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International Journal For Research in  
Applied Science and Engineering Technology



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# **INTERNATIONAL JOURNAL FOR RESEARCH**

IN APPLIED SCIENCE & ENGINEERING TECHNOLOGY

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**Volume: 7      Issue: XI      Month of publication: November 2019**

**DOI: <http://doi.org/10.22214/ijraset.2019.11018>**

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# Analyzing the Role of DNA Hydroxymethylation in BDNF Gene in case of Type 2 Diabetic Retinopathy

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## I. INTRODUCTION

Diabetic Retinopathy is the last stage of Diabetes in which eyes get affected and in advanced stage it may lead to retinal detachment causing vision loss (National eye institute). Diabetic retinopathy and neurodegeneration disease may result due to the dysregulation of 5hmC level (Tripathi et al., 2017).

In vitro studies performed by Ola et al demonstrated that, brain derived neurotrophic factor (BDNF) is the most abundant in the retina as compared to other neurotrophin. Further in vitro studies indicated that reduced levels of BDNF in diabetes may cause apoptosis and neurodegeneration early in diabetic retina, which may lead to neuro-vascular damage later in DR (Ola et al., 2013).

Another study by Liu and their coworkers reveals that the BDNF may protect retinal neurons from hyperglycemia via the TrkB/ERK/MAPK pathway and provides novel insights into the pathogenesis of DR (Liu et al., 2013). Past studies shows, a novel mechanism for mitochondrial homeostasis and mitochondrial cell death due to over expression of TET 5mC/hmC conversion at the CpG sites in BDNF (Wei et al., 2014).

A dynamic DNA methylation process of MMP-9 was shown to maintain its transcriptional activation; through the transcription factor binding sites of MMP-9 promoter are hypermethylated in diabetes, due to concomitantly increased binding of TET at same site, MMP-9 DNA remains hypermethylated resulting in its transcription activation and this continues to fuel into the mitochondrial damage (Kowluru et al., 2015).

Due to the novelty of DNA methylation, the exact mechanisms of how the DNA methylation machinery assists DR pathogenesis remains unclear.

Abnormal glucose levels enhance cellular oxidation, which triggers the progression of TET enzyme-assisted DNA demethylation, resulting in epigenetic dysregulation of damaged tissues (Cortés et al., 2017).

Carey and Xu and their coworkers demonstrate that high levels of glucose can inhibit the conversion of DNA methylation (5mC) to hydroxymethylation (5hmC) by the ten-eleven translocation (TET) dioxygenase family of proteins by influencing  $\alpha$ -ketoglutarate levels. Based on their hypothesis, high blood glucose levels would increase 5hmC due to increased TET activity, resulting in gene expression dysregulation (Xu et al., 2011; Carey et al., 2015).

Thus, it was hypothesized that over expression of TET in diseased condition may lead to 5hmC level dysregulation in its target genes such as BDNF and this gene contributes to T2DR pathogenesis.

Study shows that methylation activity of TET2 enzyme can be inhibited by using suitable methylation inhibitors which may result in the decrease of the hypermethylation of BDNF protein (Tripathi, 2017).

On the basis of literature study, DimethylallylGlycine (DMOG) is selected as ligand molecule for the controlling the level of TET2 (Zhang et al., 2017). It may regulate the level of TET2 through which BDNF level may regulate and it may help in controlling T2DR hence it can be approached towards personalized medicine.

## II. MATERIALS AND METHODS

- 1) *Sample Collection:* Human retinal cells were collected from city prestigious institute- Department of Ophthalmology, Gandhi Medical Hospital (Bhopal). Proper concern and handle must be taken for the entire provision for the collection of human retinal cells and its transportation from this source station to our labs of our institute, School of Biotechnology, Rajiv Gandhi Proudyogiki Vishwavidyalaya, Bhopal. 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. For prolonged storage, it was supplemented with 1% DMSO and kept in deep freezer between -40°C to -80°C.

- 2) **Drug Treatment:** For kinetics studies, T2DR cells were plated and treated with DMOG (Dimethylxalylglycine) for 12 hours and 24 hours with different concentration. DNA was harvested at various time points for methylation i.e. EpiMark kit analysis and real time PCR.
- 3) **DNA Isolation:** DNA was extracted from cultured T2DR cells with according to protocol (Wang *et al.*, 2012).
- 4) **Quantitative RT-PCR Analysis:** The quantitating of DNA levels was carried out by a real-time pcr. Briefly, after DNA isolation, the specific gene of interest (BDNF) and reference gene (GAPDH) were amplified by PCR. The experiment was performed in duplicate.
- 5) **EpiMark® 5-hmC and 5-mC Analysis Kit:** The EpiMark® 5-hmC and 5-mC Analysis Kit can be used to analyze and quantitate 5 methylcytosine and 5-hydroxymethylcytosine within a specific locus. The mean cytosine methylation levels of CpG sites in the fragment were determined by treatment of genomic DNA with EpiMark® 5-hmC and 5-mC Analysis Kit (Gao *et al.*, 2013). Methylation analysis was performed using Real-Time PCR for BDNF exon 4. The primer sequences were follows: for BDNF, forward- AGTTTGTGGGGTTGGAAGTG and reverse- ATACCGATATATACTCCTTCTATTCTACA. The PCR conditions were follows: 1 cycle of 95°C for 15 minutes, 40 cycles of 95°C for 15 seconds, 40 cycle of 55°C for 30 seconds, 40 cycle of 72°C form 30 seconds.

### III. RESULTS

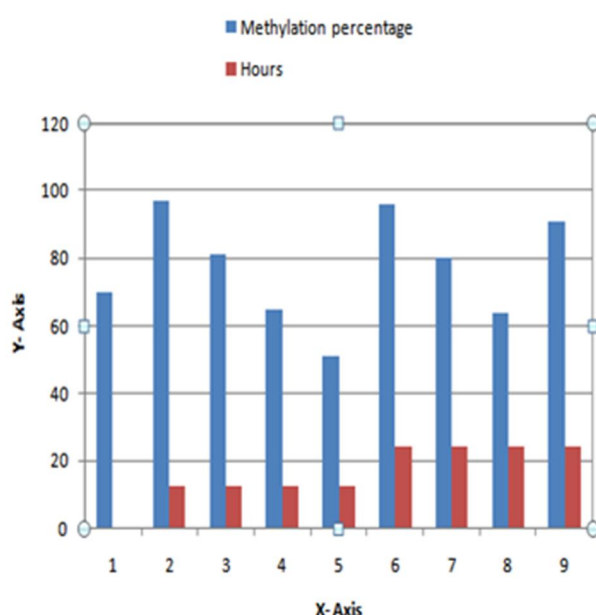
5hmC was analyzed and quantitated using the EpiMark 5hmC and 5mC Analysis Kit (NEB, #E3317S) according to the manufacturer's protocols.

Different dilutions of synthesized DNA i.e. 1X, 2X, 3X dilutions were checked with 10 pmol final primer concentration for GAPDH and BDNF expression to optimized initial template concentration. Finally 1X diluted DNA was used as a template for Real Time experiment for all DNA sample. In Real Time PCR, GAPDH and BDNF expression were successfully detected in all the dilutions of DNA. Amplification curve was found for DMOG treated DNA of T2DR.

#### A. Hydroxymethylation Percentage of DMOG

Table.1: HydroxyMethylation percentage of DMOG treated T2DR cells DNA

Concentration			
For 12 h	$\Delta CT$	$1+1/2^{-\Delta CT}$	hmc % $100/(1+1/2^{-\Delta CT})$
Control	-1.22	1.43	70%
25 $\mu M$	-5.05	1.03	97%
50 $\mu M$	-2.13	1.22	81%
75 $\mu M$	-0.9	1.53	65%
100 $\mu M$	-0.05	1.97	51%
150 $\mu M$	-	-	-
For 24 h			
25 $\mu M$	-4.89	1.033	96%
50 $\mu M$	-0.2	1.25	80%
75 $\mu M$	-0.85	1.55	64%
100 $\mu M$	-	-	-
150 $\mu M$	-3.35	1.098	91%



1	Control	Hours	70%
2	25µM	12h	97%
3	50µM	12h	81%
4	75µM	12h	65%
5	100µM	12h	51%
6	25µM	24h	96%
7	50µM	24h	80%
8	75µM	24h	64%
9	150µM	24h	91%

Figure2: Comparison of 5hmc% between DNA of healthy and T2DR patient *BDNF*

(70% of 5hmc level was observed in control (healthy patient) and in the DMOG treated diseased samples with the DMOG concentration 75µM-12h, 100 µM-12h and 75µM-24h the 5hmc level was observed to be 65% 51% and 54% respectively. This was less than the value of 5hmc level in the control sample).

This work involving exposure of human retinal epithelium cells to DMOG was found more relevant at concentration (75µM and 10075µM) in T2DR patient.

Cells were treated by DMOG with different concentration and different time which successfully inhibit TET2. Positive results were found in at DMOG concentration (75µM-12h, 100 µM-12h and 75µM-24h) in Type 2 Diabetic Retinopathy Patient retinal sample

#### IV. CONCLUSIONS

Diabetic retinopathy remains the leading cause of blindness and visual impairment in the working age population. *BDNF* is a member of neurotrophin family of growth factors and is critical for the development, differentiation, and maintenance of retina. The exact mechanism of the pathogenesis of Type 2 Diabetic Retinopathy disease till today is not well understood. Type 2 Diabetic Retinopathy (T2DR) is the leading cause of vision loss in the general population in many countries, including the adult working population and the elderly (Lee *et al.*, 2015).

The present study was focused on 5hmc analysis in *BDNF* gene to understand the mechanism of pathogenesis in Type 2 Diabetic Retinopathy which includes *in vitro* studies were performed using donated samples of human retinal tissue. Retinal epithelial cells were isolated from human retinal epithelial layer and were passaged for the growth of cells. After obtaining 82% confluency; cells were treated by different concentration of DMOG at different time intervals. 5hmc level was examined using oligo primers in *BDNF* gene region. Positive results were found in at DMOG concentration (75µM-12h, 100 µM-12h and 75µM-24h) in Type 2 Diabetic Retinopathy Patient retinal sample & at DMOG concentration (75µM-12h and 75µM-24h) in Type 2 Diabetic Mellitus Patients retinal sample.

Results conclude that, DimethylloxallylGlycine (DMOG) can be a suitable molecule to be used for the control of the level of TET2. It may regulate the level of 5hmc and thereby the *BDNF* level which in turn may help in controlling T2DR.

#### V. ACKNOWLEDGEMENT

This study was supported by the research of epigenetic modification on the basis of brain-derived neurotrophic factor that related with type II diabetic retinopathy, I am grateful to RGPV, Bhopal for offering the expertise that greatly assisted the research of epigenetics.

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