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Original Research Article

The Role of Vitamin D3 in Preventing Ethambutol-induced Toxic Optic Neuropathy on Wistar Rat Model: A Pilot Study

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Abstract

Background: Ethambutol toxic optic neuropathy (ETON) is one of the side effects of ethambutol (EMB) therapy in tuberculosis (TB) patients which can cause visual impairment. Prevention of this neurotoxicity is an effective strategy to address this condition. Vitamin D3 has the potential to prevent ETON since vitamin D3 is a neuroprotective agent.

Objective: To observe the effect of vitamin D3 in ETON Wistar rat model through its effect on the excitotoxicity pathway.

Methods: This study used a true experimental, post-test-only randomized controlled group design. Fourteen Wistar rats were randomly divided into two groups. All rats received EMB at a dose of 32 mg/ 200 g body weight (BW)/ day for 30 days. The treatment group also received oral vitamin D3 at 72 IU/ 200 g BW/ day. N-methyl D-aspartate (NMDA) receptor expression was examined using immunohistochemical (IHC) staining, and retinal ganglion cell (RGC) density was analyzed using Hematoxylin-Eosin (HE) staining. Differences in NMDA receptor expression were tested using the Mann–Whitney test because the data was ordinal type, while RGC density was analyzed with an Independent T-test because the data was numeric type and the distribution was normal. Then followed by Spearman's correlation test.

Results: NMDA receptor expression in the treatment group given vitamin D3 was lower than in the control group (62.00 ± 16.43 and 70.00 ± 7.07, respectively) with no statistically significant result (p=0.502). RGC density in the treatment group was higher and statistically significant when compared with the control group (11.36 ± 0.51 and 9.60 ± 1.14, p=0.014). The results of the Spearman's correlation test between NMDA receptor expression and RGC density were found to be p=0.380. This means that there was no significant correlation between NMDA receptor expression and RGC density.

Conclusion: Oral vitamin D3 has an effect to increase RGCs density in Wistar rat given ethambutol. However, mechanisms other than NMDA receptor-mediated excitotoxicity that may be involved in protective effect of vitamin D3 still require further study.

Keywords: *Vitamin D3; Cholecalciferol; Ethambuto; Toxic optic neuropathy; NMDA receptor; Retinal ganglion cells*
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INTRODUCTION

Tuberculosis (TB) is endemic in many countries in the world. Indonesia accounts for

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approximately 10% of the global TB incidence, according to the Global Tuberculosis Report 2024.[1,2] Globally, ethambutol (EMB) forms one of the first-line drugs for the treatment of tuberculosis. The usage of EMB has proven to be excellent when combined with other drugs including isoniazid (INH), rifampicin (RMP), pyrazinamide (PZA). The failure rate of this regimen was less than 1%, with a relapse rate of around 4%.[3] Even so, EMB also has side effect, and the most well-documented and precarious ocular side-effect is ethambutol-induced toxic optic neuropathy (ETON).[4] According to recent literature, the prevalence of ETON is 1%-2% per year, and it affects approximately 100,000 people worldwide each year.[5,6]

The toxic effect of ethambutol has been known to lead to apoptosis of retinal ganglion cell (RGC).[7] The exact mechanism of ETON is yet to be elucidated.[8] Several pathways and hypotheses were published to explain the toxicity, including the zinc chelator effects of EMB and its metabolites on metal-containing mitochondrial enzymes, activation of protein kinase C (PKC) phosphorylation, and excitotoxic pathways.[9,10] The toxicity of EMB and its metabolites via the excitotoxic pathway has been demonstrated in vitro and in vivo.[11] Ethambutol stimulates endogenous glutamate, which binds to the N-methyl D-aspartate (NMDA) receptor.[12] This results in decreased cytosolic calcium, increased mitochondrial calcium, and mitochondrial membrane potential, ultimately leading to cell death.[13]

Previously, ETON was regarded as a largely reversible condition; however, this understanding has evolved, and it is now recognized that recovery often vary between individuals or half-treated.[2] At present, no specific therapy exists other than discontinuation of the offending drug. Therefore, prevention of ocular toxicity, early detection, and prompt cessation of EMB remain the most effective approaches for managing this condition.[3,6]

Epidemiological evidence indicates that both vitamin D status and genetic polymorphisms affect the risk of developing several ocular disorders, including myopia, age-related macular degeneration, diabetic retinopathy, and uveitis.[14] At the cellular level, vitamin D has been shown to suppress inflammatory mediators, enhance barrier integrity, and promote apoptosis in cancer cells. Additionally, it mitigates excitotoxicity by modulating NMDA receptor activity.[15,16] Study by Fernandez-Robredo *et al*. stated the role of vitamin D in protecting the retina and retinal pigment epithelium from oxidative stress, inflammation and apoptosis through suppression of pro-inflammatory mediators, such as interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP)-1, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α and also acts as an antioxidant by increasing cell viability and reducing the production of reactive oxygen species (ROS) and caspase 3 activity.[17] Study by Lu *et al*. showed the effect of vitamin D on protecting retinal structure in Wistar rat model of diabetic retinopathy by inhibiting RGC apoptosis and reducing retinal blood vessel permeability through decreasing the Bax/ Bcl-2 ratio.[18]

Vitamin D has therapeutic potential in the ophthalmology field, but no studies have examined its effects in toxic optic neuropathies such as ETON. This study aimed to demonstrate the effect of vitamin D3 in ETON Wistar rat model through its effects on the excitotoxicity pathway as a first step in preventing ETON in TB patients receiving EMB therapy. We hypothesized that vitamin D3 could increase the RGC density through decreasing the expression of NMDA receptors in ETON Wistar rat model.

MATERIALS AND METHODS

This study has received approval from the Faculty of Medicine, Universitas Diponegoro ethics committee No. 41/EC/H/FK-UNDIP/V/2022 and was in accordance with the recommendations of the Association for Research and Vision in Ophthalmology. This study was an experimental study with a post-test-only randomized controlled group design. The sample of this study was the eyes of Wistar rats, which were used as ETON model given EMB 32mg/200 g body weight (BW)/day for 30 days. The inclusion criteria were male Wistar rats with body weights of 200-300 grams and ages of 2-3 months. The rats would be excluded as the subject if they had anatomical abnormalities, and on visual observation, the rats looked sick and/or inactive (weak). The rats would drop out if there was a change in behavior (weak, did not want to eat) or die during the experiment. The sample size was determined using World Health Organization (WHO) formula, with five samples in each group, considering the presence of dropouts, the number of samples was increased by 10% (2 rats) in each group. This study was a pilot experimental study, due to its exploratory nature, the findings of this study were intended to inform future larger-scale studies.

The sample size of the study was 14 rats divided into two groups of 7 rats each, which were randomly assigned using simple randomization with random number generators. All rats underwent acclimatization and received standard feed and water for 14 days. All groups were provided food and water ad libitum for 30 days. For the made ETON model, all rats were given EMB 32mg/200 g BW/day for 30 days. The drug preparation administered was ethambutol dose for humans of 25 mg/kg BW/day, which was considered to caused ETON in humans. The human dose was then converted to rats according to Laurance Bacharach, resulting in a converted dose of 32 mg/200 g BW/day. Ethambutol tablets were supplemented with carboxymethyl cellulose (CMC) as a solvent and administered orally using a sonde. Along with the administration of ethambutol, the treatment group was given 72 IU/200 g BW/day of vitamin D3, while the control group received CMC solvent. The preparation of Hi-D 1000 cholecalciferol produced by IMEDCO was administered at daily maintenance dose in humans (1000 IU), converted according to Laurance Bacharach, with a converted dose of 72 IU/200 g BW/day. Vitamin D3 tablets were supplemented with CMC as a solvent and administered orally using a sonde.

After 30 days of treatment in the experimental animal laboratory of the Faculty of Medicine, Universitas Diponegoro, the rats were enucleated. After enucleation, the eyeball was placed in a 10% formalin solution, and then a paraffin block was

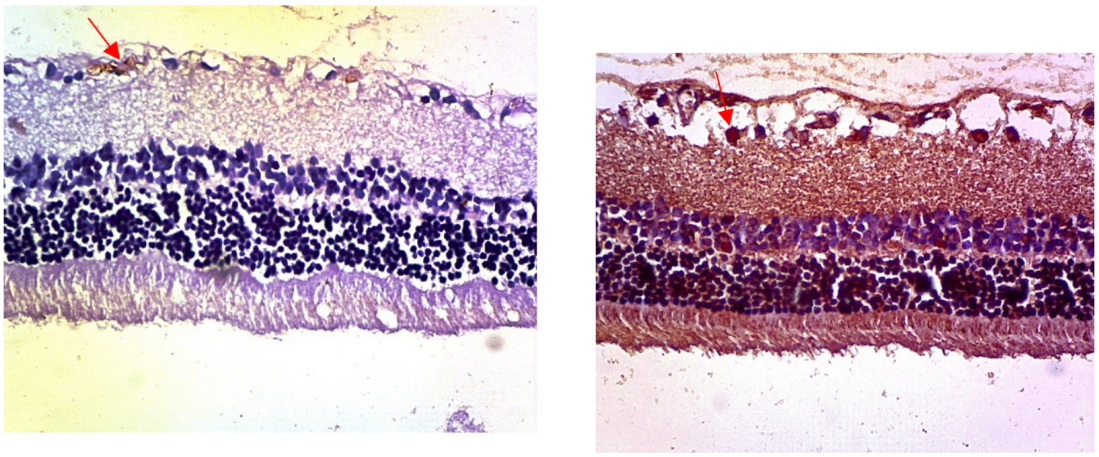
made. After cutting 4 microns of retinal tissue (including ganglion cell layer of retina) Immunohistochemical (IHC) for NMDA receptor expression and Hematoxylin-Eosin (HE) for RGC density were stained. The same region of retina was consistently used for all rats' sample. The anatomical pathology preparations showed a clear retinal ganglion layer and were taken, and the five best ones were selected. The reading of the IHC and HE results were carried out by two anatomical pathology specialists without knowledge of which group was examined.

NMDA receptor expression in RGC was measured using IHC methods by calculating the percentage of cells at each staining intensity. Assessment was done by looking at the number of cells that gave a positive reaction to anti-NMDA monoclonal

Tabel 1. Mean differences of NMDA receptor expression and RGC density between groups

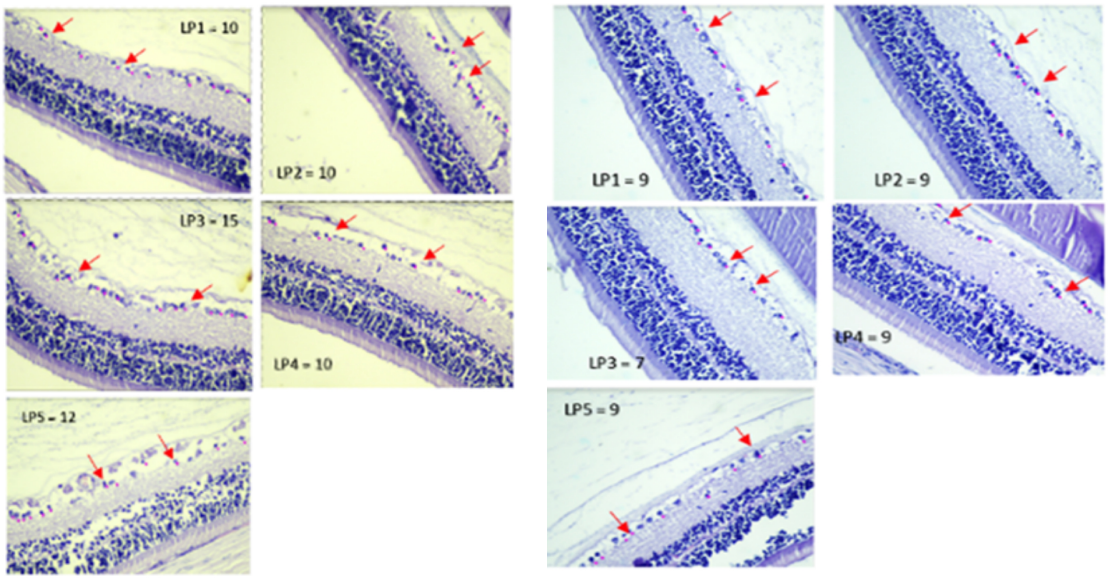
	Treatment group	Control group	p
	Mean ± SD	Mean ± SD	
NMDA receptor expression	62,00 ± 16,43	70,00 ± 7,07	0,502§
RGCs density	11,36 ± 0,51	9,60 ± 1,14	0,014*

SD=standard deviation, §Mann Whitney test, *T-test independent test, p<0,05= significant



A B

Figure 1. NMDA receptor expression is indicated by red arrows. (A) treatment group: <33% of cells were focally stained with weak intensity (light brown). (B) control group: >66% of cells were focally stained with strong intensity (dark brown).



A B

Figure 2. Retinal ganglion cell density in 5 visual fields (LP). Red arrows indicate retinal ganglion cells that did not undergo apoptosis. A) treatment group. B) control group

antibodies under a light microscope with 400x magnification in 5 fields of view. The results were observed and assessed using the Allred Score by summing the distribution and intensity of immunoexpression. The scale of immunoexpression quantity distribution consists of the following: score 0: negative; score 1: 1% focal stained cells; score 2: 2-10% focal stained cells; score 3: 11-33% focal stained cells; score 4: 34-66% focal stained cells; score 5: > 66% focal stained cells. The immunoexpression staining intensity scale consists of the following: score 0: none (not stained); score 1: weak intensity (stained light brown/pale); score 2: moderate intensity (stained brown); score 3: strong intensity (stained dark brown). The final result is a total Allred score, which is the sum of the quantity scale and the intensity scale, with the final result being the following: score 0-1: no effect; score 2-3: small effect (20%); score 4-6: moderate effect (50%); score 7-8: large effect (75%). The final score was assigned per rat, and then the average score from 7 rats was accounted for in the statistical analysis for each group.

The RGC density was assessed quantitatively by histopathological examination using HE staining and viewed under a microscope at 400x magnification. Assessment was performed by differentiating non-apoptotic cells and apoptotic cells based on the morphology. The morphology of apoptotic cells appeared round and oval, with condensed eosinophilic cytoplasm, condensed cell organelles, and dense purple chromatin fragments at the edge of the nuclear membrane. The RGC density referred the number of non-apoptotic retinal ganglion cells and was counted manually in 5 fields of view (LP). The overall average of RGC density per rat was calculated by (LP1 + LP2 + LP3 + LP4 + LP5) / 5. The average RGC density from 7 rats was accounted for in the statistical analysis for each group.

The data were processed using SPSS version 25.0. Intraclass Correlation Coefficients (ICC) test was used to assess the agreement between the two anatomical pathology specialists. The Mann-Whitney test was then performed on the NMDA receptor expression data because the data was ordinal type. The RGC density was tested for normality data using the Shapiro-Wilk test. An Independent T-Test was then performed because the distribution of the data was normal. Then followed by a Spearman's correlation test to correlate ordinal and numeric data. The difference was statistically significant if the *p*-value was <0.05.

RESULTS

The ICC test results for IHC and HE readings between the two anatomical pathology specialists were 0.878, 95% CI (0.508-0.970), and 0.865, 95% CI (0.456-0.966) indicating good agreement between the two observers. During the 30 days of treatment, no rats dropped out due to infection or death. The normality test results for RGC density data showed p=0.227 and p=0.955 for the control and treatment groups, respectively, indicating normal distribution of data for both groups.

NMDA receptor expression was lower in the treatment group (62.00±16.43) when compared to the control group (70.00±7.07), but not statistically significant, p=0.502. (Table 1). RGC density in the treatment group was higher (11.36±0.51) and statistically significant (p=0.14) when compared to the control group (9.60±1.14). The Spearman's correlation test between NMDA receptor expression and RGC density obtained $r(10) = -0.111$, $p = 0.380$. Indicating there was no significant correlation between NMDA receptor expression and RGC density in ETON Wistar rat model given vitamin D3. However, a weak negative correlation was observed: the lower NMDA receptor expression, the higher RGC density.

DISCUSSION

TB therapy was commonly initiated using the directly observed treatment short-course (DOTS) regimen, which was applied to both new and previously treated active TB cases without prior drug susceptibility testing. This regimen consists of an intensive 2-month phase using INH, RMP, PZA, and EMB, followed by a 4-month continuation phase with INH and RMP. However, due to the rise in drug-resistant TB, WHO revised treatment guidelines in 2009 to include EMB in the maintenance phase. This modification had increased the risk of ETON, as EMB toxicity was known to be both dose- and duration-dependent.[6] The reported incidence of ETON varied considerably, ranging from 1%–2.5% at a dosage of 15 mg/kg/day, 5%–6% at 25 mg/kg/day, and up to 18% at 35 mg/kg/day.[4] Typically, toxicity manifested between 3 and 5 months after initiation of therapy, though cases have been reported as early as 1 month and as late as 12 months into treatment.[3] Several studies had been conducted on providing preventive therapy to experimental animals in the ETON model, including lutein, memantine, trimetazidine, idebenone, coQ10, and zinc.[7,8,12,19,20] These studies showed that supplementation of neuroprotective agents administered alongside EMB therapy can prevent RGC damage due to EMB toxicity.

This study was the first study that examined the effect of vitamin D3 on ETON. However, the current pilot study result showed that NMDA receptor expression in ETON Wistar rat model given vitamin D3 is lower than in the control group, but was not statistically significant. Vitamin D reduced excitotoxicity through modulation of the NMDA receptor. Accumulating evidence suggests that vitamin D mitigated neurodegenerative processes by modulating key molecules and signaling pathways, such as maintaining calcium homeostasis, reducing oxidative stress, inhibiting inflammation, and preventing the formation and aggregation of pathogenic proteins. Additionally, vitamin D may exert neuroprotective effects by regulating glutamate concentrations and minimizing excitotoxicity resulting from sustained extracellular glutamate elevation and excessive NMDA receptor activation.[21] Study by Wu *et al*. showed that supplementation with vitamin D3 ameliorated nicotine withdrawal-induced anxiety, which may be related to downregulation of NR2A expression in hippocampus. Most NMDA receptors contain two obligate GluN1 subunits and two GluN2 subunits, of which there are four types (2A-D), with

NR2A and NR2B being predominant in the forebrain.[22] Margareth *et al*. demonstrated that administering vitamin D3 to NMDA-induced glaucoma model mice can maintain the thickness of the RGC layer.[23] Unfortunately, the study did not assess the expression of NMDA receptors in RGC. This finding suggested that vitamin D3 can inhibit activation of the NMDA receptor in RGC of rats given EMD; however, the effect of vitamin D3 on the excitotoxic pathway needs to be studied further. The insignificant results in this study may still be due to the insufficient vitamin D3 dosage, the small sample size, and methodological variability that could happened.

There was accumulating evidence for a possible protective role of vitamin D in the development and disease course of multiple sclerosis (MS).[24] Optic neuritis is common in MS patients and is often an early sign of MS. Vitamin D insufficiency was found in most cases of ON.[25] Agasti *et al*. found that insufficient vitamin D positively correlated with optic nerve affection severity as evidenced by significantly increased baseline thickness of retinal nerve fiber layer (RNFL) and ganglion cell layer (GCL) and more thinning of RNFL and GCL at the end of 3 months of follow-up.[26] Salari *et al.* demonstrated that vitamin D treatment reduces axonal loss in ON patients primarily by its properties to modulate inflammation and perhaps also by its neuroprotective properties.[25] This study only examined the effect of vitamin D3 on the excitotoxicity pathway via the NMDA receptor and did not examine its effect on neuroinflammation and oxidative stress. In the field of neuro-ophthalmology, research on vitamin D was still limited, but there were several studies on other eye diseases such as diabetic retinopathy. Study by Fernandez-Robredo *et al*. stated the role of vitamin D in protecting the retina and retinal pigment epithelium from oxidative stress, inflammation, and apoptosis through suppression of pro-inflammatory mediators, such as interleukin (IL)-6, IL-8, monocyte chemoattractant protein (MCP)-1, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, and it also acts as an antioxidant by increasing cell viability and reducing the production of reactive oxygen species (ROS) and caspase 3 activity.[17] Study by Lu *et al*. showed the effect of vitamin D on protecting retinal structure in Wistar rat model of diabetic retinopathy by inhibiting RGC apoptosis and reducing retinal blood vessel permeability through decreasing the Bax/ Bcl-2 ratio.[18]

RGC density in the treatment group in this study was higher and statistically significant when compared to the control group. This result is in line with other studies on the effects of vitamin D on RGC. A study by Lazzara *et al*. showed that 1.25(OH)2D3 protected RGC preserving retinal function, reducing inflammatory cytokines, and increasing expression of neuroprotective factors. Therefore, 1.25(OH)2D3 could attenuate the retinal damage in glaucomatous patients.[27] This finding indicates that vitamin D3 may exert a neuroprotective effect by preserving RGC. However, the mechanism of inhibited RGC apoptosis by vitamin D still requires further investigation. Other studies had shown that vitamin D can influence the intrinsic pathway of RGC apoptosis, neuroinflammatory pathways, and oxidative stress.[18,27]

NMDA receptor expression and RGC density were not significantly correlated in this study. This may be due to the vitamin D's primary effect on preventing RGC apoptosis rather than on the excitotoxic pathway. Yoon *et al.* reported that treatment with a glutamate antagonist, antioxidant, or cycloheximide did not reduce EMB-induced neurotoxicity, in contrast to earlier findings. They concluded that EMB-induced cytotoxicity in cultured rat retinal cells is unlikely to be mediated by excitotoxicity, oxidative stress, or apoptotic pathways. Instead, they found that chelating intracellular zinc completely prevented EMB-related cytotoxicity. In a subsequent study, they further demonstrated that intracellular zinc and lysosomal membrane permeabilization are key contributors to EMB-induced retinal cell death.[28,29] Based on those studies, the RGC apoptotic pathway that might be most influenced in ETON is the intrinsic pathway.

This study has several limitations. Neuroinflammatory and oxidative stress markers were not assessed in this study, they may represent potential therapeutic targets for vitamin D intervention in ETON. The assessment of NMDA receptors through histopathological analysis may be influenced by subjective interpretation. Utilizing a more objective method, such as RT-qPCR, could yield more reliable and significant findings. This study was a pilot experimental study with a small sample size that may have influenced the result. Inclusion of an additional healthy control group (no ethambutol, with or without vitamin D3) in future studies would allow clearer differentiation between preventive, protective, and restorative effects of vitamin D3.

CONCLUSION

Vitamin D3 led to an increase in RGC density, supporting its protective effect against ethambutol-induced neuronal damage and apoptosis. The mechanism of inhibited RGC apoptosis by vitamin D still requires further investigation, considering there was no correlation between NMDA receptor expression and RGC density. However, this study was a pilot experimental study, the findings of this study were intended to inform future larger-scale studies. Future studies should explore other apoptotic pathways in RGC that may be influenced by vitamin D3 with an appropriate sample size.

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