Correlation between Tissue Pathology and Vitreal Levels of hsCRP, IL-6 and TNF-α in Diabetic Rats Treated with Aspirin

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Abstract   Diabetic retinopathy is a progressive neurological complication of diabetes. Microvascular changes accompany diabetic retinopathy and may result in blindness. Inflammation has been shown to underlie the pathogenesis of diabetic retinopathy. We investigated the correlation between inflammatory markers and tissue pathology in diabetic rat eye. We also tested the role of anti-inflammatory drugs (aspirin) in the prevention of diabetic retinopathy. Ninety male Wistar albino rats were used in the present study. Rats were equally divided into 3 groups; control group, diabetic (streptozotocin, 50 mg/kg i.p), and aspirin-treated (ASPIRIN PROTECT, 2 mg/kg/d) diabetic group. All animals were scarified 6 months after the induction of diabetes. Histology and immunohistochemistry were conducted on the tissues of the rats' eyes, while vitreous samples were collected for the measurement of hsCRP, IL-6 and TNF-α. The levels of the measured inflammatory markers were significantly higher in diabetic rats, and notably correlated to the histo-pathological changes. Treatment with aspirin lowered the elevated levels of hsCRP, IL-6 and TNF-α with a considerable protective effect on the affected tissues. Our data suggest the possibility that hsCRP, IL-6 and TNF-α may be a cause of diabetic retinopathy progression and not necessarily a result. We also concluded that anti-inflammatory drugs which target hsCRP, IL-6 and TNF-α may play a crucial role in the prevention of diabetic microvascular complications.

Keywords   Dibetic retinopathy, Aspirin, hsCRP, IL-6, TNF-α

1. Introduction

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes and the main cause of blindness in the working-age adults [1]. DR is a progressive neurological disease which is accompanied by microvascular changes that may result in vision loss. Diabetes-induced neuronal cell loss was evident in retinas of experimental models of DR [2, 3]. The manifestations of DR are such consequences of the underlying microangiopathy; thickening of the basement membrane, accelerated apoptosis, increased permeability, formation of microaneurysms and transformation into acellular tubes [4, 5]. DR is a multifactorial complication that may involve a number of pathological pathways such as inflammation, oxidative stress, production of advanced glycation products (AGPs) and activation of protein kinase C (PKC). These pathways in turn contribute in the production of several inflammatory mediators and cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, vascular endothelial growth factor (VEGF), pigment epithelium-derived or (PEDF), P-selectin, vascular cellular adhesion molecule (VCAM)-1 and intracellular adhesion molecule (ICAM)-1 [6-8].

Being inflammatory in nature, DR and chronic inflammation share several similarities such as increased pro-inflammatory cytokines and chemokines, cell infiltration, tissue destruction, neovascularisation and altered vascular permeability [9]. Inflammation has been shown to underlie the pathogenesis of DR in experimental animals. However, the correlation between inflammatory mediators and DR in humans is quite conflicting [10, 11].

The inflammatory cytokines TNF-α, IL-6 and C-reactive protein (CRP) has been associated with metabolic syndrome [12]. TNF-α, a pro-inflammatory cytokine, has been suggested to contribute to the pathogenesis of DR. TNF-α has been found to be increased in vitreous of patients with DR [13], however, others reported the contrary [14]. IL-6 is a pleiotropic cytokine with a key impact on the pathogeneses underlying obesity, insulin resistance, β-cell destruction, type I and type II diabetes [15]. A vast number of epidemiological, genetic, rodent, and human in vivo and in vitro studies reported elevated levels of IL-6 in DR and

highlighted the putative role of IL-6 in the progression of DR [16-18]. C-reactive protein (CRP) is an inflammatory marker that has been shown to be involved in atherogenesis, endothelial dysfunction, macrovascular disorders as well as non-ocular complications of diabetes [19]. The available data on the correlation between CRP and DR are inconsistent. The Hoorn Study reported that DR was associated with higher CRP levels in diabetic subjects [20]. CRP was not found to be associated with DR progression by several researchers [10, 21]. Unexpectedly, DR was associated with lower CRP levels in diabetic patients [22].

Non-steroidal anti-inflammatory drugs remain an attractive tool for the prevention and/or treatment of DR. Diabetic patients receiving high aspirin dose daily have been shown to be less susceptible to DR [23]. Aspirin inhibited platelet aggregation and caused retardation of microaneurysm in patients with DR [24]. Results from the Early Treatment of Diabetic Retinopathy Study (ETDRS) and the Wisconsin Epidemiologic Study (WES) demonstrated that low and intermediate doses of aspirin were found ineffective in effective in DR [25]. Aspirin inhibited the diabetes-dependent degeneration of retinal capillaries in a 5-year study of diabetes in dogs [26]. Aspirin inhibits cyclooxygenase (COX)-1 and thromboxane-A2 at low doses, while intermediate doses inhibit also (COX)-2 and prostaglandins (PGs) especially PGE2 [27-29]. At high doses aspirin exerts a potent anti-inflammatory effect that is mediated through COX- and prostaglandin-independent mechanisms involving nuclear factor (NF)-κB [30].

Although the manifestations of diabetes could be found almost everywhere in the eye, previous work focused mainly on DR. The aim of the present work was to explore the effects of streptozotocin-induced diabetes on the rat retina, choroid, sclera, lens, ciliary body, iris and cornea. We also demonstrated the effects of aspirin administration on diabetic eye with correlation to the levels of pro-inflammatory markers in the rat vitreous body.

2. Materials and Methods

2.1. Animals

Ninety male Wistar albino rats, weighing approximately 200-250 g, were used in the present study. The animals were fed standard laboratory chow and allowed free access to water in an air-conditioned room with a 12 hours light-dark cycle. Animals were kept for 10 days prior to the start of the study to allow proper acclimatization. Animal care and use was approved by the University Ethics Committee.

2.2. Induction of Diabetes

Sixty rats received a single intraperitoneal injection of streptozotocin (50 mg/kg, Sigma, St. Louis, MO, USA) in 10 mM sodium citrate buffer, pH 4.5. Control (non-diabetic) animals were injected with citrate buffer alone (vehicle). All

the treated animals were selected for the study. Animals with blood glucose levels greater than 250 mg/dl 24 hours after the induction were considered diabetic. Both body weight and blood glucose level were checked regularly on weekly bases. All animals were scarified 24 weeks after the induction of diabetes and different parts of the eyes were prepared for haematoxylin and eosin stain and immunohistochemistry. Vitreous was collected for the estimation of TNF-α, IL-6 and hsCRP.

2.3. Aspirin Treatment

Aspirin treatment was started 24 hours after the induction of diabetes. Confirmed diabetic animals (60 rats) were equally randomized into two treatment groups receiving either 2 mg/kg/d aspirin (ASPIRIN PROTECT® 100, Bayer HealthCare AG, Leverkusen, Germany), or equal volumes of normal saline (vehicle) orally.

2.4. Haematoxylin and Eosin (Hx & E) Stain

Specimen from the retina, choroid, sclera, ciliary body, iris, lens and cornea were taken and fixed in 10% formol saline for 5-7 days. The specimens were washed in tap water for 10 minutes dehydrated in graded ethanol solutions (70%, 90% over night and 100% ethanol solution for three changes one hour each). The specimens were then cleared in xylene for 20-30 times according to the size of the specimen (guided by inspection at five minutes intervals). The specimens were impregnated in soft paraffin wax at 55-60°C for two hours then in hard paraffin wax at room temperature in moulds. Finally tissue blocks were cut into section of five microns thickness by using rotator microtome. Tissue sections were dipped in a warm water-bath, picked up on clean slides, and placed on hot plate for two minutes. Finally, tissue sections were stained with haematoxylin and eosin stain for general architecture of the studied tissues.

2.5. Immunohistochemistry of Tissue Samples

After dissection of the eye, tissues from the retina, choroid, sclera, ciliary body, iris, lens and cornea were immersed in 0.1 M cacodylate buffer pH 7.4 containing 4% paraformaldehyde and 5% glutaraldehyde for 10 minutes. Tissues were washed in the same buffer supplemented with 25% sucrose for 5 to 10 minutes, transferred to the same buffer supplemented with 2% glyoxylic acid and 25% sucrose for 15 min and quick-frozen in liquid nitrogen. Cryo-sectioning was performed at -30°C by adjusting the microtome at 2 to 5 μm. Three to five sections per tissue per animal group were picked up on slides, mounted and fixed for 20 minutes in 3.5% formaldehyde sodium in PBS (pH 7.2) and rinsed in PBS. Tissue sections were exposed to CD34 antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA) diluted 1:100 for 60 minutes, rinsed in PBS and incubated with anti-mouse conjugated to fluorescein diluted 1:300 for 30 minutes. All incubations

were carried out at room temperature.

2.6. Vitreous Samples Collection

All rats were scarified at 6 months after induction of diabetes. Immediately after killing both eyes from each rat were enucleated and placed in ice-cold phosphate-buffered saline (PBS; pH 7.4). Enucleation was performed with the use of iris forceps and scissors for separation of the eyes from the surrounding connective tissue, nerve, and muscles. The eyes were dried on sterile gauze, and the vitreous fluid was aspirated with a 0.5-mL insulin syringe and placed on ice in sterile tubes (Eppendorf) and kept frozen at -80°C (MAN) for further assessment of TNF-α, IL-6 and hsCRP.

2.7. Determination of TNF-α, IL-6 and CRP

At the end of the study (24 weeks following induction of diabetes), all the animals were sacrificed and vitreous samples were collected using a 33-gauge needle for determination of vitreal TNF-α, IL-6 and hsCRP levels. ELISA kits (eBioscience, San Diego, California, USA) were used for the estimation of TNF-α, IL-6 and hsCRP (eBioscience, San Diego, California, USA) levels in vitreous body. All measurements were performed with ELISA automatic optical reader with optical absorbance value of 450 nm (SUNRISE Touchscreen, TECHAN, Salzburg, Austria).

2.8. Statistical Analysis

Results are expressed as mean ± standard error (SE). Repeated-measures Analysis of Variances (ANOVA) was used for statistical analysis of the different groups using Origin® software and the probability of chance (p values). P values < 0.05 were considered significant.

3. Results

The cornea of the control (C) group (figure 1, upper panel) demonstrate normal architecture in the form of a stratified squamous non-keratinized epithelial layer, with Bowman's membrane underlying the lining epithelium. Substantia propria (90% of the thickness of the cornea) lied between Bowman's membrane and the descement's membrane (single layer of endothelial cells lying posterior to the substantia propria. The cornea of diabetic (D) rats showed abundant large fibroblasts in the substantia propria, and marked thickening of the descement's membrane. Several blood capillaries could be seen especially in the superficial part of substantia propria. The cornea of diabetic rats treated with aspirin (D+A) showed thickened and irregular Bowman's membrane and thickened descement's membrane.

Sections from the iris of the control (C) group (figure 1, 2nd panel) showed the iris being covered anteriorly with simple squamous epithelium, which was continuous with the corneal epithelium. Posteriorly, the iris was lined by two layers of epithelium called "pars iridis retina' which were in continuation with the epithelium of the ciliary body. A layer of poorly vascularized connective tissue layer separates the anterior epithelium from a more vascularized layer. The iris of diabetic (D) rats included a highly vascular connective tissue stroma. The stroma contained many dilated and congested blood capillaries, and some were of huge caliber. The iris of diabetic rats treated with aspirin (D+A) appeared similar to that of the control (C) rats to a great extent.

The ciliary body of the control (C) group (figure 1, 3rd panel) consisted of bundles of smooth muscle of different orientation. The muscle groups were present in connective tissue stroma containing blood vessels and connective tissue cells. The ciliary body formed several processes covered with double layers of cuboidal epithelial cells. The ciliary body of the diabetic (D) group showed sub-epithelial numerous dilated and congested blood vessels. The connective tissue was markedly fibrous (type III collagen fibers) when compared to the control group. The ciliary body of the aspirin treated diabetic group (D+A) showed fewer and less congested capillaries. The connective tissue was less fibrous when compared to the diabetic group.

Specimens from the lens of the control (C) rats (figure 1, lower panel) appeared of normal structure. A homogenous structure-less membrane (lens capsule) enclosed the lens. The anterior surface of the lens is thicker than the posterior surface. Under the capsule there was a single layer of cells on the interior surface, which was thicker at the equator of the lens. At the sides of the lens the epithelium transformed into lens fibers which formed the main substance of the lens. No cells were present on the posterior pole of the lens. The lens of the diabetic (D) rats showed degenerative changes in the lens capsular cells which appeared pale with unclear cytoplasm. The lens of the diabetic rats treated by aspirin (D+A) showed more homogenous lens fibers with less degenerative changes.

Histopatholocical study of the choroid and retina of the control (C), diabetic (D) and aspirin treated diabetic (D+A) rats, revealed beneficial effect of aspirin treatment. In the control (C) group, the choroid is thin, highly vascular and could be found immediately beneath the sclera (figure 2, C). The choroid consists of 3 layers; epichoroid, vessel layer (stroma containing one layer of blood vessels), and chorio-capillaries (single layer of blood capillaries underneath the retina). The choroid was separated from the retina by the Bruch's (glassy) membrane. The retina consisted of a single layer of cuboidal epithelial cells (the pigmented epithelium), and several alternating layers of fibers and nerve cells. All the retinal layers were avascular. The choroid of the diabetic rat (figure 2, D) showed marked increase in vascularity, especially in the chorio-capillaries layer which contained abundant dilated congested capillaries which sometimes appeared ballooned. The pigmented epithelial cells appeared more flattened with flattened few nuclei. The retina showed few dilated capillaries especially within the deep layers. The choroids and retina of the aspirin treated diabetic rats (D+A) (figure 2, D+A) revealed significant decrease in the vascularity

when compared to the diabetic group.

Immunohistochemical results supported the histopatholigical findings. Figure 3 shows specimen from the cornea (upper panel) iris (middle panel) and ciliary body (lower panel), stained with CD34 antibody. Sections from the cornea, iris and ciliary body of the aspirin treated diabetic rats (D+A) showed substantially less positive staining with the CD34 antibody when compared to the diabetic (D) group.
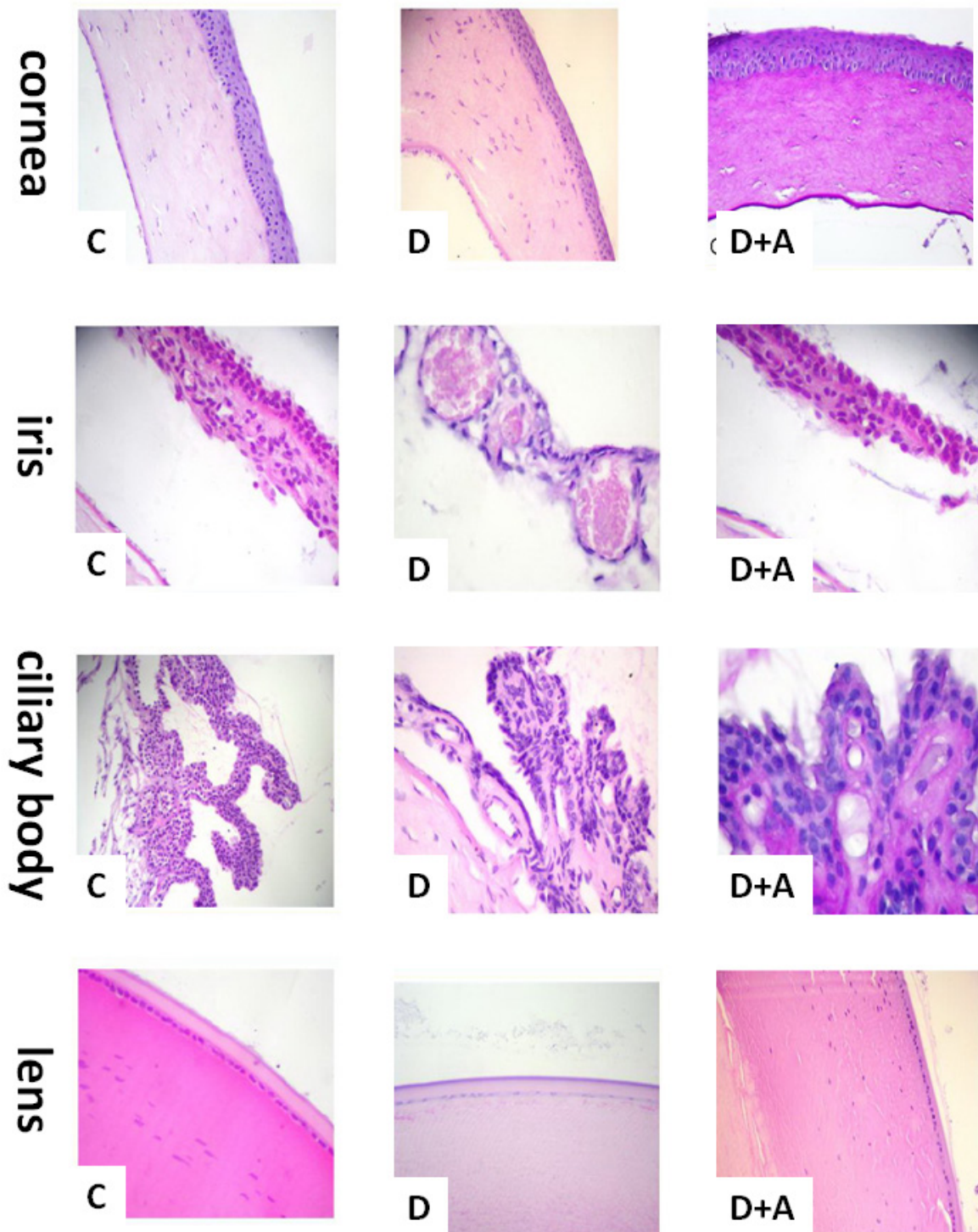

Figure 1.   Aspirin improves diabetes-induced ocular tissue changes. Photomicrograph of sections from the cornea (upper panel), iris (2nd panel), ciliary body (3rd panel) and lens (lower panel) from control (**C**), diabetic (**D**) and aspirin treated diabetic (**D+A**) rats. (Hx & E X 200, number of rats 30/group)
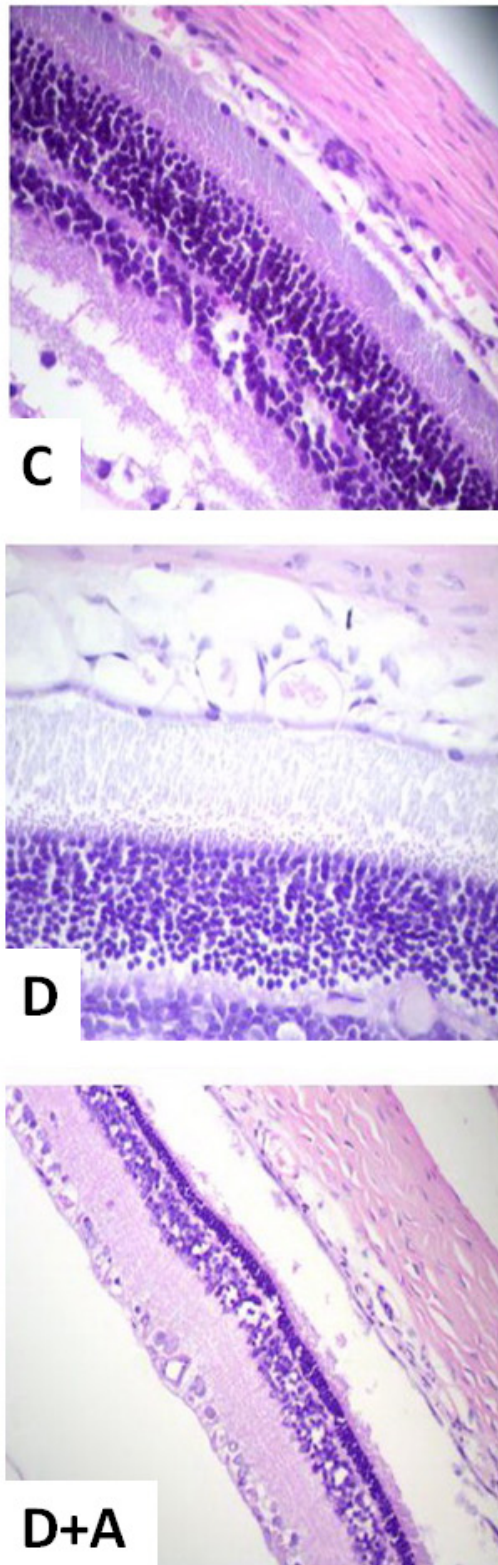
Figure 2.   **Aspirin decreases vascularity of the choroid and retina of diabetic rat.** Photomicrograph of a section of the choroid and retina of control (**C**),
diabetic (**D**) and aspirin treated diabetic (**D+A**) rats. (Hx & E X 400, number of rats 30/group)

In the present study, the vitreal level of hsCRP (high sensitive C reactive protein) was significantly higher ($p < 0.05$) in the diabetic (D) group when compared to the control (C) group (82.4 ± 9.14 and 6.92 ± 1.25 ng/ml respectively). The hsCRP level in the vitreous of the aspirin treated diabetic (D+A) group (62.3 ± 4.1 ng/ml), was significantly lower ($p < 0.05$) when compared to the diabetic (D) group, however, it was still significantly higher ($p < 0.05$) when compared to the control (C) group (figure 4 A).
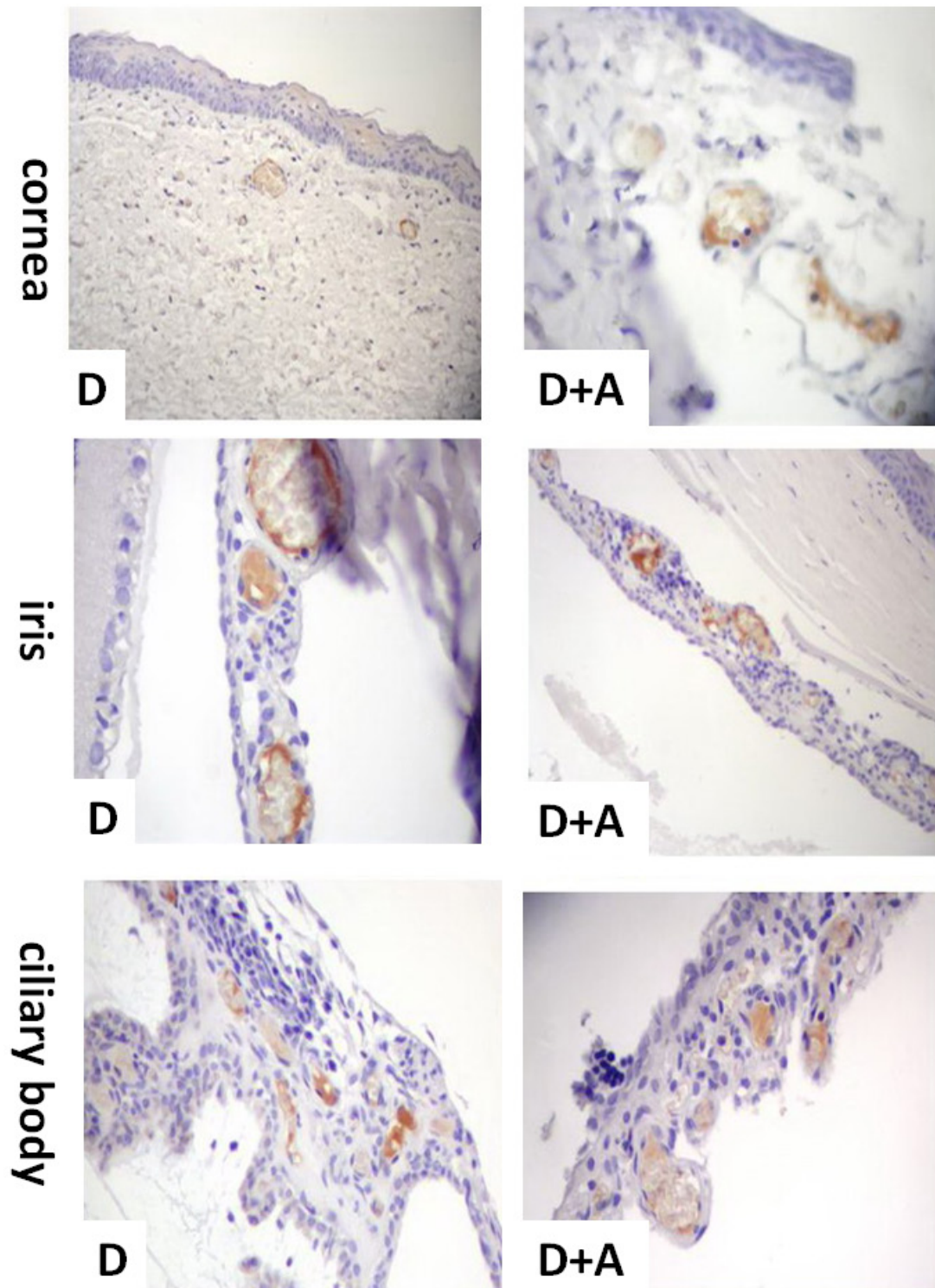

Figure 3. Aspirin attenuates diabetes-induced neovascularisation. Photomicrograph of sections from the cornea (upper panel), iris (middle panel) and ciliary body (lower panel) from diabetic (**D**) and aspirin treated diabetic (**D+A**) rats stained with CD34 antibody. Sections from aspirin treated rats showed less positive staining with CD34 antibody. (X 200, number of rats 30/group)
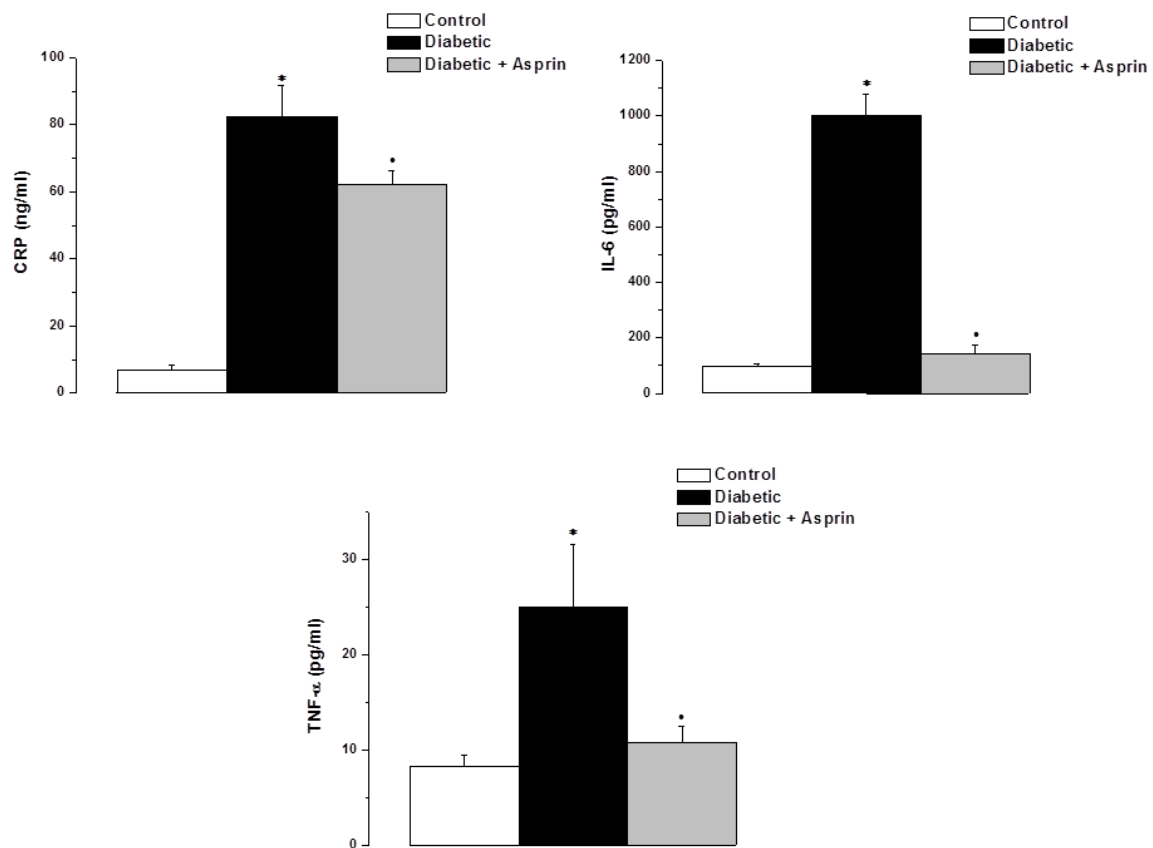
Figure 4. Aspirin reduces vitreal levels of hsCRP, IL-6 and TNF-α in diabetic rats.   A hs(high sensitive)CRP level in the vitreous of control (white column), diabetic (black column) and aspirin treated diabetic (grey column) groups. **B** IL-6 level in the vitreous of control (white column), diabetic (black column) and aspirin treated diabetic (grey column) groups. **C** TNF-α level in the vitreous of control (white column), diabetic (black column) and aspirin treated diabetic (grey column) groups * Significant when compared to the control group, ● Significant when compared to the diabetic group. (Significant = p < 0.05, number of rats 30/group)

It was also demonstrated in this work that the vitreal level of IL-6 was significantly higher (p < 0.05) in the diabetic (D) group when compared to the control (C) group (1003 ± 75.19 and 95.42 ± 12.72 pg/ml respectively). The IL-6 level in the vitreous of the aspirin treated diabetic (D+A) group (140.8 ± 34.35 pg/ml), was significantly lower (p < 0.05) when compared to the diabetic (D) group, while it was insignificant when compared to the control (C) group (figure 4 B).

Similar to the IL-6 results, the vitreal level of TNF-α was significantly higher (p < 0.05) in the diabetic (D) group when compared to the control (C) group (25.1 ± 6.65 and 8.24 ± 1.16 pg/ml respectively). Vitreal level of TNF-α in the aspirin treated diabetic (D+A) group (10.7 ± 1.77 pg/ml), was significantly lower (p < 0.05) when compared to the diabetic (D) group, while it was altered insignificantly when compared to the control (C) group (figure 4 C).

4. Discussion

The prevalence of adult-onset diabetes mellitus is expected to rise from 171 million in 2000 to 366 million in 2030. This will dramatically oblige a human and economic cost on societies [31]. Diabetic retinopathy is the leading cause of vision loss in working age adults, with vision-threatening incidence of 10% of people with diabetes [32]. The cost of diabetic care in the USA in 2010 was estimated to be $116 billion direct and $85 billion indirect costs, while in 2012 the estimated cost in the UK was £9.8 billion direct and £13.8 billion indirect costs [31, 33]. The major risk factor the progression of DR is the duration of diabetes. Microvascular changes precede DR progression. The earliest recognizable changes include death of pericytes, thickening of basement membrane, capillary occlusion and neovascularisation. Later changes result from the involvement of large vessels with subsequent release of vasoactive cytokines [34].

Ample effort has been done to identify drugs that can prevent DR, aspirin has been investigated a number of times in this context. Data available may appear inconsistent albeit aspirin remains worthy of study within growing information.

Manifestations of diabetes in the eye could be demonstrated in almost every part of the anterior or posterior segments. However, most available data have stressed mainly on retinal affection. Diabetes can influence virtually all eye tissues with subsequent deterioration of sightedness.

The corneal changes in diabetes include keratitis, corneal erosions and epithelial defects. Most importantly, other significant changes, such as increased corneal thickening and corneal oedema, are not correlated to epithelial damage and suggest corneal endothelial dysfunctioning [35]. Iris atrophy, rubiosis iridis, uveitis and glucaoma are exacerbated in diabetic patients [36]. We have shown that aspirin had insignificant effect on the diabetes-induced corneal changes, i.e. thickened Descmet's and Bowman's membranes, and dilated subepithelial capillaries (figure 1, upper panel). Nevertheless, aspirin administration averted the diabetes-dependent histological changes in the iris. Those included the increased stromal vascularity and the dilatation and congestion of the blood capillaries (figure 1, 2nd panel). Aspirin had also attenuated the pathological change in the rat eye ciliary body which included decreasing the number of dilated congested subepithelial capillaries, and reducing the connective tissue fibrous component (figure 1, 3rd panel).

In the present study, we showed that diabetes caused degenerative changes in the rat lens (figure 1, lower panel). The use of aspirin, however, rescued the rat lens from diabetes-induced lens changes. When taken altogether, aspirin could be considered to prevent or at least attenuate the alteration of the refractive state of the eye that accompanies diabetes. In this work aspirin reduced the thickening of the corneal and lens membranes (and thus curvature), attenuated the vascular changes in the iris and ciliary body, and inhibited fibroblasts infiltration. These findings are quite promising in the context of delaying the onset of visual acuity affection commonly seen in diabetic patients.

Our findings from the sections in the choroid and the retina were consistent with those seen in the anterior segment of the eye. In the diabetic rat eye, the choroid showed marked increase in the vascularity especially in the chorio-capillaries layer. Numerous and markedly dilated capillaries could be seen. Increased vascularity was also evident in the deep layers of the retina. The pigmented epithelial cell layer of the retina shoed flattened cells. Interestingly, aspirin decreased the vascularity of the choroid and the deep layers of the retina (figure 2). Our findings were further assessed by immunohistochemical studies using rat CD34 monoclonal antibody (figure 3). Diabetes induced neovascularisation and affection of endothelial cells and blood capillaries (were attenuated by aspirin treatment.

Inflammation is a nonspecific response to injury that includes a plethora of functional and molecular intermediaries. Typically, on an acute basis inflammation has favourable effects. However, inflammation comprises undesirable effects if persisting chronically. Proinflammatory proteins such as COX-2, interleukin-1, TNF-α, can contribute to cell damage and death in different tissues including the retina [37, 38]. Increased levels of inflammatory and angiogenic factors in the vitreous of diabetic patients could be a result of the breakdown of the blood-retinal barrier leading to raised levels of inflammatory and angiogenic factors [39]. A second possibility is the expression of inflammatory intermediaries by cells within the vitreous fluid, such as macrophages, monocytes, glial cells, and retinal pigment epithelial cells [40]. Retinal ischemia may cause hypoxia which in turn increases the expression of inflammatory mediators as shown in ischemia-reperfusion model [41].

Considerable data support the role of C-reactive protein (CRP) as risk factor for diabetes and macrovascular diseases including cardiovascular disorders. CRP has also been associated with polypoidal vascular vasculopathy, age-related macular oedema and microvascular diabetic complications including DR [22]. CRP was proposed to be a possible direct mediator of the disease process, because of its numerous proatherogenic effects on vascular cells [42]. In the present work the level of CRP in the vitreous of the diabetic rat eye was elevated significantly when compared to the corresponding values in the control and diabetic rats receiving aspirin (figure 4A). Our results were in agreement with several studies which reported elevated CRP levels in the serum of type I (T1DM) and type II (T2DM) diabetic patients [43].

IL-6 is a pro- as well as anti-inflammatory cytokine. We have shown here that IL-6 level in the vitreous fluid of diabetic rats was significantly increase when compared to the control as well as the diabetic group treated with aspirin (figure 4B). Elevated serum levels [44], as well as intravetreal levels [45] of IL-6 have been reported in diabetic patients with retinopathy compared with those without retinopathy. The IL-6 vitreous/serum ratio in diabetic patients was high suggesting intraocular increased production of the inflammatory cytokine [14]. IL-6 expression was increased in diabetic rat retinas [46].

TNF-α is implicated in the early inflammatory changes seen in the diabetic retina. Astrocytes and Muller cells are potential source of TNF-α [47]. TNF-α is a pro-inflammatory cytokine that is involved in the breakdown of the blood retinal barrier (BRB) breakdown by opening of the tight junctions between retinal vascular endothelial, and therefore, participating in the pathogenesis of diabetes [48]. Our results demonstrated a significant increase in vitreous level of TNF-α in vitreous of diabetic rats when compared to the corresponding values in the control group. Asprin treatment lowered the elevated TNF-α level (figure 4C). Our results are in agreement with other reports indicating that the level of TNF-α is increased in patients with DR [49].

5. Conclusions

In conclusion we report that IL-6, TNF-α and hsCRP levels were increased in the vitreous of diabetic rats. We also report a correlation between the pathological finding in the tissues from the retina, ciliary body, iris, cornea and the lens and the levels of IL-6, TNF-α and CRP. Our data suggest the possibility that IL-6, TNF-α and CRP may be a cause of DR progression and not necessarily a result, thus drugs that target IL-6, TNF-α and CRP including aspirin may play a crucial role in the prevention of diabetic microvascular

complications.

AKNOWLEGMENTS

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