SCRUTINIZING THE PROCLAMATION OF BDNF GENE IN RETINAL TISSUE LACKING EPITHELIAL LAYER IN CASE OF TYPE 2 DIABETIC RETINOPATHY

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ABSTRACT: Diabetic retinopathy (DR) is a leading cause of vision-loss globally now days bright eye are a deep subject in this new fashioned world. There are many disease which cause complications and leading to blindness. Of an estimated 285 million people with diabetes mellitus worldwide, approximately one third have signs of DR and of these, a further one third of DR is vision-threatening DR, including diabetic macular edema (DME). The identification of established modifiable risk factors for DR such as hyperglycemia and hypertension has provided the basis for risk factor control in preventing onset and progression of DR.
Additional research investigating novel risk factors has improved our understanding of multiple biological pathways involved in the pathogenesis of DR and DME, especially those involved in inflammation and oxidative stress. Variations in DR prevalence between populations have also sparked interest in genetic studies to identify loci associated with disease susceptibility.
One of which is known as diabetic retinopathy that occurs in a patient suffering from diabetes mellitus due to excessive glucose stress in the eye as a result of which, the retinal microvasculature that are responsible for proper blood flow in the four quadrants of the eyes and provide nutrients and proper oxygen supply to it and also act as a blood retinal barrier that protects the retina by excluding circulating molecular toxins, microorganisms, and pro-inflammatory leukocytes gets blocked. New blood vessel formation is quite different from angiogenesis and known as neovascularization. Neovascularization is a key process for causing diabetic retinopathy in human eyes.
At gene level in case of pathogenic condition of type 2 diabetic retinopathy, there is deregulation of various genes that are involved in causing pathogenicity. Using gene expression and epigenetic analysis, it has been possible to explore the expression profiling of various gene in the human retinal cell and contribute to understanding of the pathogenesis of these diseases. Additionally, it is possible to provide the support for the involvement of well-characterized biological molecules, and in this way, there is a chance to identify new players in retinal pathologies. It may lead to new to the design of new biological therapies.

INTRODUCTION:

1.1 TYPE 2 DIABETIC RETINOPATHY (T2DR)

Type 2 Diabetic retinopathy (T2DR) is one of the complications of diabetes mellitus. Loss of vision occurs due to diabetic maculopathy and complications of proliferative diabetic retinopathy (PDR) such as vitreous hemorrhage, fractional retinal detachment, and neovascular glaucoma. By 2030 it is estimated that developing countries will face an increase of 69% and industrialized countries by 20% of the number of patients with diabetes compared to 2010 (1, 2). A number of genetic factors account for 25-50% of the risk of developing T2DR. Therefore, the use of genetic analysis to identify those diabetic patients most prone to developing T2DR might be useful in designing a more individualized treatment. In this context, there are three main research strategies: Genome-Wide Association Studies, linkage studies and candidate gene studies. Genome-Wide Association Studies are a new tool involving a massive estimation of single nucleotide polymorphisms (SNP) in large samples. The linkage studies analyze shared alleles among family members with T2DR under the assumption that these prompt to a more aggressive development of T2DR. Finally, in the candidate gene approach, numerous genes coding proteins closely related to T2DR development have been analyzed (3).
Type 2 Diabetic Retinopathy can be divided into two types: background Type 2 Diabetic Retinopathy and proliferative Type 2 Diabetic Retinopathy. Proliferative Type 2 Diabetic Retinopathy refers to retinal neovascularization following ischemia whereas background Type 2 Diabetic Retinopathy changes occur before the onset of neovascularization (4, 5). Diabetic retinopathy is a common cause of visual loss in the world and it is a potentially blinding complication of diabetes that damages the eye's retina (6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, and 17). Non-insulin-dependent diabetes mellitus (NIDDM) may be the most rapidly growing chronic disease in the world. Its long-term complications, including retinopathy, nephropathy, neuropathy, and accelerated macrovascular disease, cause major morbidity and mortality (18, 19, 20, and 21). Initially, people can't notice any changes in their vision. But do not let diabetic retinopathy fool you. It could get worse over the years and threaten your good vision. Diabetic retinopathy is a complication of diabetes that affects the blood vessels of the retina (22). Growth of new blood vessels, known as proliferative retinopathy, may lead to blindness through hemorrhage and scarring. A deterioration of retinal blood vessels causing loss of blood vessels and leakage into the retina is known as maculopathy and leads to visual impairment and may progress to blindness.

Diabetic retinopathy falls into two main classes: non-proliferative and proliferative. The word "proliferative" refers to whether or not there is neovascularization (abnormal blood vessel growth) in the retina. Early disease without neovascularization is called non-proliferative diabetic retinopathy (NPDR). As the disease progresses, it may evolve into proliferative diabetic retinopathy (PDR), which is defined by the presence of neovascularization and has a greater potential for serious visual consequences (23).

In retinal diseases such as Diabetic Retinopathy vision loss is due to retinal vascular dysfunctions. Angiogenic stimulators and angiogenic inhibitors play important role in regulation of vascular functions. Under normal conditions balance is maintained between stimulators and Inhibitors. During pathological conditions like Diabetic Retinopathy this balance is disturbed due to the overproduction of angiogenic stimulators and decreased production of angiogenic inhibitors. With the number of diabetes increasing at an alarming rate, the number of people with retinopathy is expected to increase from 126.6 million in 2011 to 191 million by 2030, and the vision- threatening retinopathy during this period will increase from 37.3 million to 56.3 million (24). Due to high circulating glucose, the tiny blood vessels that nourish the retina are damaged, and in the early stages of the disease, microaneurysms, hemorrhages, intra-retinal microvascular abnormalities result in bleeding. If not controlled, this non-proliferative stage could progress to proliferative stage where the new vessels begin to grow, ultimately resulting in retinal detachment and blindness (25). The pathogenesis of diabetic retinopathy is complex; although hyperglycemia is considered as the leading cause of the development of diabetic retinopathy, however, hypertension and dyslipidemia are also some of the major risk factors associated with the disease (26, 27). A number of metabolic abnormalities initiated by hyperglycemia are implicated in the development of diabetic retinopathy. Therefore, the regulation of the histone sequences through the epigenetics modification, in chromatin, DNA base pairs are organized around core histones, and those histones are subject to multiple post-translational modifications that regulate many aspects of cell viability, including gene expression, apoptosis, and DNA replication and repair (28, 29). Dysregulation or unbalanced levels of histone modifications are involved in or associated with human diseases, including cancer, neurodegeneration, and inflammation (100%) (30, 31).

1.4 BRAIN DERIVED NEUROTROPHIC FACTOR

Brain-derived neurotrophic factor (*BDNF*) is a member of the neurotrophic factor family, which plays a major role in legislation of the survival, growth and maintenance of neurons (32). Brain-derived Neurotrophic Factor (*BDNF*) family of growth factors and is essential in modulating memory-associated neuroplasticity through regulating cell survival, proliferation, and synaptic growth in the developing central nervous system (33, 34). *BDNF* can induce long-term potentiation (LTP), which is considered to be the neurophysiological basis for learning and memory (35). Three studies have shown alterations in *BDNF* levels in the blood of T2DM patients. Interestingly, both fasting glucose levels and diabetes duration was negatively associated *BDNF*, suggesting that the decreased *BDNF* levels in T2DM may be related to higher glucose levels and longer diabetes duration previous studies have revealed that *BDNF* finds great application in photoreceptor cells and the repair of damage in the optic nerve and the retina. *BDNF* stimulates survival in injured RGCs prompted by axotomy or retinal ischemia and also supports regeneration of the nerve fibber (36, 37).

MATERIALS AND METHODOLOGY

Human retinal tissues (Donated) are collected from city prestigious institute **Department of Ophthalmology, Gandhi Medical College (Bhopal).** Proper care and handle must be taken for the entire provision for the collection of Retinal tissues and its transportation from these source station to our labs of our institute, School of biotechnology, RGPV Bhopal. Collection of donated human retinal endothelial cell has been approved by Institutional Ethics Committee for Human Research (IECH) VIDE letter no. 7824-26/MC/IEC/2016 dated on 22/03/2016.

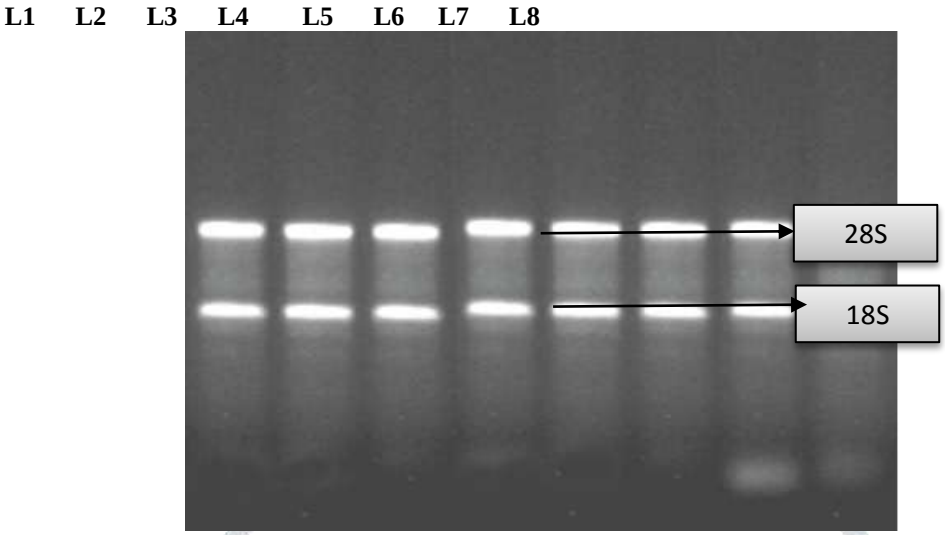
Samples were collected in M-K medium (a media formulated by McCarey and Kaufman) that has been kept in triple layer casing filled with dry ice. Samples were also collected directly in RNA later. For a prolonged storage, it was supplemented with 1% DMSO and kept in deep freezer between -40^0C to -80^0C.

Sample size includes (08) donors with (04) normal tissues, (02) Type 2 Diabetes Mellitus, (02) Type 2 Diabetic retinopathy.

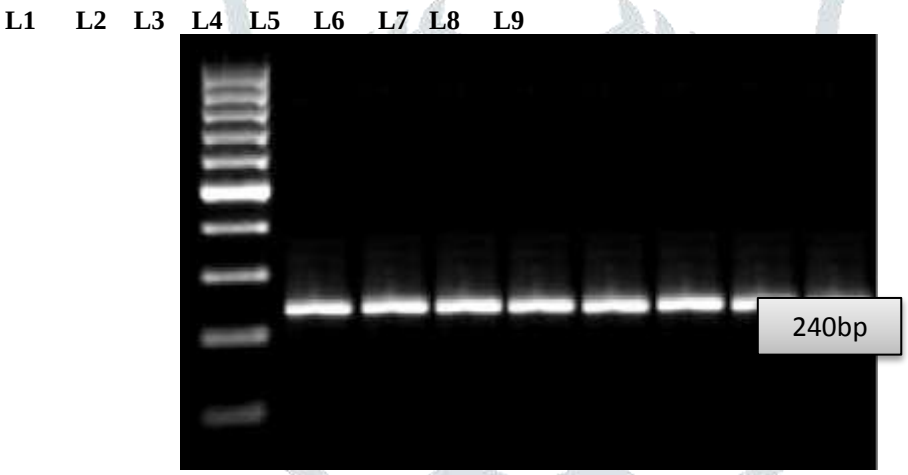
Total RNA has been isolated from human retinal tissues by using RNA-Xpress reagent (MB601 MolBioHimedia) and performed as per the manufacturer's protocol. Later the integrity was checked by running agarose gel (1.2%). Purity and concentration was checked by using Nanodrop ND-1000 spectrophotometer V 3.5. The total RNA was reverse transcribed by oligodT primers by using ProtoScript^R first strand cDNA Synthesis kit (BioLabs) in a thermal cycler **(**Gradient Palm Cycler from Corbett Life Sciences**)** and performed as per the manufacturer's protocol. The integrity and quality of cDNA was checked by performing gene specific PCR for GAPDH (housekeeping gene). The sequence of the primers was F: AGCTGAACGGGAAGCTCACTGG R: GGAGTGGGTGTCGCTGTTGAAGTC which amplified the region of 240bp. Later on gene specific PCR for *BDNF* has been performed by using oligo primers that has customly synthesized. The sequence of the primer was F: CTTCCCCTTTTAATGGTC R: CAGGGGCATAGACAAAA which amplified the region of 167bp. The Real time PCR had also been performed by using SYBR Green (LightCycler^R 480 SYBR Green I Master from Roche Diagnostics).

RESULTS:

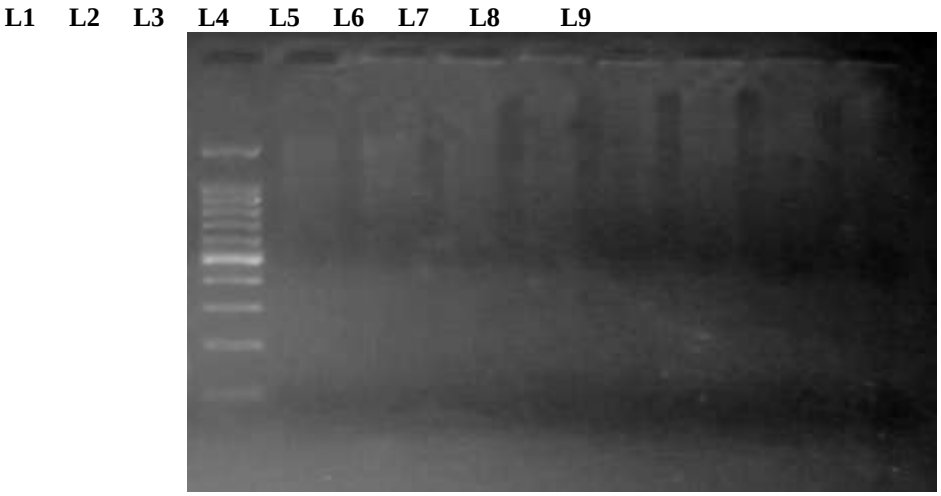
Total RNA were isolated from human retinal tissues and the bands were visualized by running agarose gel (1.2%). Two intact bands i.e. 28S and 18S were visible which shows the good quality RNA as shown in fig.



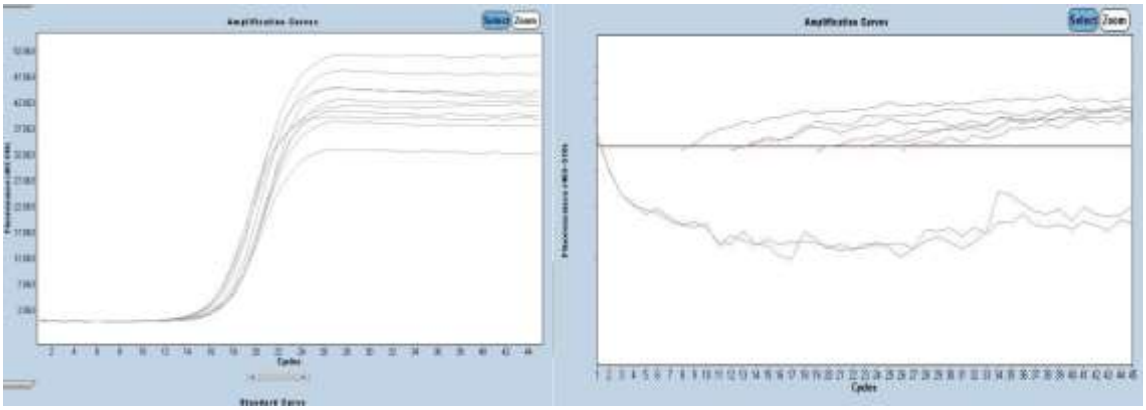
The reverse transcription PCR was performed by using oligo dT primers and further the integrity and genomic DNA contamination was checked by performing gene specific PCR for *GAPDH.* Amplified product of 240bp of *GAPDH* were visible by running PCR product along with 100bp DNA ladder on 2% agarose gel as shown on fig.



Gene specific PCR for *BDNF* gene with annealing temperature 50°C was performed for each sample through the manually synthesized oligo primers from cDNA prepared from Retinal RNA of human. On agarose gel electrophoresis of amplified PCR product of *BDNF* gene along with 100bp DNA ladder not revealed a single band of 167bp.



Real time PCR analysis of gene *GAPDH* and *BDNF* expression in human retinal tissue was performed which shows amplification as shown in fig.



CONCLUSION

Till today, the exact mechanism of the pathogenesis of this disease is not well understood. There are some key players that are responsible for causing this is never unfolding or remains underrated. The available method of treatment whether it is photocoagulation method, anti VEGF blockade, steroids have certain limitation in regards with the cure of this disease.

As of the available literature till now, there are some key holes that have to be studied in a sequence to understand the pattern of this disease and give a way for the ultimate cure of the disease.

In the present study, total RNA from the retinal tissue has been successfully isolated, and the cDNA synthesis with the oligo dT primer has also been done successfully. In order to confirm the quality of a cDNA, PCR amplification of *GAPDH* gene has also been done with the help of oligo primers that shows good quality cDNA and free from genomic DNA contamination. Expression of *BDNF* in human retinal tissues lacking epithelial and neuronal cell has not been confirmed by normal PCR as well as with Real time PCR. All the samples taken *viz* healthy person, type 2 Diabetes Mellitus and human suffering from type 2 Diabetic Retinopathy displayed negative amplification of 167bp PCR product specific for *BDNF* gene.

(*BDNF*) is a member of the neurotrophic factor family, which plays a major role in legislation of the survival, growth and maintenance of neurons (32). Brain-derived Neurotrophic Factor (*BDNF*) family of growth factors and is essential in modulating memory-associated neuroplasticity through regulating cell survival, proliferation, and synaptic growth in the developing central nervous system (33, 34). Finally, full interpretation of our findings is hindered by the lack of knowledge regarding the expression of BDNF in retinal endothelial cells it might be possibilities that the size of *BDNF* is so small that it could not found in PCR and in real time PCR. But in future there is might be possibility that the current method is insensitive to detect the low level expression of *BDNF* in this retinal tissue. Several methods are available which is more sensitive than the method we use such as

Micro assays, ELISA, Western blot, SDS DIGE, RNA seq., Tiling arrays

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