The effect of vitamin D and melatonin on the ocular tissues in streptozotocin – induced diabetes model in rats

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Abstract. – OBJECTIVE: The aim of the study was to investigate the effectiveness of vitamin D and melatonin on the ocular tissues in streptozotocin (STZ)-induced diabetes.

MATERIALS AND METHODS: In this study, a total of 45 male Wistar rats were randomly divided into five groups as follows: 1) non-diabetic rats (control group); 2) untreated STZ-induced diabetic rats; 3) STZ-induced diabetic rats treated with vitamin D; 4) STZ-induced diabetic rats treated with melatonin; 5) STZ-induced diabetic rats treated with the combination of vitamin D and melatonin. After six weeks of treatment, all rats were sacrificed for post-mortem analyses. Retinal and corneal samples were obtained and analyzed for glial fibrillary acidic protein (GFAP) expression. Retinal and corneal thicknesses in addition to morphological changes were assessed via hematoxylin and eosin staining.

RESULTS: Untreated diabetic rats revealed retinal disorganization, atrophy, increased vascularization along with more GFAP staining. However, all the treated groups exhibited more regular retinal layers and minimal GFAP staining. Additionally, treatment groups showed more uniform corneal layers with minimal GFAP staining.

CONCLUSIONS: Melatonin and vitamin D could be used as an additional complementary treatment in diabetes. Developing treatment protocols involving supplementation, as well as informing patients about the potential benefits of vitamin D and melatonin could be impactful in the treatment process of diabetes.

Key Words: Retinal inflammation, Corneal inflammation, Vitamin D, Melatonin.

Introduction

The pathogenesis of diabetic injury is believed to involve oxidative stress and hyperglycemia but most of its aspects are still under investigation. Hyperglycemia-induced oxidative stress is one of the most common pathological mechanisms in diabetes mellitus, and it results in various complications, including diabetic keratopathy and retinopathy. Also, the increased inflammatory process is another important factor responsible for diabetic damage. In uncontrolled diabetes, oxidative stress leads to free radicals to accumulate, and the retina is highly susceptible to oxidative damage caused by these free radicals. Oxidative stress also causes an increase in vascular endothelial growth factor (VEGF) levels in the diabetic retina. Melatonin, a strong antioxidant and anti-inflammatory agent, is mainly synthesized and released from photoreceptors in the retina. As well as being an effective antioxidant, it is also considered to be a direct free radical scavenger and stimulates several antioxidative enzymes. There are some studies affirming that melatonin reduces retinal VEGF levels. An alternative treatment method is vitamin D, which plays an important role in normal insulin release and maintenance of glucose tolerance. There is ample evidence suggesting that vitamin D has a role in insulin secretion, which includes the presence of the 1,25(OH)2D3 receptor in β cells and vitamin D-dependent calcium-binding proteins in pancreatic tissue. Furthermore, vitamin D can decrease nitric oxide production, inhibit VEGF, downregulate the IGF-1 pathway, and upregulate the cellular transporters of sulfate. All these molecules have been implicated in the pathogenesis of DR. Similarly, calcium homeostasis and calcium-induced signal pathways have an important role in the development of retinal hypoxia. Glial fibrillary acidic protein (GFAP) is an intermediate filament protein present in numerous cell types including muller cells and supports the mechanical strength of astrocytes. Increased
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GFAP production is a key indicator of glial reactivity\textsuperscript{15}. Likewise, it is a biomarker for the process of immune inflammation\textsuperscript{16}. The oxidative stress that occurs in DM causes dysfunction and structural abnormalities in Müller cells and upregulation of GFAP\textsuperscript{17}. In addition to the central nervous system (brain, spinal cord, retina), GFAP has been observed in corneal stromal cells. However, there is limited information in the literature regarding corneal GFAP. In few studies\textsuperscript{18,19}, an increase in the corneal GFAP has been noted yet the mechanism is unclear.

So, in this study, we aim to evaluate the effectiveness of vitamin D and melatonin treatment, separately and in combination, on retinal inflammation and structural changes in diabetic rats.

Materials and Methods

In this study, a total of 45 male Wistar rats (10 weeks old) weighing between 200 and 250 grams were divided into 5 groups of 9 rats each. The animals were housed in well-ventilated 750 cm\textsuperscript{2} polypropylene cages, each containing a maximum of 3 rats, in a room at 20-25°C with 60% humidity and 12 h light/dark cycle, with standard rat pellets and ad libitum access to water. They were treated in accordance with the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Vision Research. The study protocol was approved by the Institutional Animal Care and Use Committee.

Streptozotocin (STZ), an antibiotic produced by Streptomyces achromogenes, was used to induce experimental diabetes.

The animals were randomly divided into 5 groups as follows:
1. Control group: non-diabetic rats (CG);
2. Untreated diabetic rats: STZ-induced diabetes, no treatment;
3. STZ-induced diabetic rats treated with vitamin D (STZ+D);
4. STZ-induced diabetic rats treated with melatonin (STZ+M);
5. STZ-induced diabetic rats treated with combination vitamin D and melatonin (STZ+D/M).

Diabetes was induced in groups 2-5 using a single dose of 60 mg/kg STZ dissolved in 0.01 M citrate buffer pH 4.5 injected intraperitoneally. Rats in the control group received a single intraperitoneal injection of 0.9% NaCl.

At 72 hours after STZ injection, a blood sample was taken from the tail artery of each rat to diagnose diabetes. Rats with fasting blood glucose (FBG) levels of 250 mg/dl or higher were accepted as diabetic. For the next 6 weeks, the rats in groups 3 and 5 were treated with intraperitoneal vitamin D at a dose of 0.5 µg/kg/day and the rats in groups 4 and 5 were treated with intraperitoneal melatonin 10 mg/kg/day (Sigma-Aldrich, M5250, St. Louis, MO, USA) which was dissolved in 1% ethanol. Melatonin injections were performed daily in the evening, between 20:00 pm-22:00 pm. FBG in the diabetic rats was measured at 2-week intervals. At the end of 6 weeks, the rats were killed by cardiac puncture under ether anesthesia and transcardially perfused with heparinized saline followed by 10% formalin in phosphate buffer. Retinas and corneas were obtained from the rats by an ophthalmologist, subsequently stored at -70°C until analysis. For histopathological examinations, corneal and retinal specimens taken from the rats were fixed in 10% formalin solution and embedded in paraffin. Two slides of 4-µm thick corneal and retinal sections were prepared for analysis using the fully automated Leica ASP300S. For each tissue type, one slide was stained with hematoxylin and eosin and the other was stained with anti-GFAP antibody (Dako, clone 6F2, ready-to-use) using a Dako Autostainer 48 Link immunohistochemical staining machine. Retina and corneal thickness were measured (in µm) from the hematoxylin-stained retinal tissue using Olympus image analysis software (DP21). The proportion of GFAP-positive cells was calculated based on a count of 100 cells in the most intensely stained area and graded as 0 (no stained cells), 1 (1-5% stained cells), 2 (6-10% stained cells), or 3 (>11% stained cells).

Statistical Analysis

Data were reported as mean ± standard deviation. All data were analyzed using the nonparametric Kruskal-Wallis test, and subsequent individual comparisons were performed by Mann-Whitney U-test. The significance level was set at a $p$-value of $<0.05$.

Results

Retinal GFAP and Histopathological Results

The retinal layers showed normal organization in the control group (Figure 1a). Retinal GFAP staining was significantly intensified in
untreated diabetes (STZ) group compared to the control group. Correspondingly, in the untreated diabetes group, the retina appeared to be disorganized, with irregular and atrophied layers (Figure 1b, 1c).

In all treatment groups, the retinal layers appeared normal and there was minimal GFAP staining (Figure 1d, 1e). The STZ+D, STZ+M, and STZ+D/M groups had significantly less retinal GFAP staining compared to the untreated diabetic group. There was also less GFAP staining in the STZ+D group than the STZ+M group, and even less in the STZ+D/M group than the other two groups (Table I). However, retinal GFAP staining did not differ significantly between rats in the STZ+D, STZ+M, and STZ+D/M groups (Table I).

**Corneal GFAP and Histopathological Results**

Corneal samples taken from the control group and all treatment groups showed regular layers with minimal GFAP staining (Figure 2a, 2b, 2c, 2d). However, in the untreated diabetic group, corneal irregularity, vascularization, and more stromal GFAP staining were observed (Figure 2e). Untreated diabetic rats had the highest rate of corneal GFAP staining, while rats in the STZ+D/M group showed the least GFAP staining. Corneal GFAP staining was significantly lower in the STZ+D, STZ+M, and STZ+D/M groups compared to the untreated diabetic group (Table I).

**Discussion**

Despite the various medications and surgical methods, millions of people worldwide persistently develop sight-threatening DR. Therefore, receiving supportive treatment in the early stages, prior to the progression of the disease is crucial. On this subject, a great deal of work has been done by researchers. However, there is no prior information on the combined use of vitamin D and melatonin.

In a study conducted by Jiang et al., melatonin reduced the amount of VEGF, thus they stated that melatonin has a protective role for diabetic retinopathy. Baydas et al. reported that reactive gliosis was prevented by melatonin administration. Ozdemir et al. showed that melatonin diminished the number of molecules responsible for oxidative damage, such as nitrotyrosine and malondialdehyde, as well as the VEGF level which is responsible for vascular changes and microvascularopathy.

Similarly, we found that melatonin treatment either alone or in combination with vitamin D had a significant effect on retinal inflammation. Also, combination treatment had better results.

Vitamin D treatment has been shown to be effective in the treatment and prevention of diabetes mellitus in both human and animal models. Many studies have reported an inverse relationship between vitamin D deficiency (VDD) and DR. Aksoy et al. reported an association between lower levels of active vitamin D (1,25-dihydroxyvitamin D3) and increased retinopathy.
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Similarly, Ashinne et al.28 demonstrated that lower serum 25(OH)D was associated with increased severity of DR, and the presence of vitamin D deficiency was associated with a two-fold increased risk of proliferative DR. Furthermore, Albert et al.29 showed calcitriol (1α,25-dihydroxyvitamin D3) has an inhibitory effect on retinal neovascularization, which may also be beneficial in the treatment of ocular diseases with the neovascular component. In contrast, some studies30,31 in the literature failed to detect an association between vitamin D and DR. Patrick et al.32 found a correlation between the severity of diabetic retinopathy and prevalence of VDD, but they declared that findings were inconclusive about the existence of a relationship between retinopathy severity and serum 25-hydroxyvitamin D concentration.

Table 1. Comparisons of retinal and corneal GFAP staining between groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n (%)</th>
<th>Retinal GFAP</th>
<th>Corneal GFAP</th>
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<tr>
<td></td>
<td></td>
<td>G.Median [Min. / Max.]</td>
<td>G.Median [Min. / Max.]</td>
</tr>
<tr>
<td>1 (control)</td>
<td>9 (20)</td>
<td>1.6 (1 / 2)</td>
<td>0.7 (0 / 2)</td>
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<tr>
<td>2 (STZ)</td>
<td>9 (20)</td>
<td>2.4 (2 / 3)</td>
<td>1.9 (1 / 3)</td>
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<tr>
<td>3 (STZ+D)</td>
<td>9 (20)</td>
<td>1.3 (1 / 2)</td>
<td>0.7 (0 / 2)</td>
</tr>
<tr>
<td>4 (STZ+M)</td>
<td>9 (20)</td>
<td>1.5 (0 / 2)</td>
<td>0.7 (0 / 2)</td>
</tr>
<tr>
<td>5 (STZ+D/M)</td>
<td>9 (20)</td>
<td>1.1 (0 / 2)</td>
<td>0.6 (0 / 2)</td>
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<tr>
<td>Total</td>
<td>45 (100)</td>
<td>1.6 (0 / 3)</td>
<td>0.9 (0 / 3)</td>
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*p-value*<0.001 0.015

<table>
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<tr>
<th>Pairwise Comparisons</th>
<th>p-value</th>
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<tbody>
<tr>
<td>1→2</td>
<td>0.013* 0.010*</td>
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<tr>
<td>1→3</td>
<td>0.444 1.000</td>
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<tr>
<td>1→4</td>
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<tr>
<td>1→5</td>
<td>0.176 0.744</td>
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<tr>
<td>2→3</td>
<td>0.001* 0.010*</td>
</tr>
<tr>
<td>2→4</td>
<td>0.007* 0.009*</td>
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<tr>
<td>2→5</td>
<td>&lt;0.001* 0.004*</td>
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<tr>
<td>3→4</td>
<td>0.576 0.962</td>
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<td>3→5</td>
<td>0.556 0.744</td>
</tr>
<tr>
<td>4→5</td>
<td>0.251 0.781</td>
</tr>
</tbody>
</table>

Kruskal-Wallis Test (Monte Carlo) - Post Hoc Test: Dunn’s Test, G. Median: Grouped Median, Min.: Minimum, Max.: Maximum, *statistical significant (*p<0.05).

Figure 2. Example corneal samples from the study groups. (a) Normal corneal tissue in the control group (H&E x 40); (b) Regular corneal tissue (arrow) in treated rats (H&E x 40); (c) Corneal stromal 1(+) GFAP staining (arrow) in treated rats (x 100); (d) Corneal 1(+) GFAP staining (arrow) in treated rats (x 100); (e) High intense 3(+) GFAP staining (arrow) and vascularization in corneal stroma in diabetic rats (x 100).
In this study, we showed that vitamin D treatment either alone or in combination with melatonin was effective on retinal inflammation caused by diabetes. Although combined therapy made a minor difference, nevertheless it was more effective.

Another sight-threatening complication of diabetes is keratopathy manifesting with delayed cornea wound healing, recurrent corneal erosions, and neurotrophic ulcers. However, there is significantly less attention to diabetic keratopathy compared to retinal complications. It has been shown in some studies, that the cornea is more structurally damaged in diabetic rats. Similarly, the corneal layers were more irregular and vascularized in diabetic rats in our study. Unfortunately, there were insufficient data collected to statistically quantify the vascularization.

A study conducted by Jing et al showed that calcitriol treatment could significantly suppress the inflammatory markers, such as IL6, TNFA, and IL1B in the rat corneas. Similarly, Saadettin et al demonstrated that 1,25-dihydroxyvitamin D3 inhibits wound healing by decreasing the IL6 level. These studies have focused on the anti-inflammatory aspect of vitamin D treatment.

Another agent with anti-inflammatory and antioxidant properties is melatonin, and its receptors are also present in the cornea. Melatonin has been shown to affect corneal hydration and corneal wound healing.

In our study, corneal GFAP expression was most pronounced in the untreated diabetic group. When the diabetic rats were treated with vitamin D and melatonin either separately or in combination, GFAP staining decreased dramatically, and more regular corneal layers were observed. Both melatonin and vitamin D can prevent diabetic damage and neuronal injury related to oxidative stress, which may explain the reduction in corneal GFAP with treatment.

The present study has some limitations. Unfortunately, vitamin D and melatonin serum concentrations were not assessed. Also, we have limited data to evaluate vascularization and structural changes. So, further research on vascular and structural parameters would be more fruitful.

Conclusions

To our knowledge, this is the first study that has assessed the effects of vitamin D and melatonin applications when used separately and in combination on eye tissues in STZ-induced diabetes. Our results suggest that vitamin D and melatonin may have therapeutic and protective effects against retinal and corneal damage in diabetic patients. Therefore, this combination could be used as a complementary treatment of diabetes.

Authors’ Contribution
Conceptualization: S.B; methodology: S.B, O.N.S., investigation: S.B., E.C., writing-original draft: E.C., S.B., writing-review and editing: S.B., O.C.

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Conflict of Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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