptosis (Katai and Yoshimura, 1999; Doonan and Cotter, 2002). Moreover, caspase-3 has been studied to play an important role in diabetes and its complications (Kern et al., 2000; Kowluru and Koppolu, 2002b). Similarly, in the present study, we have seen an increased expression of caspase-3 in nerve fibre layer and along Müller cell processes as compared to normal retina (Fig. 10B). However, treatment with Hsp inhibited the expression of caspase-3 as compared to diabetic retina (Fig. 10C). Our findings are consistent with earlier studies that anti-oxidant play important role in inhibiting caspase-3 mediated retinal apoptosis (Kowluru and Koppolu, 2002b).

The Müller (radial glial) cells are the principal glia of all vertebrate retinas. They stabilizes the retinal architecture, helpful in fluid homeostasis and support neuronal survival and information processing. It has been studied that Müller cell destruction causes retinal dysplasia, photoreceptor apoptosis and, finally, retinal degeneration (Mizutani et al., 1998; Bringmann, et al., 2004; Dubois-Dauphin et al., 2000). In the present study, diabetic retina showing edematous Müller cell end feet in nerve fibre layer (Fig. 21B & 12F), ganglion cell loss (Fig. 20), increased intercellular spaces in inner-nuclear layer and outer nuclear layer, degenerated outer retina due to apoptotic cell death as a result of over-expression of caspase-3 (Fig. 10), and also Photoreceptor layer degeneration due to inflammation induced apoptosis and retinal detachment (Fig. 10 and 11). However, LM images of toluidine and H&E stained normal retina showing normal Müller cell structures and all nuclear layers without any obvious damage or degeneration (Fig. 19A and 21A). Similarly, Hsp-treated retina showing

### Table 5. Gene nomenclature, GenBank accession code, primer sequences and predicted size of the amplified product for rat genes.

<table>
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<tr>
<th>Unigene Symbol</th>
<th>Description</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
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<td>CGCCCCACCTTGATTTTGGGA</td>
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lesser edematous Müller cell end feet in nerve fibre layer and

![Flow chart showing antiangiogenic effects of Hsp in prevention of diabetic retinopathy.](image)

Figure 27. Flow chart showing antiangiogenic effects of Hsp in prevention of diabetic retinopathy.

normally organised inner-nuclear layer (Fig. 21C and D). The preventive effects observed in Hsp-treated retina are due to its potential anti-oxidant, anti-inflammatory and anti-apoptotic properties.

Further, Increased expression of GFAP is the hallmark of glial cell reactivity. Earlier various studies have shown significantly increased expression of GFAP in diabetic retina (Feit-Leichman, et al., 2005; Mizutani, et al., 1998). Similarly, in the present study we have observed increased GFAP expression along Müller cell processes. However, Hsp-treated retina showed insignificant expression of GFAP (Fig. 11).
Previous studies have also shown inhibitory effects of flavanoids on GFAP activation in Müller cells (Yang, et al., 2009).

AQP4, a water specific membrane-channel protein, is more specifically expressed in Müller cells and astrocytes, and its overexpression has been implicated in neuronal and glial swelling (Da and Verkman, 2004; Nagelhus et al., 2004; Bringmann et al., 2005; Liu et al., 2007). Earlier studies have shown that intense expression of AQP4 at Müller cell end feet (Nagelhus et al., 1998; Da and Verkman, 2004). Similarly, we have found increased intensity of AQP4 at Müller cell end feet in diabetic retina as compared to normal rat retina (Fig. 12F and C). However, Hsp treated retina showed decreased expression of AQP4 at Müller cell end feet (Fig. 12I and L). Further, various authors have suggested that AQP4 inhibition could be considered as novel target for therapeutic intervention (Nagelhus et al., 1998; Da and Verkman, 2004; Bringmann et al., 2005).

Thickened BM of retinal capillary is hallmark of retinopathy in patients with type I and Type II diabetes (Roy and Sato, 2000). The microvascular BM acts as a barrier to prevent vascular permeability, therefore any changes to the BM structure results in increased vascular permeability. The BM consists of various components that are arranged in a highly organized fashion, such as type IV collagen, fibronectin, laminin, and heparan sulfate proteoglycans (Roy et al., 2011). During hyperglycemic state there is increased formation and decreased degradation of these components results in the thickening of vascular BM. Earlier studies have suggested role of growth factors, PKC and VEGF, in the over-expression of various BM matrix proteins and extracellular matrix related gene expression (Kuiper et al., 2007; Studer et al., 1993). Since PKC activation has been shown to be a precursor for triggering different diabetic complications. Therefore, PKC has been already targeted for the treatment of DR because its inhibition cause down-regulation of extra-cellular matrix protein as a result prevents thickening of BM (Clarke and Dodson, 2007). Similarly, in the present study diabetic retina shows significantly thicker BM as compared to normal retinae.
(Fig.25 and 26). However, Hsp treatment was found to be effective in preventing BM thickening possibly by inhibiting pro-angiogenic parameter expressions (PKC & VEGF).

A recent study has shown transcorneal permeation of Hsp across isolated rabbit cornea (Sriramgam and Majumdar, 2010). Various pharmacokinetics studies have shown that permeability of Hsp in various ocular tissues (Chroidal, Sceeral etc) (Franke et al., 2005). Therefore, ocular bioavailability is very well established. Further, the dose range of Hsp (100 and 200 mg/kg BW) are well within the feasible range as already studied for its anti-oxidant action in healthy volunteers (Majumdar and Srirangam, 2009).

Therefore, the present study is first to clearly demonstrates the therapeutic benefits of Hsp in rescuing retinal vasculopathy, neuroinflammation, oxidative stress,
apoptosis and edema as a result of chronic uncontrolled hyperglycaemic state. In conclusion, it can be postulated that dietary flavanoids, like Hsp, can be very effective for the prevention of diabetes induced neurovascular complications such as DR.

**Summary**

In the present part, we studied the protective effects of hesperetin on diabetes induced neurovascular degeneration in rats. In the first part, the role of hesperetin in the prevention of retinal vasculopathy has been evaluated in diabetic rats. Various parameters evaluated like effect of hesperetin on basement membrane thickness, dilatation of retinal vessels, vascular leakage and angiogenic factors (VEGF and PKC-α). Further, the results clearly showed that hesperetin could be a potential candidate for the prevention of microvascular degeneration and vascular leakage.

In the later study, we studied the effects of hesperetin on retinal neuroinflammation, oxidative stress and apoptosis in diabetic rats. We found that hesperetin acts as good anti-inflammatory agent (via inhibition of TNF-α and IL-1β), anti-oxidant (as it positively modulated retinal levels of GSH and anti-oxidant enzymes; SOD and CAT) and anti-apoptotic agent via inhibition of caspase-3 activity. Apart from this, hesperetin prevented formation of edema in muller cell endfeet in diabetic rats. The present study also showed significant protective effects of hesperetin on diabetes induced photoreceptor degeneration.

We also evaluated effects of hesperetin on gene expression of tight junction proteins (Occludin, Claudin-5 and ZO-1) and protein expression of extracellular matrix (Collagen-IV and Fibronectin) in diabetic rat retina. Results showed that hesperetin prevents inhibition of gene expression of tight junction proteins and inhibits increase expression of collagen-IV and fibronectin in diabetic retinæ.