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An Epigenetic Approach to the Treatment of Type 2 Diabetic Retinopathy: The Experience with DNMT Demethylated Agents- Decitabine and Zebularine

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I. INTRODUCTION

Diabetes, it is a chronic metabolic disorder in which the amount of insulin makes by the body falls underneath the normal range. In body, Insulin is usually accountable for lowering the blood sugar level. In the lack of sufficient quantity of insulin, the blood sugar levels raise leading to symptoms like extreme thirst, frequent urination and increase hunger (Adapa and Tk, 2015). DR (diabetic retinopathy), it is a neurovascular disorder and the leading reason of blindness and visual impairment amongst working-age Adults (Lee et al., 2015)(Solomon et al., 2017). Even though extended diabetes period, high blood pressure and poor glycemic control are the foremost risk factors of DR (diabetic retinopathy) (Lee et al., 2015)(Cardoso et al., 2017). Epidemiological data support the hypothesis of differential genetic susceptibility to this chronic complication (Priščáková et al., 2016). Intonation of modification of histone (methylation, phosphorylation, acetylation) and methylation of DNA are the chief driving forces behind the epigenetics phenomenon (Materials et al., 1998)(Jones et al., 2013)(Bestor, 2000). In Epigenetic, for instance histone post-translational modifications in chromatin, non-coding RNAs and DNA methylation, arbitrate the interaction between environmental and genetic risk factors. Determination of changes in epigenetic might contribute to the oxidative stress and the metabolic memory phenomenon, extracellular matrix accumulation and inflammation, all of which guide to the progress of DR (diabetic retinopathy) (Marpadga A. et al., 2016)(Enoia et al., 2018). As per the available literature, the diagnosis of T2DR with the help of regulating level of 5mC and BDNF on retinal epithelial cell has not been studied so far. However, it was not known whether BDNF protects retinal epithelial cells exposed to hyperglycemia in vitro by maintaining the normal level of 5mC DNA methylation. The methylation pattern on BDNF gene predicted the DNMT1 role, as DNMT1 plays an important role in DNA methylation and in case of diabetes and diabetic retinopathy DNMT1 level increases. Interaction pattern of the proteins i.e. BDNF protein and DNMT1 protein was studied with the help of different docking tools i.e. swissdock, autodock, clusproserver. On the basis of result obtained from bioinformatics tools best ligand molecules were selected i.e. decitabine and zebularine (Singh and Tiwari, 2018). Zebularine and decitabine are two of the most well-known DNA methylation inhibitors. Both drugs are nucleoside analogs which have been widely used for studying the role of DNA methylation in biological processes as well as for clinical treatment of patients with acute myeloid leukemia and in human bladder cancer cell (Amatori et al., 2009)(Cheng et al., 2004). No studies of decitabine and zebularine have been reported in case of diabetic retinopathy. Decitabine and zebularine, which may control the level of dnmt1 through which BDNF level may get controlled and it may help in controlling T2DR so it can approach 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 -40ºC to -80ºC.
2) *Drug Treatment:* For kinetics studies, T2DR cells were plated and treated with zebularine and decitabine for 12 hours and 24 hours with different concentration. The medium was changed every 4 hours, 8 hours. 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- TAACGGCGGCAGACAAAAAGA and reverse- GAAGTATTGCTTCAGTTGGCCT. The pcr conditions were follows: 1 cycle of 95ºC for 15 minutes, 40 cycle of 95ºC for 15 seconds, 40 cycle of 55ºC for 30 seconds, 40 cycle of 72ºC for 30 seconds.

III. RESULTS

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 zebularine treated DNA and decitabine treated DNA of T2DR.

A. *Methylation Percentage of Zebularine*

Concentration	ΔCT	½ $^{-\Delta CT}$	Methylation percentage 100/ (1 + ½ -ΔCT)
10 µM (12 h)	-1.05	0.48	67 %
5 µM (12 h)	-0.99	.53	65%
1 µM (12 h)	-	-	-
10 µM (24 h)	-3	0.125	88 %
5 µM (24 h)	-0.05	0.97	50 %
1 µM (24 h)	-4.76	0.037	96%
Control	-1.2	0.43	70%

Table 1: Methylation percentage of zebularine (T2DR)

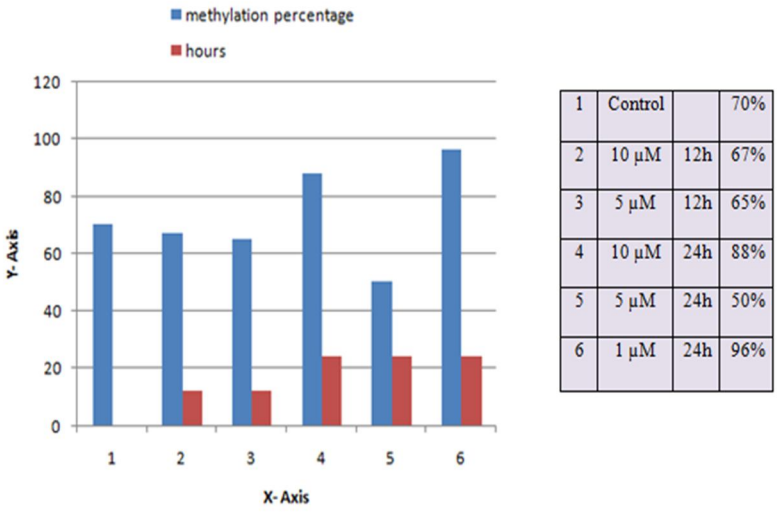
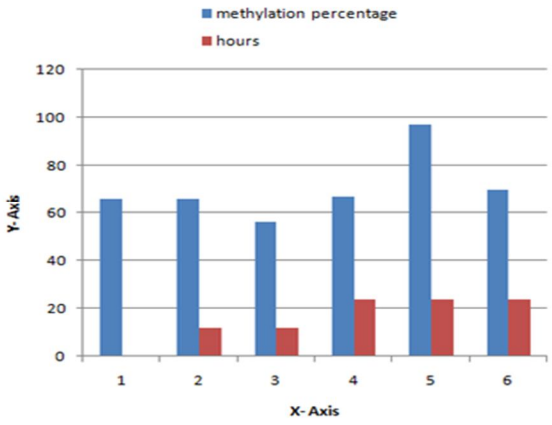


Figure 1: Methylation percentage of zebularine treated DNA of T2DR - Methylation percentage were seen with low concentrations of zebularine – 10 µM (12h), 5 µM (12h), 5 µM (24h) but 10 µM (24h) and 1 µM (24h) were showed high methylation percentage than control which was 70%.

B. Methylation Percentage Of Decitabine

Concentration	ΔCT	½ $^{-\Delta CT}$	Methylation percentage 100/ (1 + ½ -ΔCT)
1 µM (12 h)	-0.95	0.51	66%
'5 µM (12 h)	-0.35	0.78	56%
'3 µM (12 h)		-	-
1 µM (24 h)	- 1	0.5	67%
'5 µM (24 h)	-5.01	0.03	97%
'3 µM (24 h)	-1.75	0.42	70%
Control	-0.95	0.51	66%

Table 2: Methylation percentage of decitabine



1	Control		66%
2	1 µM	12h	66%
3	.5 µM	12h	56%
4	1 µM	24h	67%
5	.5 µM	24h	97%
6	.3 µM	24h	70%

Figure 2: Methylation percentage of decitabine treated DNA of T2DR - Methylation percentage were seen with low concentrations of decitabine – 1 µM (12h), .5 µM (12h) but 1 µM (24h), .5 µM (24h) and .3 µM (24h) were showed high methylation percentage than control which was 66%.

Activity of DNMT inhibitor – zebularine at the following concentration along with selected time interval at 10 µM (12 h) -67 %, 5µM (12 h) – 65% and 5µM (24 h) – 50% were showed the positive results in T2DR. Hence the zebularine acting as an inhibitor as it methylation percentage is below 70% of the normal. Activity of DNMT inhibitor – decitabine at the following concentration along with selected time interval at 1 µM (12 h) - 66% and .5 µM (24 h) – 56 % were showed the positive results in T2DR. Hence the decitabine acting as an inhibitor as it methylation percentage is below 66 % of the normal.

IV. CONCLUSIONS

Diabetic retinopathy, it is a microvascular complication of diabetes and it is the leading cause of vision loss in the general population in many countries, including the adult working population and the elderly. BDNF is a part of the neurotrophin family of growth factors and it is critical for the differentiation, development and maintenance of the retina. The exact machinery of the pathogenesis of T2DR disease till today is not well understood. Earlier studies have reported that T2DR is related with neurodegeneration. BDNF factor, it is constructing from muller cell and glial cell in the eye play significant role in retinal neuronal cells and plays a role in neoangiogenesis. Current development in understanding of T2DR pathogenesis has lead to important advances in available pharmacotherapy. On the other hand, a cure of T2DR has yet to be unclear. This study propose that a new sign in the field of retinopathy; previous study shows that epigenetically regulated gene BDNF inversely correlated with T2DM present. Relative studies have exposed that zebularine is much less effective than decitabine as a demethylating agent and it have shown dissimilar effects of both agents on transcription of gene. The machinery of action of zebularine and decitabine is not identical. DNA methylation may be a hopeful target for latent therapeutic strategies and demethylating drugs may be beneficial in the treatment of T2DR. This study describes new findings on epigenetic regulation of BDNF genes by DNA methylation in T2DR
Different dilution of drug treated DNA i.e. 1X, 2X, 3X dilutions were checked with 10 pmol primer concentration for BDNF and

GAPDH expression to optimized initial template concentration. In real time PCR, BDNF and GAPDH expression were successfully detected. Finally 2X diluted DNA were used as a template for real time PCR experiment for the drug treated DNA. This study concludes that the expression of BDNF and GAPDH gene were detected in the retinal epithelial cells. The molecular compound zebularine and decitabine should be validated with *in vivo* techniques. The technique used to validate the compound is molecular targets program (MTP). Through MTP a molecular compound will be validated for a drug which may control the level of dnmt1 through which BDNF level may get controlled and it may help in controlling T2DR and it can approach towards personalized medicine but further study will required to validate the drugs.

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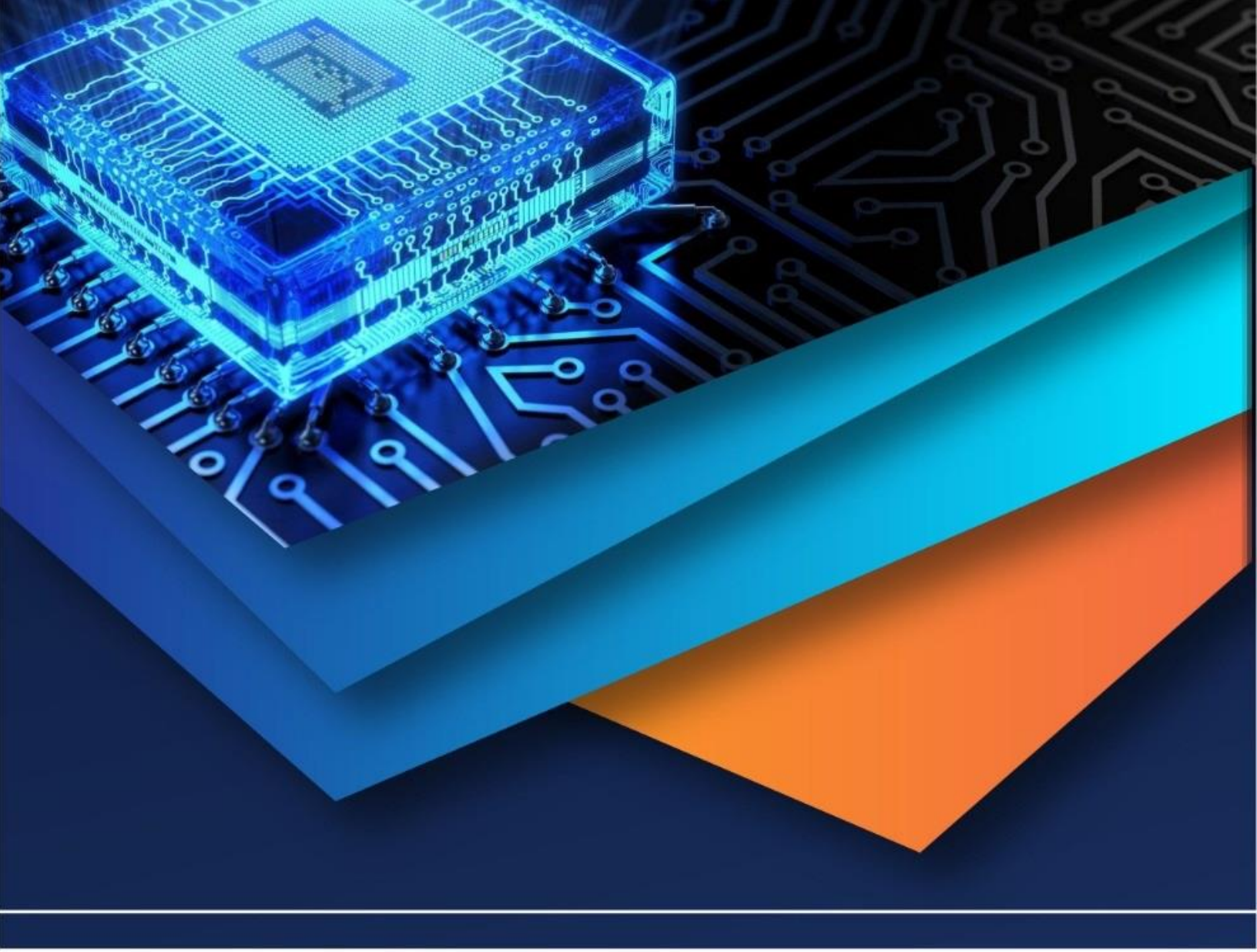

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