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An Investigation of the Retinal Layers in an Experimental Model of Multiple Sclerosis; a Histopathological and Immunohistochemical Study

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Authors' contributions

This work was carried out in collaboration between all authors. Author LR designed the study, wrote the protocol and wrote the first draft of the manuscript. Authors NA, AM and MF managed the literature searches and analyses of the study. Authors LR and JSR managed the experimental process. Authors HT and BN identified the clinical sign of MS model in mice. All authors read and approved the final manuscript.

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ABSTRACT

Background: Animal model of multiple sclerosis is a demyelinating and inflammatory disorder of central nervous system and eye. Histological evaluation in eyes in experimental autoimmune encephalomyelitis (EAE) models demonstrated evidence of retinal atrophy and inflammation in late stage of disease. Deciphering the relationships between the retinal atrophy and proliferation on retinal layers may help us in understanding the factors that drive atrophy and proliferation in multiple sclerosis.

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Aims: The aim of the present study was to determine alterations in thickness of retina and its sub-layers in MS induced mice model in comparison with control group.
Study Design: Experimental study.
Methodology: EAE was induced in female C57BL/6 mice using Hooke kits. Animals were scored for clinical signs of the disease according to 10-point EAE scoring system. At 35th day after immunization, mice (n=15/group) were deeply anesthetized and eyes were removed.
Morphometric study of proliferation in retinal sub-layers were assessed by Hematoxilen and Eosin staining and expression of Ki67. The proliferating marker was performed by Ki67analyzing kit. All measurement obtained by Motic image analyzer software 2 and analyzed spss 18, respectively.
Results: Here we reported that retinal thickness in MS group including total retinal layer, especially photoreceptor layer, ganglion cell layer and neural layer reduced in comparison to control group ($p< 0.001$). In Ms Group proliferation rate is also decreased in comparison to control group (0.05).
Conclusion: Our results show that ki67 expression, as an indication of proliferation, decreased in retinal layers in MS group. Furthermore, our data revealed that retinal thickness also decreased in MS group.

Keywords: Retina; multiple sclerosis; mouse; EAE.

1. INTRODUCTION

The main cause of inflammatory debilitating disease of the central nervous system (CNS) as multiple sclerosis is axonal degeneration (MS) [1]. Sometimes, MS disease is called complex genetic disease, because of interaction between heritability and multigenetic and environmental factors. One of the most important genes that strongly related with MS is HLA-DRB1 [2].

The visual pathway and histopathological structure of retina is a major frequent site of damage during the course of multiple sclerosis. [3,4].

Although damage to the optic nerve that affects white matter is reasonably well characterized, the pathology of the retina is poorly understood.

Retinal periphlebitis, optic disc atrophy and localized damage in the retinal nerve layer can be detected on routine ophthalmoscopy in multiple sclerosis [5]. Optic disc atrophy and retinal periphlebitis and other retinal associated neuropathy with rapidly appearing apoptosis shows thinning of retinal nerve fiber layers [6-10].

Experimental autoimmune encephalomyelitis (EAE) a demyelinating animal model mimics many of the clinical and pathologic features.

Ki67 is a member of the cellular proliferation marker family, that is usually present during all active phases of the cell cycle [11-14].

Alterations of retinal thickness is a complex process and as a novel criterion could be used for determination of retinal damage in multiple sclerosis as EAE.

This study is performed through the use of histological and histochemical characteristic of retinal layer in EAE.

The aim of the present study was to determine alterations in thickness of retina and its sub-layers in MS induced mice model in comparison with control group.

2. MATERIALS AND METHODS

2.1 Animals

For this experimental study, thirty C57BL/6 healthy female adult mice (8-10 weeks old) were purchased from Pastore institute (pasture institute, IRAN) and housed under standard humidity, 22-23' c temperature , and 12/12 (7am-7 pm) dark/light cycle on pathogen free animal laboratory condition at Tabriz University of medical sciences animal care services(ACS) facility. Animals were housed in cages, four in one cage, and maintained one week for acclimate.

All the experiments were approved by the ethical committee and their guidelines set of Tabriz University of medical sciences and that involved animal be performed in compliance with the ARVO statement for use of animal in ophthalmic and vision research.

For EAE induction mice were immunized with Hooke kits (Hooke laboratories, EK-0115, Lawrence, MA, USA) according to the manufacturer's instructions and described in [15]. Mice were randomly divided into control (n=15) and MS (n=15) groups.

All animals were housed separately in a cage with access to food and water ad libitum.

Clinical evaluation were evaluated and scored [16].

Animals were evaluated and scored for clinical signs of the disease from day 7 to day 35 post immunization by a single researcher blind to the treatments using the 10 point EAE scoring system as follows:

0, no clinical sign; 0.5, partial tail paralysis; 1.0, complete tail paralysis; 1.5, complete tail paralysis and discrete hind limb weakness; 2.0, complete tail paralysis and strong hind limb weakness; 2.5, unilateral hind limb paralysis; 3, complete hind limb paralysis; 3.5, hind limb paralysis and forelimb weakness; 4.0, complete paralysis (tetraplegia); 5.0, moribund or dead [16,17]. The length of treatment (35 day) post immunization was selected according to previous studies [16,17].

2.2 Immunohistochemistry Staining

Eye tissues were obtained from EAE induced and control groups, 35 days after immunization. Tissue preparation was performed routinely and then 5 µm thickness cross sections were prepared and picked up on poly-l-lysine coated slides. For evaluation of thickness of retinal layer, as an indication of their physiologic function, proliferation of cells at retinal sublayers was determined. Proliferation was assessed using Ki67 (Dako Denmark A/S) Antibody Staining Protocol for Immunohistochemistry performed according to previous report [18]. Goldish brown staining pattern of nuclei is considered as ki67 positive cells.

2.3 Histopathological Analysis

After 35 days post immunization, mice were anesthetized with ketamine/xylazine (5/1) and perfused with cold PBS then sacrificed and eyes were removed and immersed in 4% paraformaldehyde for 72 hours. Fixed tissues were paraffin-embedded and serial sections with 5 µm thickness were prepared from retina. All sections selected for light microscopy evaluation contained optic nerve tissue landmarks. The histological sections that demonstrated the appearance of the optic nerve head were considered histological inferior point.

The sections were deparaffinized through xylol and stained with H&E and toluidine blue with routine tissue passage protocol. A number was selected randomly, and 5 sections from every 10 section were selected from the retina in each animal (using systematic random sampling technique). The sections were from the same position in both control and experimental groups. The sections were evaluated histologically and retinal layer thickness was measured by morphometric techniques with manual caliber motic image analyzer version 2 imaging software. Total retinal thickness was derived from sum of all measured histological layers up to but not including retinal pigment epithelium.

All data were analyzed with spss software version 18.

3. RESULTS

3.1 Morphometrical Study of Retinal Layers

The overall total retinal sub layers are shown in Fig. 1 from control group. Mean thickness of each retinal sub-layers and total retinal thickness (TRT) is demonstrated in Table 1. Total retinal thickness in all animals in MS group decreased significantly ($p<0.0001$) as compared to control group 227.9±2.982 µm vs. 177.2±2.478 µm. Retinal nerve fiber layer (RNFL) in treated group (5.691±0.4698 µm) was decreased in comparison to control group (9.976±0.8748 µm) and RNFL thickness was significantly different between two groups ($p<0.0002$).

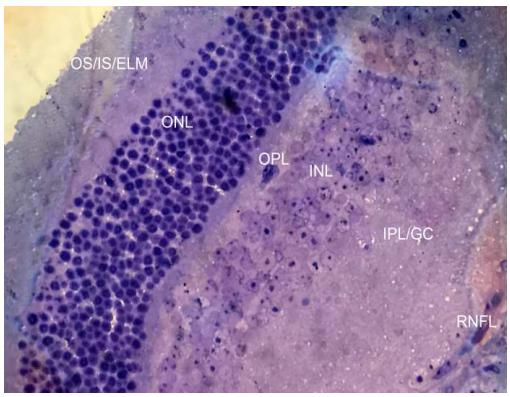

Fig. 1. Photomicrograph of a retinal sub layers, from control group: OS/IS/ELM, outer segment/inner segment/external limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL/GC, inner plexiform Layer/ganglion cell; RNFL, retinal nerve fiber layer. Toluidine blue staining

Table 1. Mean thickness (in µm) of retinal layers in MS and control groups for each animal

Sub layers	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6	Animal 7	Animal 8	Animal 9	Animal 10
Control group										
TRT	223.21	208.25	230.01	225.07	235.11	219.37	239.74	227.50	234.00	236.71
RNFL	13.17	14.81	10.25	11.01	9.00	10.03	7.57	7.03	10.27	6.09
IPL/GC	65.72	61.28	70.95	71.76	68.32	71.67	69.07	59.00	60.00	70.27
INL	32.16	36.33	35.21	34.00	37.00	30.33	31.11	30.02	30.07	30.44
OPL	14.78	15.29	16.17	15.43	14.27	15.88	16.62	14.38	14.31	15.78
ONL	55.70	43.98	61.78	60.27	60.77	59.05	64.02	62.18	65.17	67.32
OS/IS/ELM	43.72	38.53	44.17	39.72	41.28	44.70	43.12	41.02	38.04	44.77
MS group										
TRT	185.11	184.00	189.08	180.77	174.70	165.55	174.08	168.41	181.18	169.61
RNFL	7.05	7.00	7.11	8.22	5.11	4.52	4.22	4.70	4.21	4.77
IPL/GC	45.20	48.29	50.00	53.15	62.19	44.11	45.07	48.22	49.11	50.00
INL	29.00	25.12	24.01	22.00	24.77	20.11	30.17	21.75	21.64	20.08
OPL	14.17	16.52	17.11	15.70	14.91	14.89	14.18	17.11	15.66	14.77
ONL	52.70	41.28	45.32	47.34	39.79	39.99	44.27	44.87	40.45	40.44
OS/IS/ELM	41.18	35.11	43.27	41.16	41.27	35.11	34.24	32.67	33.11	32.77

OS/IS/ELM, outer segment/inner segment/external limiting membrane; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL/GC, inner plexiform Layer/ganglion cell; RNFL, retinal nerve fiber layer; TRT, total retinal thickness

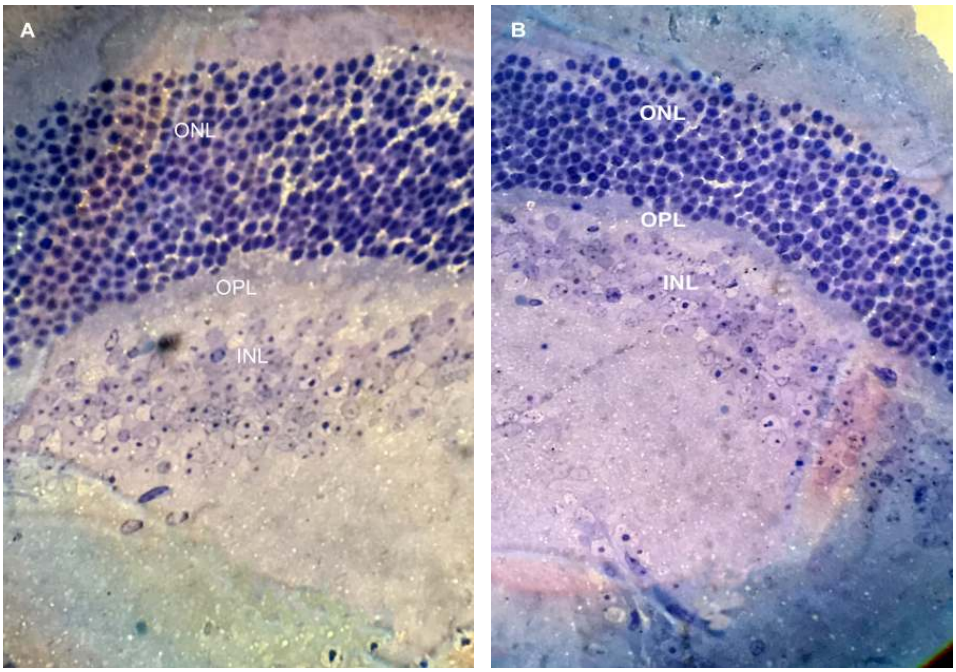

Fig. 2. Photomicrograph of retinal sub layers from control (A) and multiple sclerosis (B) mouse eye samples

Layers of retina are as follows: INL = inner nuclear layer; OPL = outer plexiform layer; ONL = outer nuclear layer. Toluidine blue staining

Table 2. The number of ki67 positive cells in sub layers of retina in MS and control groups

sub-layers	Control group	MS group	P-value
Outer nuclear layer(ONL)	2.40±0.65	0.50±0.26	0.007
Inner nuclear layers (INL)	4.90±0.76	1.10±0.23	0.0002

Inner plexiform layer/ganglion cell (IPL/GC) and Inner nuclear layer (INL) and outer nuclear layer (ONL) thickness is significantly decreased in MS group. However, histological evaluations of outer plexiform layer (OPL) and Outer segment/Inner external limiting membrane thickness in MS and control groups showed no differences (Figs. 2,A and B).

3.2 Proliferation Expression

Proliferation was assessed by ki67 kit; Immunohistochemistry (Dako Denmark A/S) staining was performed with ki67 kits on control and MS groups. Expression of ki67 positive goldish brown cells in retinal layers demonstrated in Fig. 3. The numbers of ki67 positive cells in sub layers of retina are demonstrated in Table 2. Proliferation rate in outer nuclear layer (ONL) in MS group (0.50±0.26) was significantly lower than in control group (2.40±0.65). Also in Inner nuclear layers (INL) proliferation rate in MS group (1.10±0.23) decreased significantly in comparison to control group (4.90±0.76).

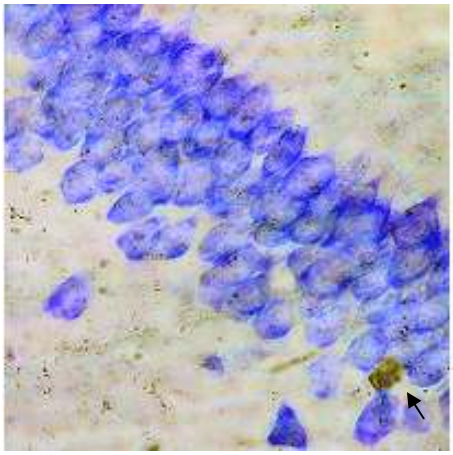

Fig. 3. Photomicrograph of a section of retinal inner nuclear layer showing a Ki67 positive cell with goldish color (Arrow)

4. DISCUSSION

The present study revealed thinning of total retinal thickness, especially some of its layers including retinal nerve fiber layer, inner plexiform layer, and ganglion cell layer, inner and outer nuclear layers. These findings were associated with decreasing of proliferation rate, meaning that the main mechanism of retinal thinning could be lack of cellular replacement and or cell loss via apoptosis. These findings could be correlated with clinical symptoms such as visual loss in MS patients. There are evidences that retinal glial cells have an important role in cellular maintenance and metabolism of retinal cells [19]. This is done by glial cell proliferation and their additional support for retinal cells even their regeneration in lower animals [20,21]. Glial cell proliferation occurs in response to retinal cell damages and cause retinal thickening. However, it appears that in case of MS disease, for any reason glial cell proliferation does not occur and this along with increasing cell loss due to decreasing of glial support, leads to thinning of retinal thickness.

In animal study of retinal disease, ocular histology is still considered to be the gold standard for assessing structural morphology [22]. Histology provides a better view of the microstructural feature of the retina by staining and tissue preservation techniques [23]. The drawback of histology in humans is difficult to conduct longitudinal studies to describe the course of a disease process. However, in animal studies, the animals can be sacrificed sequentially to get serial data to improve our knowledge of many retinal disorders such as multiple sclerosis (MS) [24]. Studies on the measurement of retinal layers are scarce [25,26]. The aim of the present study was to compare thickness of total retina and its sub-layers in MS induced mice model with control group. The histological method appears to be the most reliable method. Unfortunately, histological characteristics of retina have not always been clearly described in other publications.

Blumenthal EZ in 2009 could not detect an association between RNFL thinning and visual function disability and axonal loss in MS patient [1,6]. Our findings showed that the thickness of total retina in MS group is decreased significantly. The latter parameter could be proposed as a novel criterion for showing the association between retinal thickness and Ms related retinal disorders. In addition, thickness of retinal nerve fiber layer (RNFL) and inner plexiform layer, ganglion cell layer and inner nuclear layer, in MS group are obviously lower than in control group. These changes are an indication of cellular damage in treated group and are in line with several previous studies [26,27]. However, Horstmann et al. [28] despite of increased apoptosis in EAE group, have not shown a decrease in retinal thickness. Probably they have measured the retinal thickness 23 days after immunization but in the present study the measurement is carried out 35 days after immunization. We found higher RNFL measures in control group as compared to MS group, which is striking in accordance with previous reports [26,29].

These findings may point to a more severe RNFL damage in MS group. Our finding are in line with clinical feature of MS with a lower proportion of visual loss, less frequent optic nerve attacks [30,31]. Interestingly, an analysis of the combined ganglion cell layer and inner plexiform layer measure lower in MS group with optical coherence tomography [32]. Based on published data form cross-sectional studies in MS patients with disease duration, it appears that atrophy rate is ten times higher than what happens in normal ageing [31]. Furthermore, in previous cross sectional studies significant inverse correlations of RNFL thickness [30] and disease duration could be established by some authors [33,34].

5. CONCLUSION

According to the results it is concluded that decreasing of thickness of total retinal and every retinal sublayers in MS group correlates well with decreasing of proliferation rate in some retinal sublayer. Indicating that decreased retinal thickness may mainly due to reduced proliferation of retinal glial cells and consequently retinal cell loss.

CONSENT

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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