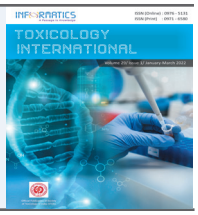
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Influence of Vitamin D on Ethanol Induced Retinal Changes in Experimental Rats

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Abstract

Retina is a neurosensory tissue of ocular system having highly unique lipids which are susceptible to free radical injuries. Chronic ethanol consumption can initiate a wide range of disorders in the CNS and also alteration in the visual system via oxidative pathway. Our study was designed to evaluate the role of vitamin D, a well-known antioxidant and neuroprotective steroid hormone, in the maintenance of ocular health in ethanol induced toxicity. In our present study 4 months old Wistar rats were exposed to ethanol, daily for 6 weeks, while another set of rats received ethanol daily, along with vitamin D for the same duration. A control group consisting of normal rats without any treatment was also included in this experiment. After 6 weeks, Total Anti-Oxidant (TAO) level of eyeball and retinal thickness was measured and statistically analyzed. We observed significantly decreased total antioxidant level in whole eye homogenate in ethanol administered rats which was associated with morphometric changes in the retina. Whereas, vitamin D treated rats showed near to normal retinal morphology as well as TAO level suggesting that vitamin D has the potential to reverse retinal damages caused by ethanol toxicity induced by long term consumption of it.

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1. Introduction

Excessive consumption of alcohol can be a root for serious public health problem that exerts different impact on individuals, depending upon their age and sex. Ethanol intoxication has shown mild to severe injury in the liver and kidney in a dose dependent manner[1]. Chronic alcohol intake induces cognitive impairments, brain damage[2], and neurodegeneration[3]. However, the effect of alcohol on retina has not been reported much according to our literature survey. Retina, the inner nervous layer of eyeball has highly unique lipids which play an important role in the retinal disease and function[4]. It is a neurosensory tissue which is developmentally a part of the brain and derived from diencephalon[5]. Chronic consumption of alcohol has shown decreased GSH and increased MDA level in rat optic nerve[6]. Excessive alcohol intake induced elevated MDA level and considerably declined GSH in rat retina[7]. A study on zebra fish embryo demonstrated inhibition of photoreceptor development and hypoplasia of optic nerve induced by high level of ethanol[8]. Chung and his co-workers observed ethanol induced cell death and suppressed neuronal differentiation in the retina and brain of zebra fish embryos[9]. Since vitamin D enhanced neurogenesis and improved cognitive impairment in a mouse model of Alzheimer's disease[10], we hypothesized that vitamin D might influence the microstructure of rat retina as eyeball is a part of brain as well as TAO level of the rat eyeball that underwent long term ethanol consumption. Vitamin D at the cellular level exerts antioxidant property and was able to induce apoptosis of cancerous cells, enhance barrier function and reduce inflammatory mediators[11]. Lu in his report stated that vitamin D decreased diabetic induced ROS in retina along with retinal recovery and decreased apoptosis of its vascular capillary cell which was detected by fluorescence staining[12].

2. Material Methods

2.1 Animal Groups

Female albino rats of Wistar strain were used in the present study. The rats were maintained in 12 hours light and 12 hours dark cycle and also in temperature and humidity controlled environment. Animals were provided with free access to water and rat pellets, which were provided in a container inside the cage *ad libitum*. Rats were housed in polypropylene cages with paddy husk. Rats were obtained by the central animal house of our own institution. This study was approved and reviewed by the Institutional animal ethics committee (Reference: KMC/MNG/IAEC/06-2019).

The experiment consists of following animal groups (n=6).

- Group 1: Control rats received tap water,
- Group 2: Rats received Ethanol 5g/kg bw/day for 6 weeks (orally),
- Group 3: Rats received Vitamin D 20 µg/kg bw/day for 6 weeks (orally), and
- Group 4: Rats receive Ethanol 5g/kg bw/day + Vitamin D 20 µg/kg bw/day for 6 weeks (orally).

2.2 Biochemical Study

2.2.1 Estimation of Total Anti-Oxidants (TAO)

Principle of the assay: The TAO level was estimated according to the method described by Koracevic and his co-workers[13]. A standardized solution of Fe-EDTA complex reacts with hydrogen peroxide by a Fenton - type reaction, leading to the formation of hydroxyl radicals. These reactive oxygen species degrade benzoate, resulting in the release of Thio-Barbituric Acid-Reactive Substances (TBARS). Antioxidants from the added sample (eyeball homogenate) cause the suppression in the production of TBARS. The reaction can be measured spectrophometrically and the rate of inhibition of colour development is proportional to the concentration of anti-oxidative activity.

Assay procedure: Each sample had its own control in which Fe-EDTA mixture, H_2O_2 and sodium benzoate were added after 20% acetic acid. For each series of analysis, a negative control was prepared containing the same reagents as A, except that eyeball homogenate was replaced with 0.1M sodium phosphate buffer, pH 7.4. Uric acid (1 Mm/L) was used as a standard. The reaction mixture was incubated at 37°C for 60 min. Then 20% acetic acid and 0.8% TBA were added and incubate for 10 min at 100°C, then cooled in ice bath. The absorbance

was measured at 532 nm against deionised water using Systronic-117 UV-Visible spectrophotometer. The total antioxidants level in whole eyeball was expressed as mmoles/L.

2.3 Histomorphometric Study

2.3.1 Quantitative Analysis of Rétinal Changes

Soon after the treatment rats were deeply anesthetized with ether and trans-cardiac perfusion with 0.9% saline and 10% formalin was carried out. Following perfusion, the eyeball was removed and kept in 10% formalin for 48h. Paraffin blocks were made and sections of 4-5µm thickness were cut using a rotary microtome at the level of optic disc either transverse or sagittal. The sections were stained with haematoxylin and eosin stain. Total thickness of retina in each eyeball was measured as the distance from the retinal pigment epithelium up to ganglion cell layer, in µm using NIS Elements Br Version 4.30 software. The outer nuclear level thickness measurement is considered to be the most convenient method to evaluate photo receptor cell damage[14].

2.4 Statistical Analysis

The data is expressed as mean ± SD. The significance of differences among the groups was assessed using one-way analysis of Variance (ANOVA) test. Probability (P) values <0.05 was considered as significant.

3. Results

3.1 Total Anti-Oxidants (TAO)

Ethanol administered rats showed significant reduction ($p<0.001$) in the level of TAO in the eyeball tissue than that of the control rats. Where as, vitamin D treatment in ethanol administered rats demonstrated significant enhancement ($p<0.001$) in the level of TAO in the eyeball when compared to that of rats receiving ethanol alone. vitamin D alone treated group has also shown a highly significant ($p<0.001$) increase in TAO level when compared to control and ethanol induced rats (Figure 1).

3.1.1 Morphometric Evaluation of Various Layers of Retina

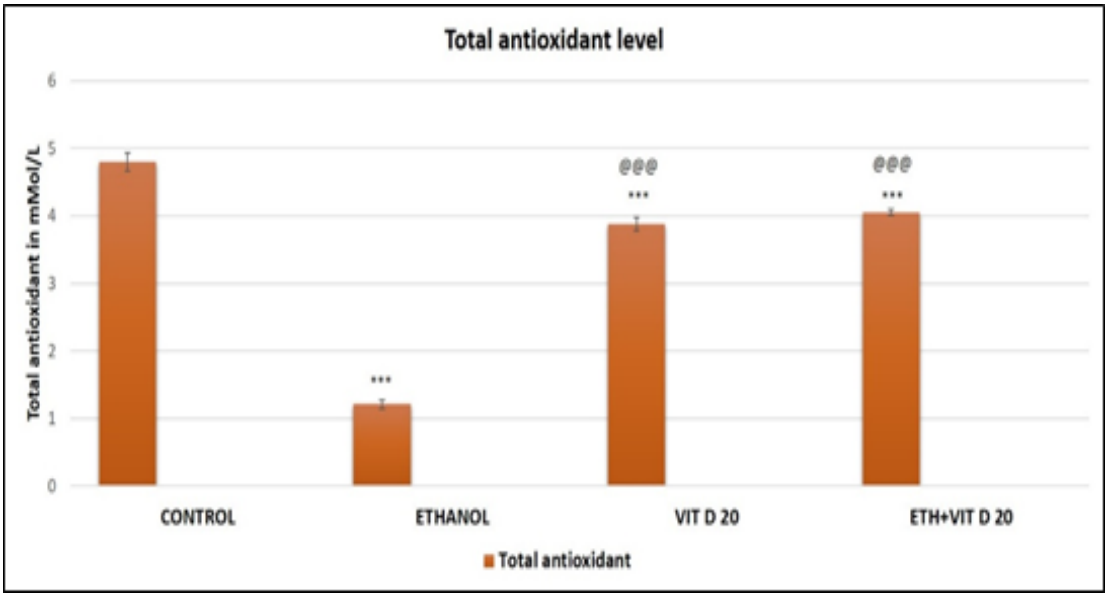


Figure 1. Comparison of total antioxidant level in rat eyeball of different group. Values are expressed as mean ± SD (n=6); Control vs Ethanol, Vit D20, Eth+Vit D20 is *** = $p<0.001$; Ethanol vs Vit D20, Eth+Vit D20 is @@@ = $p<0.001$.

3.1.2 Total Thickness of Retina

It was found that the entire thickness of retina increased significantly ($p<0.001$) in ethanol administered rats in comparison to that of control rats. But in vitamin D treated ethanol administered rats retinal thickness decreased significantly ($p<0.001$) when compared to ethanol treated rats. However, there was no significant difference between control and vitamin D treated rats (Figure 2).

3.1.3 Thickness of Different Layers of Retina

The following figures illlustrate improvement in the thickness of different layers of retina in vitamin D treated rats.

3.1.4 Rods and Cone Layer (RCL)

A significant ($p<0.01$) increase in retinal rod and cone layer was observed in the rats administered with ethanol

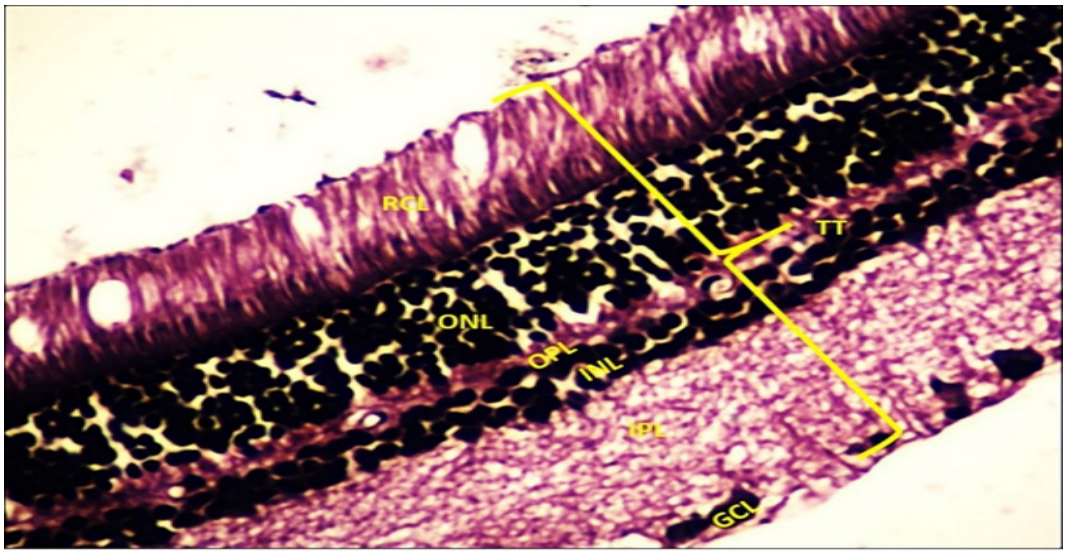


Figure 2. Photomicrograph of rat retina showing different layers under 20X (H & E stain), RCL – Rods and cone layer, ONL – Outer nuclear layer, OPL – Outer plexiform layer, INL – Inner nuclear layer, IPL – Inner plexiform layer, GCL – Ganglion cell layer, TT – Total thickness.

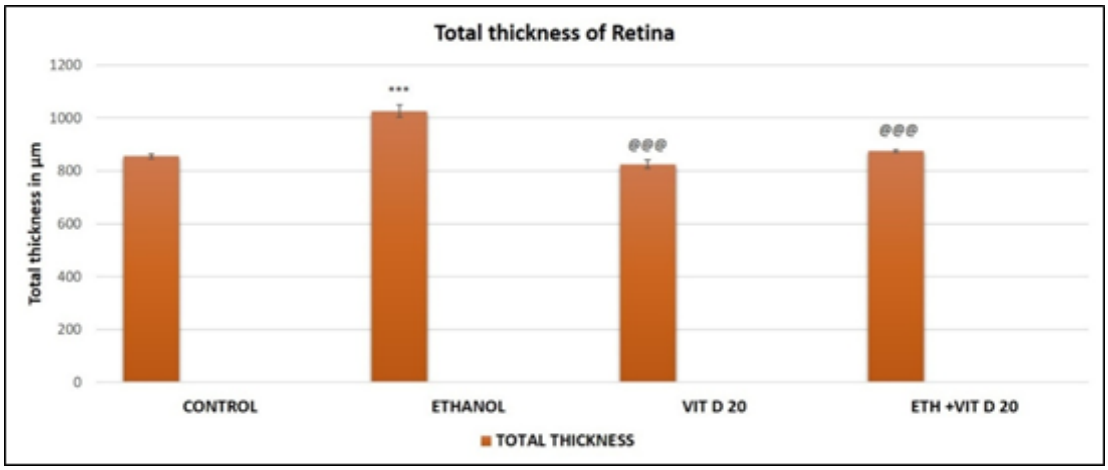


Figure 3. Effects of ethanol and Vitamin D 20µgm administration on the Total thickness (µm) of rat retina. Values are expressed as mean ± SD (n=6). Comparison of Control Vs Ethanol *** = $p<0.001$. Comparison of Ethanol Vs Vit D20, Eth+Vit D20 @@@ = $p<0.001$.

when compared to that of control rats. In rats exposed to ethanol along with vitamin D treatment the thickness of RCL was decreased significantly ($p<0.001$) in comparison to rats who only recieved ethanol. Similarly, the thickness of RCL of ethanol administred vitamin D treated rats significantly decreased ($p<0.05$) in comparison to that of control (Figure 3).

3.1.5 Outer Nuclear Layer (ONL)

There is a significant increase ($p<0.05$) in the thickness of outer nuclear layer in ethanol administered rats when

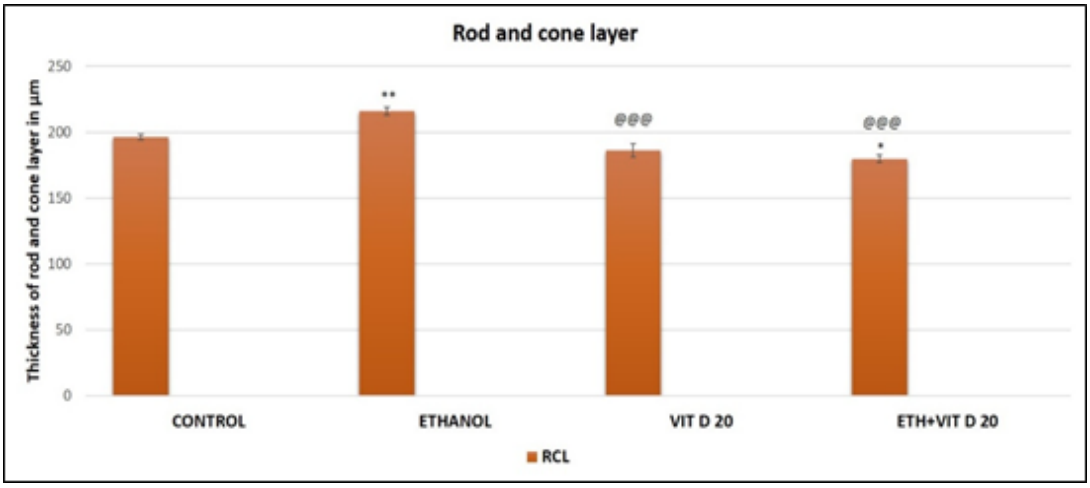


Figure 4. Effects of ethanol and Vitamin D 20µgm administration on the RCL (µm) of rat retina. Values are expressed as mean ± SD (n=6). Comparison of Control Vs Ethanol ** = $p<0.01$, Control Vs Eth+Vit D20 *= $p<0.05$. Comparison of Ethanol Vs Vit D20, Eth+Vit D20 @@@ = $p<0.001$.

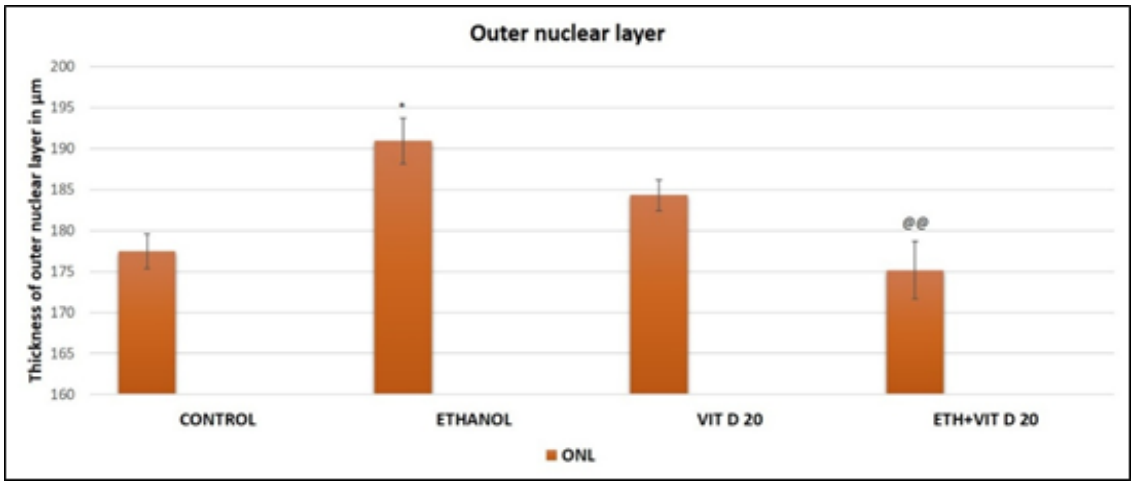


Figure 5. Effects of ethanol and Vitamin D 20µgm administration on the ONL (µm) of rat retina. Values are expressed as mean ± SD (n=6). Comparison of Control Vs Ethanol *= $p<0.05$. Comparison of Ethanol Vs Eth+Vit D20 @@ = $p<0.01$.

compared to that of control rats. However, rats exposed to ethanol along with vitamin D treatment showed significant decrease (p<0.01) in the ONL in comparison to that of ethanol administered rats. (Figure 4).

3.1.6 Outer Plexiform Layer (OPL)

The thickness of OPL is increased significantly (p<0.01) in ethanol treated rats in comparison to that of control rats. Whereas, in rats exposed to ethanol along with vitamin D treatment the thickness of OPL was decreased significantly (p<0.01) in comparison to rats who only recieved ethanol. The thickness of OPL in vitamin D alone treated rats had significant difference (p<0.001) when compared to ethanol induced rats (Figure 5).

3.1.7 Inner Nuclear Layer (INL)

In ethanol administered rats INL exhibited a increase in its thickness significantly (p<0.001) in comparison

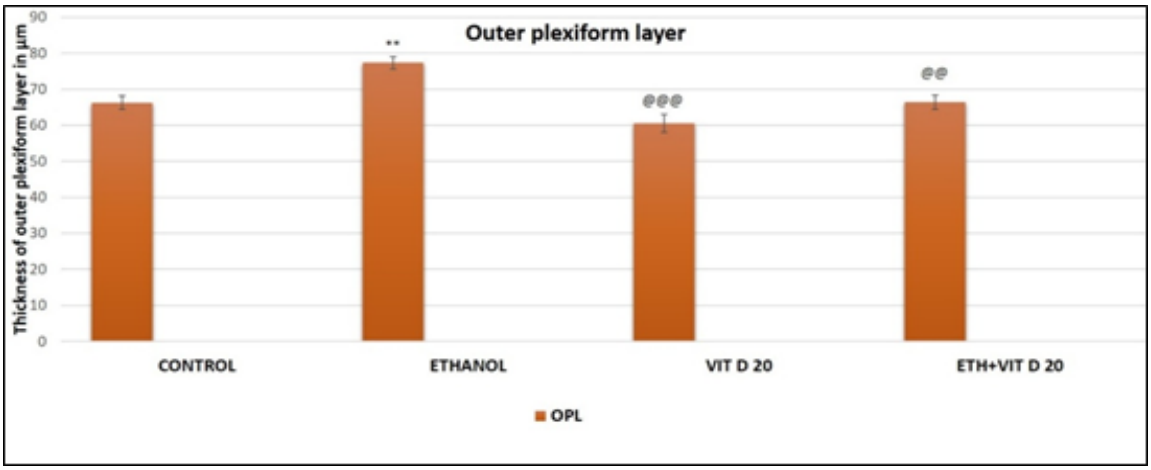


Figure 6. Effects of ethanol and Vitamin D 20µgm administration on the OPL (µm) of rat retina. Values are expressed as mean ± SD (n=6). Comparison of Control Vs Ethanol**= p<0.01. Comparison of Ethanol Vs Vit D20 @@@ = p<0.001, Ethanol Vs Eth+Vit D20 @@ = p<0.01.

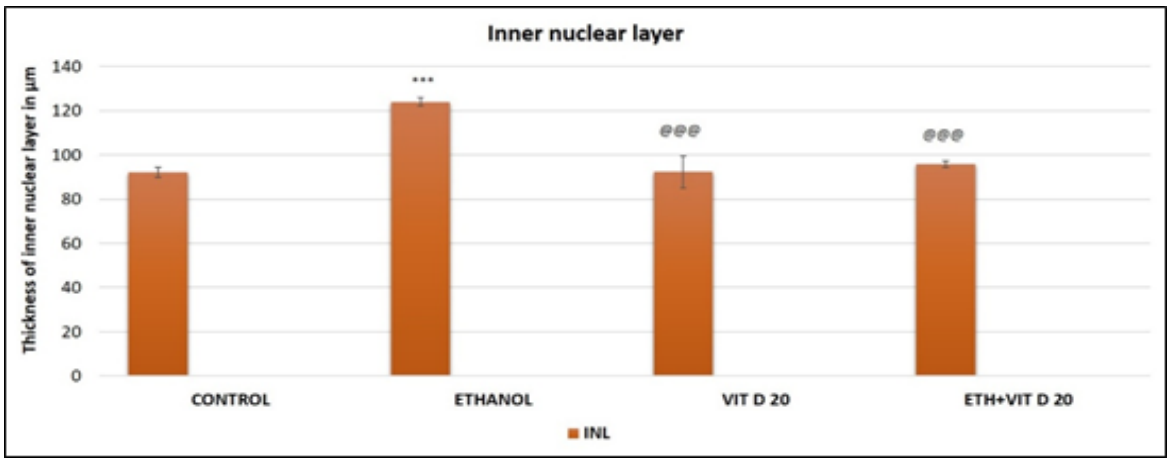


Figure 7. Effects of ethanol and Vitamin D 20µgm administration on the INL (µm) of rat retina. Values are expressed as mean ± SD (n=6). Comparison of Control Vs Ethanol ***= p<0.001. Comparison of Ethanol Vs Vit D20, Eth+Vit D20 @@@ = p<0.001.

to that of control rats. In rats exposed to ethanol along with vitamin D treatment the thickness of INL decreased significantly ($p<0.001$) in comparison to rats who only recieved ethanol. The thickness of INL in vitamin D treatment alone has a significant difference ($p<0.001$) when compared to rats who recieved only ethanol (Figure 6).

3.1.8 Inner Plexiform Layer (IPL)

The thickness of IPL was increased significantly ($p<0.001$) in ethanol administered rats in comparison to that of control rats. Whereas, the thickness of IPL decreased significantly ($p<0.001$) in rats exposed to ethanol along with vitamin D treatment in comparison to rats who only recieved ethanol. The thickness of IPL showed significant reduction ($p<0.001$) between vitamin D treated rats when compared to rats induced with ethanol alone (Figure 7).

3.1.9 Ganglion Cell Layer (GCL)

The ganglion cell layer showed significantly increased thickness ($p<0.001$) in ethanol administered rats in

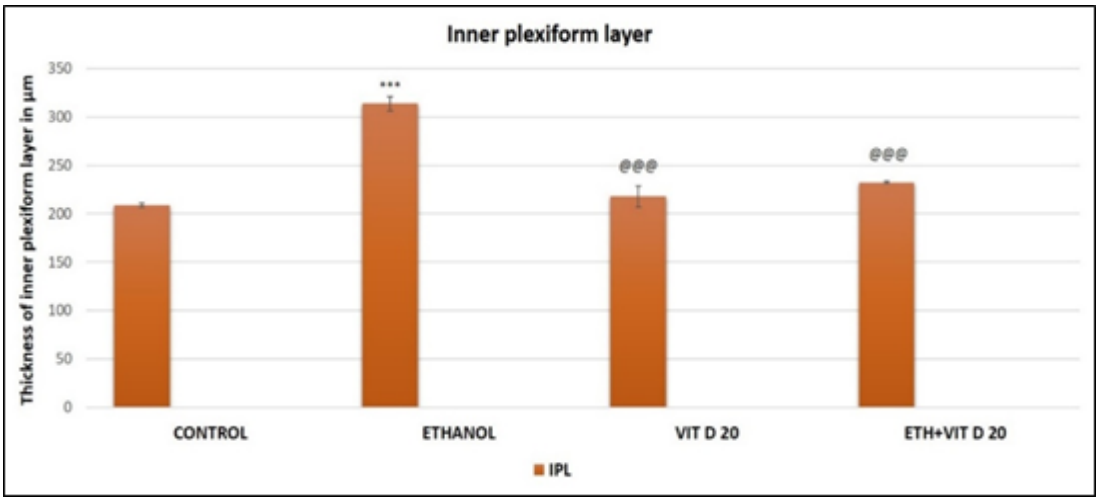


Figure 8. Effects of ethanol and Vitamin D 20µgm administration on the IPL (µm) of rat retina. Values are expressed as mean ± SD (n=6). Comparison of Control Vs Ethanol ***= $p<0.001$. Comparison of Ethanol Vs Vit D20, Eth+Vit D20 @@@ = $p<0.001$.

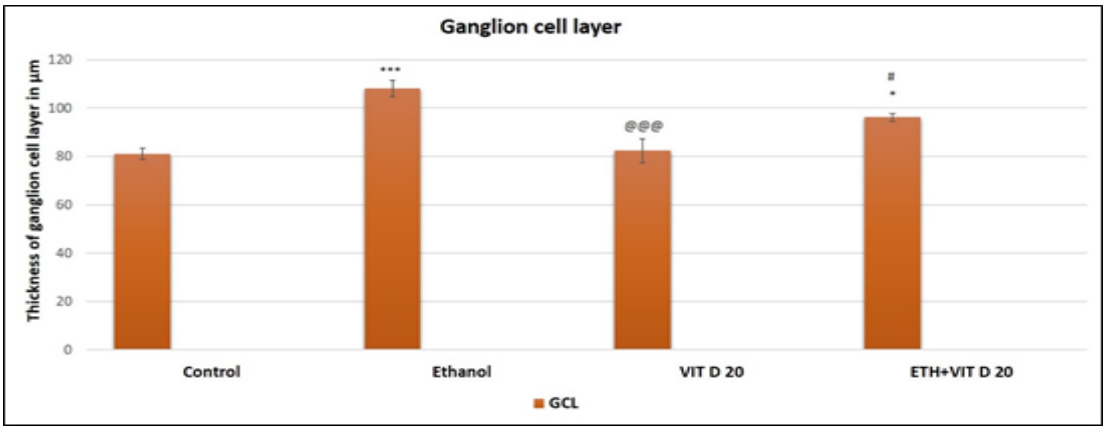


Figure 9. Effects of ethanol and Vitamin D 20µgm administration on the GCL (µm) of rat retina. Values are expressed as mean ± SD (n=6). Comparison of Control Vs Ethanol ***=$p<0.001$, Control Vs Eth+Vit D20 *= $p<0.05$. Comparison of Ethanol Vs Vit D20 @@@ = $p<0.001$. Comparison of Vit D Vs Eth+Vit D # = $p<0.05$.

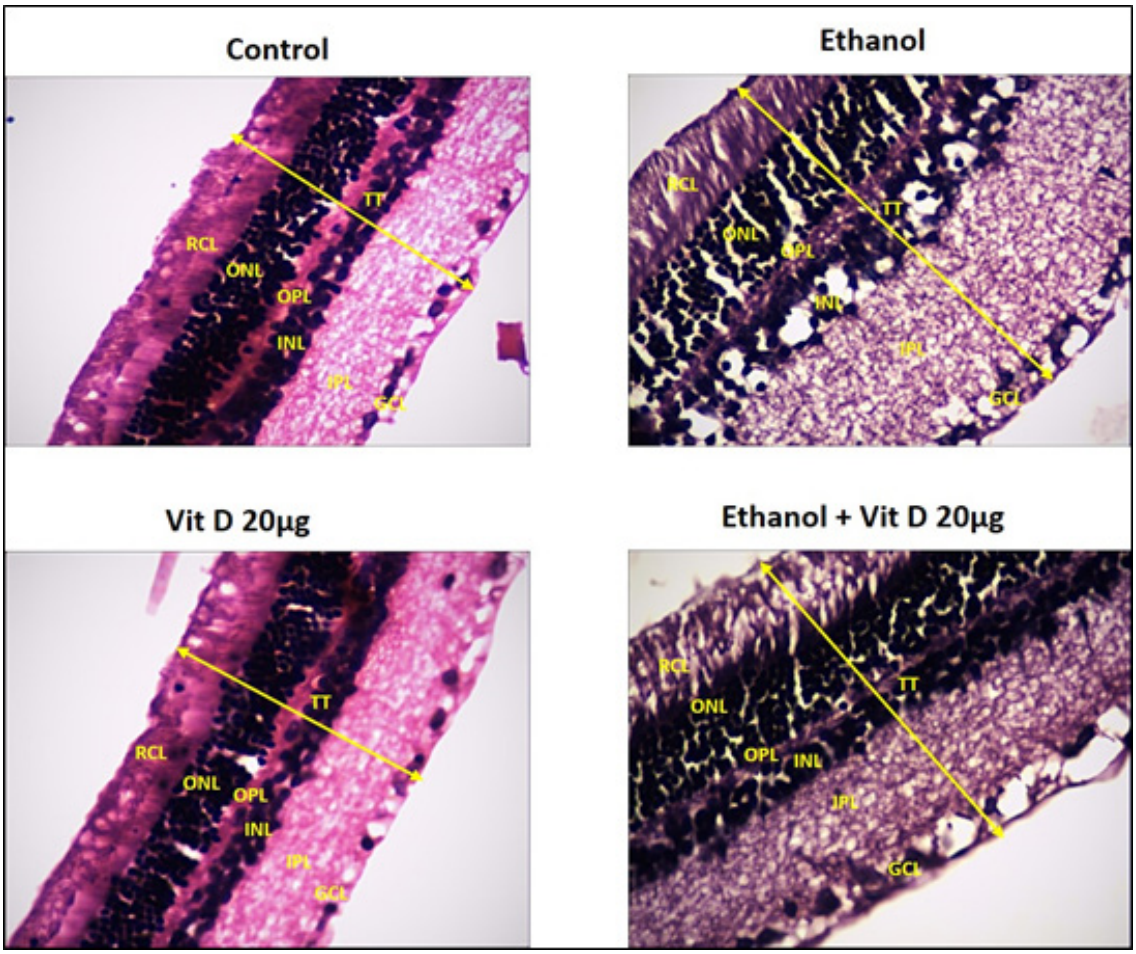


Figure10. Photomicrograph of rat retina in different groups under 20X (H & E stain) - Control Vs others.

comparison to that of control rats. Rats exposed to ethanol along with vitamin D treatment showed significant reduction ($p<0.05$) in the size of the GCL when compared to control rats. The thickness of IPL showed significant reduction ($p<0.001$) between vitamin D treated rats when compared to rats induced with ethanol alone (Figure 8).

4. Discussion

Effects of ethanol toxicity on target organs can be determined using biochemical tests which can caution against its potentially harmful changes[15], including change in visual function and morphometry[16]. The major advantage of evaluating TAO, as in the present study, is to assess the potential of all antioxidants in a given tissue instead of a single component[17]. Several authors have shown neurodegenerative changes and neuro-inflammation in CNS induced by chronic ethanol intake due to decrease in oxidative defense mechanisms[18,19]. Alteration in antioxidant mechanism has been reported in brain[20], liver and kidney[21], peripheral nerve[22] and optic nerve[6] after chronic administration of ethanol. A study by Sancho-Tello revealed that chronic ethanol intake in rats caused impairment of Electro-Retino-Gram (ERG) and Bcl2 (anti apoptic protein) over expression in retina suggesting ethanol toxicity on retina[16]. Similarly, in the present study the TAO activity was severely affected after ethanol administration, demonstrating that retina is quite susceptible to oxidative stress induced by alcohol

intoxication and this effect was reversed to a larger extent by vitamin D treatment (Figure 9).

In addition, our study includes micro-anatomical morphometry of each layer of the retina in terms of its thickness. Protective effect of vitamin D receptor in retinal ganglion cell damage against high level glucose induction has been revealed by Luand co-workers[12]. Changes in Retinal Ganglion Layer (RGC) density and cell size along with increased intraocular pressure using different drug treatments in eye is documented by several authors[23-25]. In our study ethanol induced rats exhibited a significant increase in all the layers which was subsequently decreased by vitamin D treatment (Figure 1-8). Vitamin D has proven to be effective in inhibiting tumor cell growth in retinoblastoma induced rats[26]. Additionally, Graffe opine that age related macular degeneration is associated with vitamin D insufficiency[27].

5. Conclusion

Vitamin D treatment on ethanol toxicity induced rats showed improvement in the thickness of the retina thus bringing it back to normalcy as well as enhanced TOA level in the eyeball in the present study. Therefore, it can be advocated that vitamin D exerts considerable beneficial property against ethanol induced retinal changes, thus adding to the existing therapeutic use of vitamin D in future.

6. Conflict of Interest

The authors declare that they have no conflict of interest.

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