

Review Article**Pathogenesis of Diabetic Retinopathy: Biochemical Aspects and Therapeutic Approaches****Mr. Bhaskar Gaonkar¹, Dr. KrishnanandaPrabhu²**¹PhD Scholar, Department of Biochemistry, Kasturba Medical College, Manipal, Manipal University, Udupi-576104, Karnataka, India.²Professor and Head, Department of Biochemistry, Kasturba Medical College, Manipal, Manipal University, Udupi - 576104, Karnataka, India***Corresponding author**

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Abstract: Diabetic retinopathy is one of the reasons for severe loss of visual acuity in diabetic patients. Exposure to hyperglycemic environment is the root cause, which activates or increases the rate of several biochemical pathways such as, polyol pathway, PKC pathway and formation of advanced glycation end products in the tissues leading to hypoxia, followed by tissue damage. Oxygen deprivation stimulates the retina to release growth factors causing growth of undesirable blood vessels in the retina, which is the central cause for retinopathy. Laser and anti-VEGF therapies are being used worldwide as a mode of treatment. But newer approaches to an effective treatment with less side effects is always a matter of concern. So several therapeutic agents to block the basic biochemical pathways of pathogenesis are designed and under research. This review focuses on the basic biochemical pathways for the pathogenesis of retinopathy and the drugs designed to block those pathways for the treatment of diabetic retinopathy.**Keywords:** Diabetic Retinopathy, Treatment, polyol pathway, Advanced glycation end products, PKC pathway, Leukostasis.

INTRODUCTION

Diabetic retinopathy (DR) is considered to be the most serious complication and one of the major causes of blindness in diabetic patients. It is a vascular disorder affecting the microvasculature of the retina. Usually, retinopathy was not found to be clinically significant during early years of diagnosis of diabetes. It has been found that 25-50% of the type1 diabetic population showed some degree of retinopathy within 10 to 15 years and 75% within 15 to 20 years of diagnosis of type 2 diabetes mellitus. However 20% of type2 diabetic patients found to have retinopathy by the time they are diagnosed with diabetes [1,2]. In addition, several factors such as, duration of diabetes mellitus, hypertension, hyperlipidemia, nephropathy, poor glycemic control, anemia, alcohol consumption and pregnancy can influence the development and progression of diabetic retinopathy [3-6].

Hyperglycemia is a key factor in the development of diabetic retinopathy which can activate multiple biochemical pathways involved in the pathogenesis of diabetic retinopathy. It causes increased flux of glucose through several pathways mainly, hexosamine pathway, aldose reductase and protein kinase C (PKC) pathway. Activation of hexosamine

pathway can further causes altered glycosylation of transcription factors and activation of inflammatory genes [7]. Non enzymatic glycosylation can leads to functional alteration of several proteins. Increased formation of sugar alcohols (such as sorbitol) from aldose reductase pathway can deplete NADPH required for the cell to fight against oxidative stress [8]. Protein kinase C pathway activation can trigger changes in retinal blood flow, thickening of the basement membrane, extracellular matrix expansion and increase in vascular permeability [7]. All these factors finally cause endothelial cell dysfunction, pericyte apoptosis, microvascular leakage and microaneurysms leading to retinal ischemia which can trigger angiogenesis in retina which is a hallmark of diabetic retinopathy.

Microvascular leakage caused by increased expression of potent vasoactive molecules such as Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor- β (TGF- β), Interleukin-1 (IL1), Tumor Necrosis Factor-(TNF- α), Matrix Metalloproteinases (MMPs), etc. can result in development of macular edema (ME). The same vasoactive molecules can also trigger hypoxia induced neovascularization in the retina [9-13]. The new blood vessels thus formed are thin and fragile. They can

rupture and bleed into the vitreous cavity leading to loss of visual acuity, contraction of the vitreous, retinal detachment and severe loss of vision [14-17].

Laser photocoagulation therapy has been used worldwide as the most effective course of treatment in preventing severe vision loss in diabetic patients [18,19]. Anti-VEGF agents are also in use to trap VEGF and to stop macular edema as well as angiogenesis [20,21]. In recent years, several anti-angiogenic molecules such as PKC inhibitors, tyrosine kinase receptor blockers etc. have been tried and are under clinical trial for the treatment of angiogenesis in diabetic retinopathy.

Pathogenesis of diabetic retinopathy a biochemical view:

One of the most important factors in the pathogenesis of diabetic vascular complications is hyperglycemia induced abnormal activation of several metabolic pathways. A major part of it comprises of hyperactive polyol pathway, increased flux of glucose through hexosamine pathway, oxidative stress and increased activation of PKC pathway.

Polyol pathway

Cells or tissues in the hyperglycemic milieu which are independent of insulin [nerve fibers, retina, lens, kidney etc.] can freely uptake glucose, which will be acted upon by aldose reductase that reduces glucose

to sorbitol and NADP⁺. Further sorbitol dehydrogenase oxidizes sorbitol to fructose and NAD⁺. This reaction consumes NADPH and NADP⁺ starts accumulating with compromising anti-oxidant defense system. The excessively produced sorbitol simultaneously starts accumulating in these tissues also cause osmotic stress [22-26]. Aldose reductase (AR) is the rate limiting enzyme in polyol pathway. Expression of aldose reductase protein have been shown to be elevated in nerve fibers, ganglion cells and Muller cells derived from diabetic patients with retinopathy when compared to no diabetic individuals [7,26,27]. Rate of polyol formation in *ex vivo* rat retinas have shown increased flux of glucose through polyol pathway which increase with the duration of diabetes and hyperglycemia [28]. Julia V. Busik, *et al.* 2002 showed that hyperglycemia itself causes increased uptake of glucose by retinal endothelial cells and pigment epithelial cells in culture, coupled with accumulation of sorbitol in tissues [29].

In addition to decrease cytosolic NADPH, polyol pathway also found to increase the ratio of NADH/NAD⁺. The remarkable detrimental effects of this pathway also include, decrease in the activity of Na⁺/K⁺ ATPase enzyme, activation of PKC pathway, a compromise in the antioxidant defense, which collectively can cause micro vascular damage, leading to diabetic retinopathy and other complications of diabetes [8,30,31][Fig. 1].

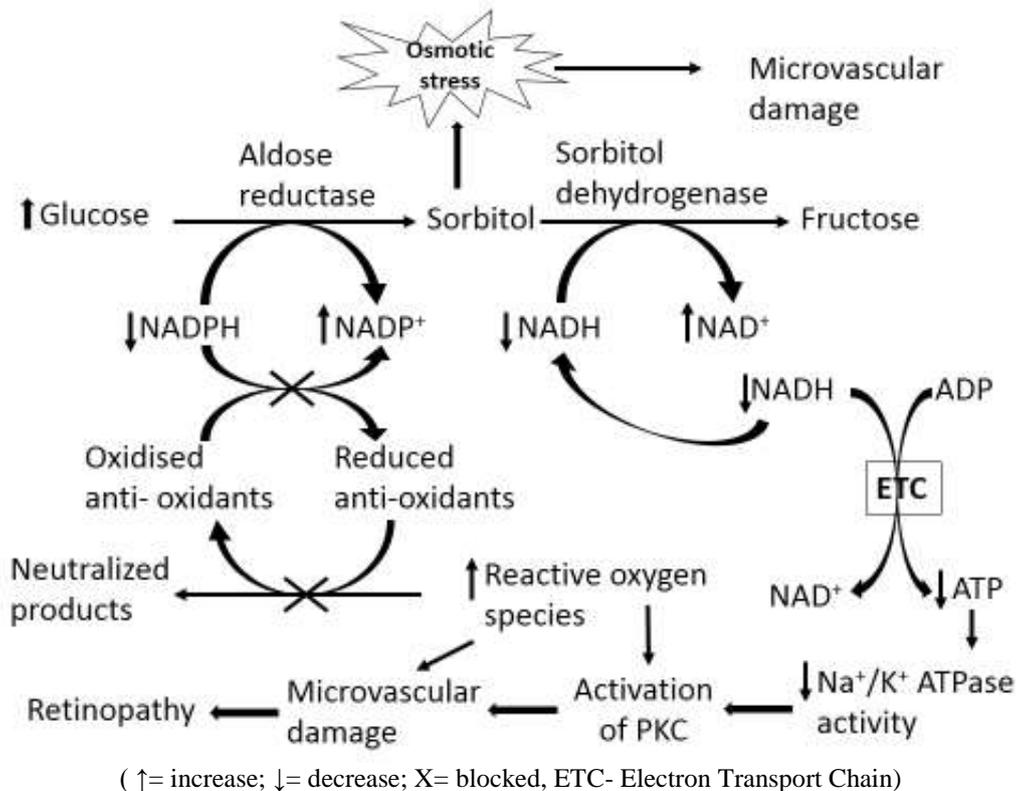
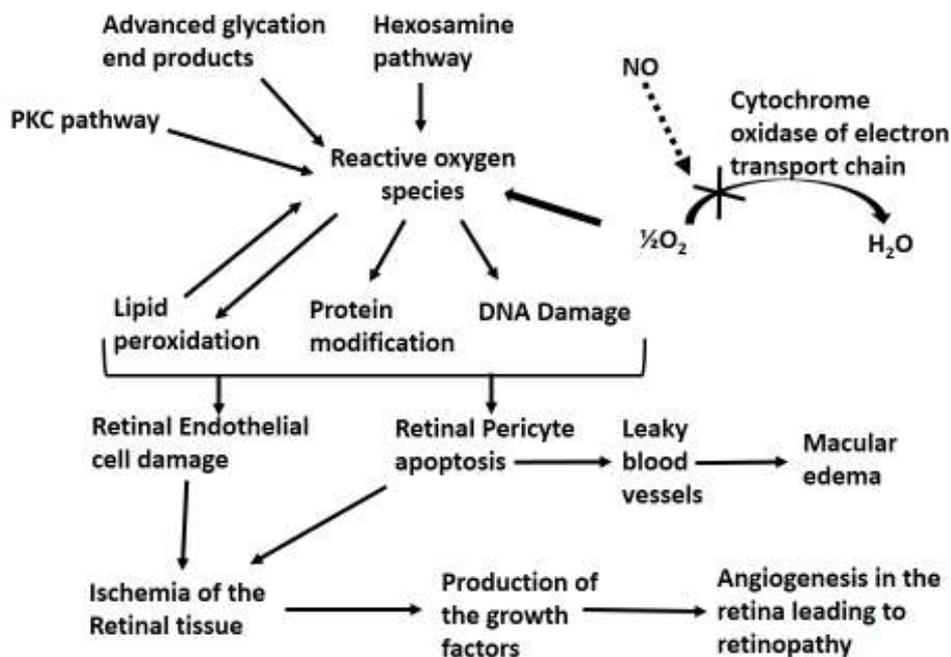


Fig-1: Showing Polyol pathway and its effects

Oxidative stress

Another pathophysiological condition in diabetes is an increase in the oxidative stress. When compare to other tissues retina has a high oxygen uptake and lipid peroxidation as its polyunsaturated fatty acids content is high. This is the reason why retina is more susceptible to oxidative stress [32]. Several studies have indicated that, reactive oxygen species (ROS) activate signaling pathways that promote angiogenesis [33-35]. Vascular endothelial cells and

smooth muscle cells contain NAD(P)H oxidases and are found to be good source of ROS formation from molecular oxygen [36,37]. NO is a vasodilator, synthesis of which is upregulated in diabetes. Nitric Oxide has been shown to promote ROS production. Possible mechanism could be interference of nitric oxide (NO) in reaction of mitochondrial cytochrome c oxidase enzyme complex preventing oxygen reduction to water, thereby promoting ROS production by the reactive oxygen molecule [38-39].



(X = blocked, -.- = Inhibition)

Fig-2: Showing Oxidative stress; sources and consequences

Hyperactive aldose reductase, PKC pathway, hexosamine pathway, variation in the metabolism of lipoproteins, advanced glycated end products (AGE), induced by hyperglycemia could be the major sources for ROS [40]. Structural changes in the biomolecules (DNA and protein modification and lipid per oxidation) due to ROS attack decide the functional status and thereby integrity of all the cellular reactions. Among all the body cells pericytes were found to be highly responsive to oxidative stress which was proven by several *in vivo* studies where lack of their function or loss of pericytes itself have been shown to augment endothelial proliferation [28, 41-46]. When the loss crosses a particular limit blood vessels become fragile and start bleeding leading to macular edema. This may end up in development of diabetic retinopathy [Fig. 2].

Non-enzymatic glycation reactions and formation of advanced glycated end products

Advanced glycated end products are formed by the Maillard reaction. It is a non-enzymatic reaction between aldehyde group of a glucose molecule and amino group of a protein. Advanced glycated end

products have been implicated in the aging of proteins and alteration in their function [49,50]. These complex molecules induce specific effects by binding to specific receptors on the target tissue called receptors for advanced glycated end products (RAGEs). There were two types of receptors found. One that is anchored on cell membrane and another freely circulating form lacking transmembrane domain. Membrane bound form of RAGE are involved in pathogenic effects of AGEs. In contrast the soluble extracellular ligand binding domains formed by alternative splicing of RAGE mRNA, scavenge AGEs and prevent damages caused by them [51-53].

In pericytes AGEs found to induce apoptosis with an increased activity of caspase-3, caused due to a decreased Bcl-2/Bax ratio and nuclear factor-κB (NF-κB) activation [54,55]. Since pericytes play an important role in the maintenance of micro vascular homeostasis, loss of pericytes can induce, endothelial cell (EC) injury in the retinal blood vessels and angiogenesis, leading to diabetic retinopathy [Fig. 3] [48].

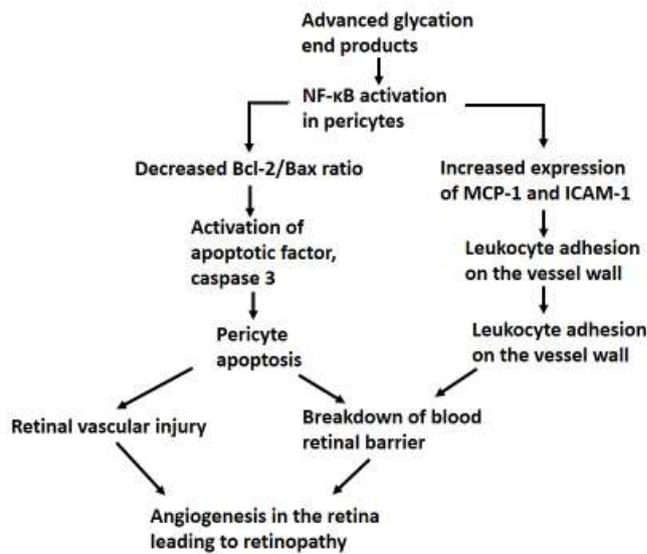


Fig-3: Showing Advanced glycation end product induced angiogenesis in the retina

Literature show, through the intracellular ROS generation AGEs can induce the expression of intracellular cell adhesion molecule-1 (ICAM-1) and monocyte chemo attractant protein-1 (MCP-1) by micro vascular ECs. These events can induce leukocyte adhesion on ECs, leading to breakdown of blood retinal barrier (BRB) (consisting of retinal vascular endothelium and retinal pigment epithelium which maintain the transport of substances between blood and retinal tissue) [7,53,56]. Ming Lu, 1997 in an *in vitro* study found that AGE-induces VEGF expression in a dose and time-dependent manner. Anti-VEGF antibody has shown to block capillary endothelial cell proliferation in these tissues exposed to AGE [57]. As AGEs have the ability to increase retinal VEGF gene expression they might be involved in the pathogenesis of diabetic retinopathy.

Hexosamine pathway

Hexosamine pathway was proposed to be one of the culprits for insulin resistance, growth factors expression and there by diabetic vascular complications. This pathway involves the conversion of fructose-6-phosphate to glucosamine-6-phosphate by glutamine: fructose -6-phosphate amidotransferase (GFAT). This is a rate-limiting step in this pathway. Glucosamine-6-phosphate will be rapidly metabolized to UDP-N-acetyl-glucosamine. Hexosamines found to be involved in the synthesis of glycoproteins, gangliosides, proteoglycans, glycolipids etc.[58-61]. Altered glycation of the transcription factors found to alter the expression of inflammatory genes. Studies regarding the role of GFAT in ophthalmic complications of diabetes are lacking. But it is hypothesized that undue channeling of glucose via hexosamine pathway might affect neuroprotective effect of insulin thereby inducing apoptosis of retinal neurons.

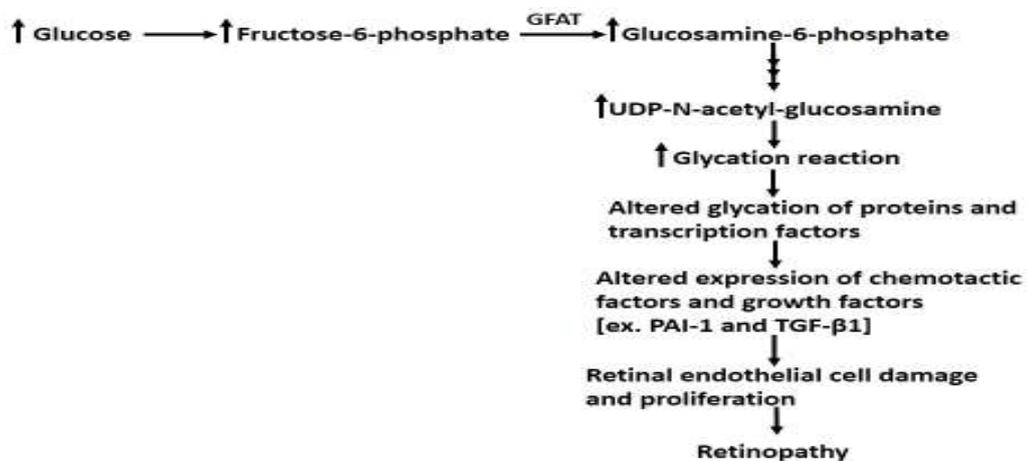


Fig-4: Showing Association of hexosamine pathway with diabetic retinopathy

This could be mediated by protein kinase B and possibly linked with altered glycosylation of

proteins [62,63]. Reports have shown involvement of hexosamine pathway in the expression of transforming

growth factor-β1 (TGF-β1), plasminogen activator inhibitor-1 (PAI-1) etc. which are already proved to be the culprits for several vascular complications in the diabetes [7,64] [Fig 4].

Increased PKC activation

The protein kinase C is a large group of structurally related family of serine/threonine kinases involved in cell signaling cascade that mediates several unique functions. The family of PKCs includes at least eleven isoforms (α, β1, β2 etc.) and are the key targets for lipid second messengers in the downstream signaling cascade [65,66]. Ca²⁺ ions, diacylglycerol (DAG), phosphatidylserine are found to be some of the activators of these molecules. Increased *de novo* DAG synthesis has been seen in animal models due to the

inhibition of glyceraldehyde-3-phosphate dehydrogenase or accelerated reduction of dihydroxyacetone phosphate to glycerol-3-phosphate. Researchers stated that it would even activate PKC in cultured vascular cells, retina and glomeruli *in vivo* [62,67]. The activities of PKC-α, -β1, -β 2, and -δ were found to be stimulated immensely with DAG and found to be linked with pathogenesis of diabetic retinopathy [58]. PKC activation can cause vasoconstriction in the retina by increasing the expression of endothelin-1 (ET-1) [68]. Consequences of PKC activation would be an increase in blood flow through capillaries, increased vascular permeability, cytokine activation, basement membrane thickening, angiogenesis, which ends with retinopathy and other complications tangled with diabetes [7,68-70] [Fig 5].

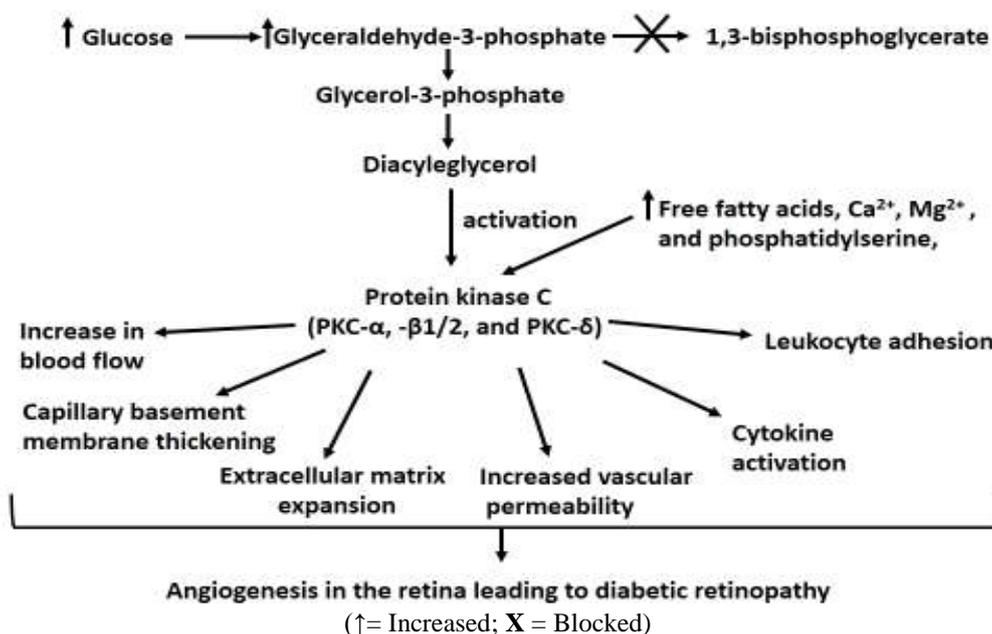


Fig-5: Showing PKC activation in the pathogenesis of diabetic retinopathy

Leukostasis

Aggregation of leukocytes on the vessel wall was found to be one of the causes and earlier events in the pathogenesis of diabetic retinopathy. Leukocytes have a capacity to adhere to the vascular endothelium, and can produce highly toxic superoxide radicals and proteolytic enzymes [71]. Adhesion process was shown to be mediated by a member of immunoglobulin supergene family of cellular adhesion molecules called, vascular cell adhesion molecule (VCAM) which acts as a chemotactic factor for leukocytes, help in adhesion to vascular wall, and if required migrating to adjacent tissue [72].

Vascular endothelial growth factor is shown to promote leukostasis and vascular leakage, through the activation of intracellular cell adhesion molecule-1 (ICAM-1) in the diabetic retinas [73]. In addition,

AGEs were also been demonstrated to increase leukostasis by increasing the expression of ICAM-1 in retinal microvascular endothelial cells in culture [53,74].

Blockages in capillaries and formation of acellular capillaries found to be associated with leukostasis in the diabetic retina [75]. Blockage of ICAM-1 by monoclonal antibodies prevented leukocyte adhesion and found to maintain the BRB, which would indicate one of the primary events in diabetic retinopathy is ICAM-1 upregulation [76]. Also, inhibition of the AGE receptor (RAGE) with Pigment Epithelial Derived Factor (PEDF) or pyridoxal phosphate had been shown to inhibit leukostasis and expression of ICAM [77]. So inhibition of leukostasis may prevent endothelial cell and pericyte death which may prevent diabetic retinopathy.

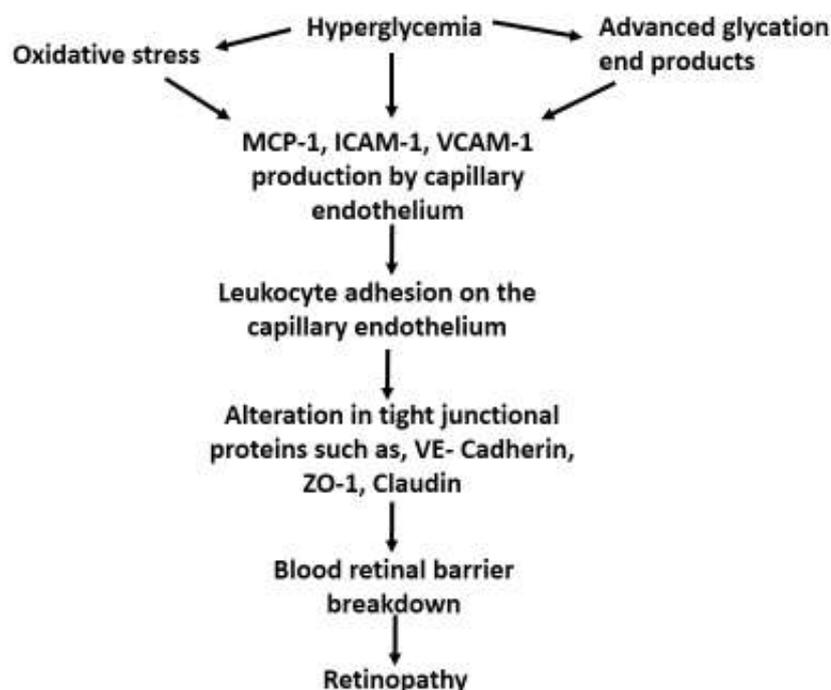


Fig-6: Showing Involvement of leukostasis in the pathogenesis of retinopathy

It is found that, expression of MCP-1 in vascular endothelium is increased due to hyperglycemia. AGEs were also found to trigger the expression MCP-1 gene by inducing ROS generation [72]. Increased numbers of leukocytes, in patients with retinopathy found to be significantly correlated with an increased expression of retinal ICAM-1 and CD18 in retinal endothelium. The CD18 molecules on the leukocytes helps leukocytes to attach themselves to intercellular adhesion molecule-1 (ICAM-1) on the surface of retinal endothelium in diabetic animals [78].

The increased leukostasis and recruitment of macrophages and other inflammatory cells mediated by over expression of cell adhesion molecules shown to cause alteration of tight junctional proteins such as, VE-Cadherin, ZO-1, Claudin. This causes infiltration of inflammatory cells into the retinal tissue, leading to breakdown of the blood retinal barrier which is a hallmark of diabetic retinopathy [Fig 6] [79].

THERAPEUTIC APPROACHES

Inhibitors of Polyol pathway

Trials carried out by inhibiting aldose reductase yielded inconsistent results. The long-term sorbinil (an inhibitor of aldose reductase) trial also indicated that sorbinil found to reduce the rate of progression of microaneurysms, but failed to check the worsening of retinopathy [80]. Trials with zenarestat (aldose reductase inhibitor), gave hopeful results with diabetic neuropathy, which need to be validated for future use for retinopathy [62]. Six months double blind study with tolrestat was failed to show any clinically significant cure for retinopathy [81]. The trial carried out by Dagher Z, 2004 showed that apoptosis of pericytes

and endothelial cells, activation of complement system in the capillary wall thereby formation of acellular capillaries can be prevented by the use of aldose reductase inhibitors [24].

PKC inhibitors

PKC inhibitors were well studied and showed certain hope in treatment of diabetic retinopathy. This was supported by several studies in which, leukostasis, endothelial cell proliferation, angiogenesis, and permeability induced by VEGF were suppressed by the use of PKC- β selective inhibitor ruboxistaurin or LY333531 [82-84]. New phase III clinical trial (Protein Kinase C- β Inhibitor-Diabetic Retinopathy Study) with ruboxistaurin reported decrease in the progression of retinopathy, reduced requirement for Laser photocoagulation and improved visual acuity in diabetic retinopathy patients [85]. Ruboxistaurin also found to act as an anti-angiogenic agent via suppression of phosphorylation of ERK1/2 and Akt [86]. Treatment of human retinal microvascular endothelial cells with Rottlerin, transfection of PKC- δ -DN, or siRNA for PKC- δ all found to decrease the vascular leakage and restored lost tight junctional proteins, ZO-1 and ZO-2. Which showed that inhibition of PKC- δ can prevent breakdown of blood retinal barrier [87]. This indicates PKC inhibitors could be used for the treatment of diabetic retinopathy.

Advanced glycosylated end product inhibitors

Inhibitors of Advanced glycosylated end product formation had also shown a promising results in improvement of diabetic retinopathy. A recently found inhibitor LR-90 had shown to reduce the death of retinal capillary pericytes and endothelial cells by preventing

AGE formation, thereby reducing the formation of acellular capillaries in streptozotocine induced diabetic rats [88,89]. A RAGE-Ig fusion protein in diabetic rats shown to inhibit capillary degeneration, accumulation of albumin in the neural retina, nitration of retinal proteins, retinal leukostasis and ICAM-1 expression in streptozotocine induced diabetic rats [90]. The systemic administration of sRAGE in diabetic rats found to significantly inhibit blood-retinal barrier breakdown, leukostasis, and expression of ICAM-1 in the retina [91]. Olmesartan, angiotensin II type 1 receptor blocker shown to inhibit the AGE-induced NF-kappaB promoter activity and expression of RAGE gene in cultured micro vascular endothelial cells. It was also shown to block AGE-induced up-regulation of VEGF mRNA levels and further increase in DNA replication in the endothelial cells [92]. Sulforaphane and RAGE-Ab shown to inhibit the AGE-induced decrease in DNA synthesis, apoptotic cell death, and up-regulation of monocyte chemo attractant protein-1 mRNA levels in pericytes [93].

Antioxidants

As a therapeutic strategy to specifically target ROS in patients with PDR, Yamagishi S, *et al.* 2011 proposed that, PEDF could be a best treatment option for retinopathy as it has an anti-oxidative, anti-angiogenic, anti-inflammatory and neuroprotective effects [94]. Vitamin C and E supplementation in type 2 diabetic retinopathy patients found to reduce NO induced stress in the eyes [95]. Pazdro R, *et al.* demonstrated a protective role of Vitamin E against lipid peroxidation, however its effects on protein and DNA oxidation is yet to be evaluated [96]. Treatment of diabetic rats with retinopathy, with lipoic acid had demonstrated a reduced pericyte loss and formation of acellular capillaries in the retinal capillary endothelial cells. This had also shown to reduce the expression of angiogenic factors, angiopoietin-2 and VEGF [97]. An intracellular labile iron chelator, salicylaldehydeisonicotinoyl hydrazine has been shown to reduce the apoptosis of retinal pigment epithelial cells induced by hydrogen peroxide *in vitro* have been proven to be useful in several disease conditions [98]. So anti-oxidants have been found to be useful for the management of retinopathy. However appropriate dose and combination of antioxidants, better route for the administration and their side effects are yet to be addressed.

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