·Basic Research·

# The correlation between rat retinal nerve fiber layer thickness around optic disc by using optical coherence tomography and histological measurements

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## Abstract

**• AIM: To explore the correlation between the retinal nerve fiber layer (RNFL) thickness by using optical coherence tomography (OCT) and by histological measurements in normal adult rats and optic nerve transected rats.**

**• METHODS: The RNFL thickness of 36 rats was scanned in a circle 3.46mm far from the optic disc by OCT. The two experimental groups were the normal group ($n$=20 rats) and the optic nerve transected group ($n$=16 rats). The latter group included 4 groups ($n$ =4 /group) surviving for 1 day, 3, 5 and 7 days. Then the RNFL thickness of the same retina area was also measured by NF-200 immunohistochemical staining method. Linear regression was used to analyze the correlation between the data obtained from these two methods.**

**• RESULTS: The RNFL thickness of normal right eyes around optic disc by OCT was 72.35±5.71μm and that of the left eyes was 72.65±5.88μm ($P$=0.074). The RNFL thickness of the corresponding histological section by immunohistochemistry was 37.54±4.05μm (right eyes) and 37.38±4.23μm (left eyes) ($P$=0.059). There was a good correlation between the RNFL thickness measured by OCT and that measured by histology ($R^2$=0.8131). After optic nerve transection, the trend of the RNFL thickness was thinner with the prolonged survival time. The correlation of the thickness detected by the above two methods was approximately ($R^2$=0.8265). Value of the RNFL thickness in rats around optic disc measured by OCT was obviously higher than that measured by common histological measurement in normal adult rats and optic nerve transected rats.**

**• CONCLUSION: The RNFL thickness measured by OCT has a strong correlation with that measured by histological method. Through OCT scanning, we found that the thickness of RNFL gradually becomes thinner in a time-dependent manner.**

**• KEYWORDS:** retinal nerve fiber layer thickness; optical coherence tomography; optic nerve transection; immunohistochemistry staining; relevance analysis; rat
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## INTRODUCTION

The pathological changes of retinal disease are mostly viewed by its morphology variation after Haematoxylin-Eosin or immunohistochemical staining. Unfortunately, these methods can't be applied *in vivo*. Optical coherence tomography (OCT), which has high resolution to the histological micro-structures, can be performed in the living eye tissues without the need for animal tissue sacrifice. Although some previous studies have already reported significant correlation between the OCT thickness of different experimental animals' retinae (monkey, rabbit and

guinea pigs, *et al*) and their histological thickness, the use of OCT in animal retina remains at experimental level even as to date[1-12]. In some eye diseases leading to blindness such as glaucoma and optic nerve contusion, damage to retinal nerve fiber layer (RNFL) always appears earlier than the loss of visual function [13]. Therefore, the measurement of thickness of RNFL by OCT can play a critical role in the early diagnosis and treatment of these diseases. Some studies reported that OCT thickness of RNFL showed alteration in retinae injured with light, acute high intraocular pressure (aHIOP), and also following optic nerve neurodegeneration [8,14,15]. However, there is still lack of evidence about the correlation between the RNFL thickness measured by OCT and histological methods in either healthy rats or RNFL damaged rats with transected optic nerve. The present study measured the RNFL thickness around optic disc of healthy rat retina as well as rats with damaged RNFL, and correlated with their corresponding histological measurements. Linear regression analysis was used to quantify these correlations. Furthermore, the effect of optic nerve transection on RNFL thickness was also studied over time.

## MATERIALS AND METHODS

**Materials** Thirty-six SD rats aged 2 to 3 months were purchased from the Experimental Animal Center of Xiang-ya School of Medicine, Central South University. The weight was about 180g-250g. The pupils of all rats were round and reactive to light, and they had transparent ocular media. The rats were divided into two groups namely: normal group ($n$= 20 rats) and transected optic nerve (ON) group ($n$=16 rats). The rats undergoing ON transection were randomly assigned into 4 sub-groups ($n$=4 rats in each sub-group) surviving for 1 day, 3, 5 and 7 days after ON transection. The laboratory animals were housed in a constant temperature at 24℃ with relative humidity at (55±10)%. All experiments were carried out according to the principles outlined in the NIH Guide for Care and Use of Laboratory Animals [16,17]. This study was approved by Animal Ethics Committee of Xiang-ya School of Medicine, Central South University.

**Methods OCT Scanning Procedures for Normal Rat** All the normal rats were anaesthetized with intraperitoneal injection of 0.5mL/100g wt chloral hydrate (10%), and Tropicamide eye drops (0.5%, Hubei Qianjiang Pharmaceutical Co., Qianjiang, China) were used for mydriasis. After shearing the whisker, the rat was placed on a horizontal table in its natural state, then the eye was opened and the infratemporal fossa was pressed to cause proptosis of the eyeballs. The jaw was adducted slightly and the head was skewed about 15°-25° in order to allow the penetration of light to the dioptric media vertical to the cornea from the temporal side. The direction of the incoming light was close to the axis oculi. Besides, a black eyeshade was used to cover the white fur of the rat in order to decrease the interference of the catoptric light. The RNFL of rat was scanned by OCT (Cirrus HD-4000, Carl Zeiss Meditec Inc., Germany), which could automatically identify the center of the (optic disc) OD and fix the position of the calculation circle (using RNFL Thickness Analysis and the system will define the diameter of the calculation circle as 3.46mm). The center of the calculation circle was located in OD and the signal strength was ensured at least 5 levels. After taking at least 3 clear captures by OCT, the rat was sacrificed and the retinal tissues were collected for further use.

**Transected Optic Nerve Animal Model Preparation** Sixteen animals were anesthetized with intraperitoneal injection of 0.5mL/100g wt chloral hydrate (10%). Subsequently, the heads of each rat were fixed in stereotaxic apparatus by using 0.5% lavo-ofloxacin to keep the eyes from drying. With the use of a binocular operating microscope, the superior conjunctiva was incised, the muscles and connective tissue were separated, and the intraorbital optic nerve was exposed. With a diamond knife, the optic nerve was transected 1mm to 2mm behind the globe, taking care not to interfere with the blood supply. The eye was dressed with antibiotic ointment. After cleaning the operation areas, the lavo-ofloxacin was used again to moisten the cornea. At the same time, the left eye served as time-matched control (Non-operated) group in ON transected animal model without any treatment. After the operation, the rats were kept as before until sacrifice. Before our sacrificed (1 day, 3, 5 and 7 days), we did OCT scanning first.

**OCT Scanning Procedures for TON Rat** We carried out OCT scanning on operated eye and non-operated eye at each survival time point after anesthesia. On the scanning image of RNFL by OCT, three groups of RNFL thickness were demonstrated. The one on the top was the average value of data from all the measuring sites on the circle. In the middle was the average value of the data from measuring points of the quadrants of the top, the bottom, the nasal side and temporal side. The thickened image on the bottom, which was chosen in the next step, was the average of data from all the measuring points at intervals of 30°. Three images, with good clarity and strong signal intensity (>5), were chosen for each eye and for statistical analysis. The RNFL thickness in the top and bottom hour clocks of histological measurement site (in Figure 1, 1:30, 4:30, 7:30 and 10:30 hour clock) had been calculated. Consequently, the average value of RNFL thickness in all four quadrants had been calculated, which was the final result of the RNFL thickness around the optic disc of rat retina.

**Samples Collecting** After OCT scanning, the rat was punctured through the left ventricle, rinsed by 37℃ saline for 1-3min and then quickly perfused by 4% paraformaldehyde (PF) at 4℃ and then slowly for another 20min. The eye was enucleated carefully after the perfusion,

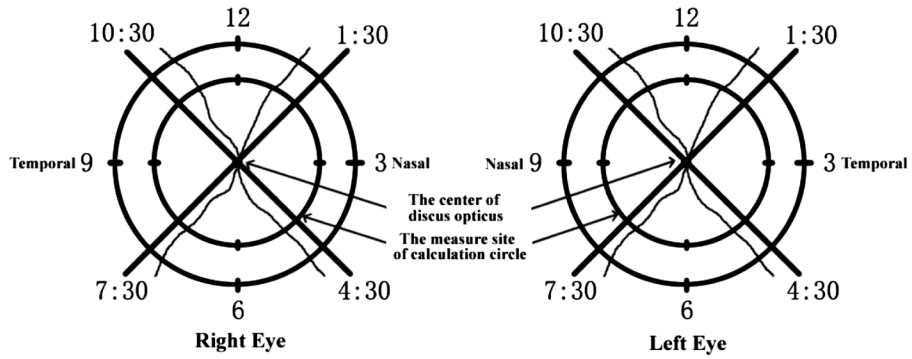


**Figure 1 Schematic diagrams of rat retina tissue collection and histological measuring areas.**

leaving markers to identify the direction of 3, 9 and 12 hour clock and put into 4% PF for 2 hour for post-fixing. After the cornea, lens and the vitreous body were removed, the eye cup intersected the OD from 1:30 to 7:30, cutting the retina and len radially to OD from 4:30 to 10:30, a circle 1.75mm far from the optic disc was left. Then the samples were put in PF again for fixing overnight at 4℃ for further morphological studies [1,2,6]. The retina was embedded in paraffin wax after dehydration and clearing. Subsequently, the paraffin embedded block was cut into 3μm sections from 1:30 to 7:30 and 4:30 to 10:30 by a microtome (AO, USA). After finishing these procedures, the sections were floated on water at 40℃ for stretching and then sections were mounted on Superfrost Plus slides (VWR, PA, USA). The slides were dried in the oven at 70℃ for 2h for further immunohistochemical staining.

**Immunohistochemical Staining and Haematoxylin Count-staining** After rinsing the paraffin wax by dimethylbenzene and alcohol, the sections were heated for antigen retrieval (1mmol/L EDTA buffer, pH8.0, 92℃ -98℃ , 15min), hydrogen peroxide (3%) was used to remove peroxidase for 10min and 5% bovine serum albumin (BSA) to block the endogenous nonspecific antigen for 30min. Then, the sections were incubated in primary antibody (monoclone, mouse anti NF-200, 1:200, BM0100, Boster, Wuhan, China) overnight at 4℃ , in the biotinylated secondary antibody (goat anti mouse, 1:200, Vector, CA, USA) for 2h and then with ABC complex (1: 200, Vector, CA, USA) for 1h. DAB (Sigma, MO, USA) was used for visualization. After haematoxylin (Sigma, MO, USA) counter-staining, the sections were dehydrated and mounted with cover slips. Between each procedure, sections were rinsed by phosphate buffered saline (PBS), and all steps were achieved at 24℃ except some special situations. BSA was used to replace the primary antibody for negative control.

**Histological Measurement** The pictures of retina were taken by light microscope (20× objective lens, Nikon 80i, Japan) at 1:30, 4:30, 7:30 and 10:30 hour clock which corresponded to superior temporal, inferior temporal, superior nasal quadrant and inferior nasal quadrant. Ten well-structured sections (5 pictures were picked from each side of the eye tissues) whose center was the OD. The radius was about 1.73mm and pictures were picked in order to correspond to the same area by OCT (Figure 1). Forty pictures were taken from each eye and the thickness of RNFL was measured by Photoshop CS 7.0 (Adobe, CA, USA). Histological measurement used the staff gauge in our microscope, and OCT measurement used the staff gauge in OCT equipment. The thickness in each picture was obtained by calculating the average of five different measurements chosen randomly, and the average RNFL thickness for each eye was the mean of the values from 40 pictures.

**Statistical Analysis** In normal control eyes, the RNFL thickness values of OCT scanning and histological measurement were analyzed by paired *t* test. The RNFL thickness values of left and right eyes with same method measurement were analyzed by paired *t* test. Between operated groups and non-operated groups, the RNFL thickness values in a single time-point by two methods were analyzed by paired *t*-test. The operated groups in different surviving time points were analyzed by one-way ANOVA using SPSS 19.0 (SPSS, CA, USA). All data were presented as mean±SD. $P<0.05$ was considered statistically significant. Besides, Excel 2010 (Microsoft, WA, USA) was used to analyze the linear regression of the normal groups and the transected optic nerve groups.

## RESULTS

**Rat RNFL Thickness Around the Optic Disc by OCT** We have 3 sets of average RNFL thickness values, which were calculated through the whole calculation circle, the quadrant and the hour clock respectively (Figure 2A). The first one was chosen to represent the OCT measurement average values. The RNFL thickness around OD was 72.35±5.71μm for the right eye and 72.65±5.88μm for the left eye without statistically significant difference between them ($P$=0.074, Figure 2B).

**Rat RNFL Thickness Around the OD by Histological Measurement** The pictures of retina histological staining by light microscope were shown in Figure 3. The positive staining of NF-200 was restrictively located in ON and

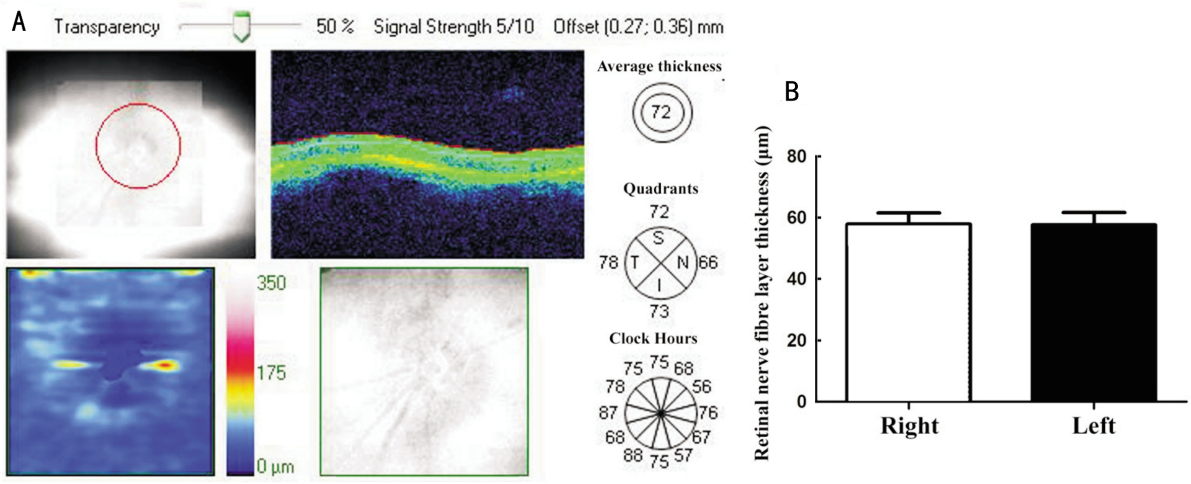


**Figure 2 OCT color photography and statistical analysis of RNFL thickness in rat** A: OCT color photography of RNFL thickness; B: Statistical analysis of RNFL thickness. There is no statistical significant difference between the bilateral RNFL thickness ($P$=0.074).

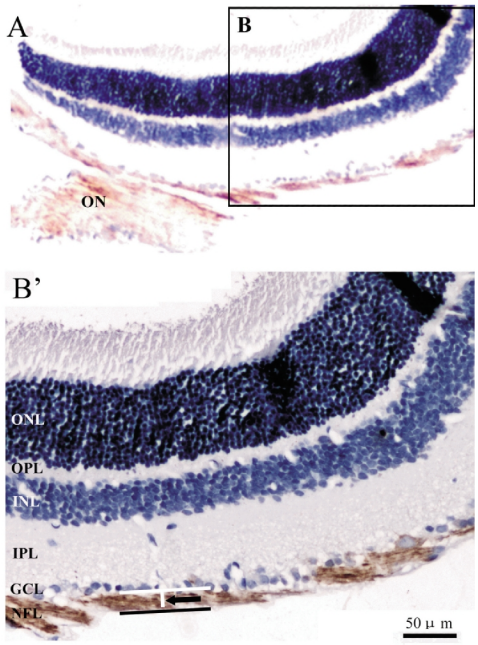


**Figure 3 Immunohistochemistry staining of NF-200 in rat retina** A: Picture of retina in low magnification; B: The measured area corresponding to A; B': The higher magnification picture of panel B, two lines and an arrow in panel B show the measurement process used in digital images. (ON: optic nerve; NFL: nervous fibre layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer. Scale bar=50μm in Panel B', 100μm in Panel A).

RNFL shown as fabric morphology. The RNFL thickness around the OD by histological method was 37.54±4.05μm for the right eye and 37.38±4.23μm for the left eye with no statistical significant difference ($P$=0.059, Figure 4).

**Rat RNFL Thickness Around the OD by OCT Had Positive Correlation Measured by Histological Method** The linear regression analysis (Figure 5) of RNFL thickness around the OD suggested there was a good correlation ($R^2$= 0.8131, $n$=40) between OCT and histological measurements. In addition, OCT measurements were larger than histological measurements ($P$=0.000).

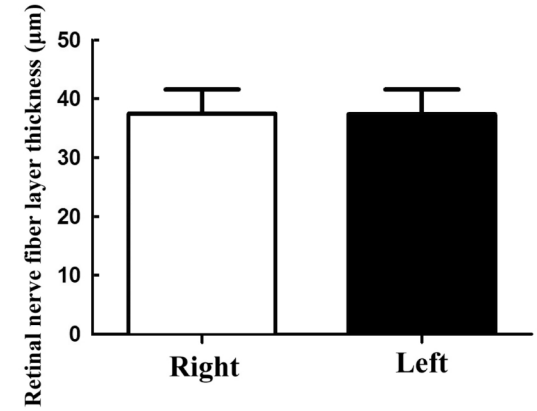


**Figure 4 Statistical analysis of RNFL thickness around the OD by using histology in rat** There is no statistical significant difference between bilateral RNFL ($P$=0.059).

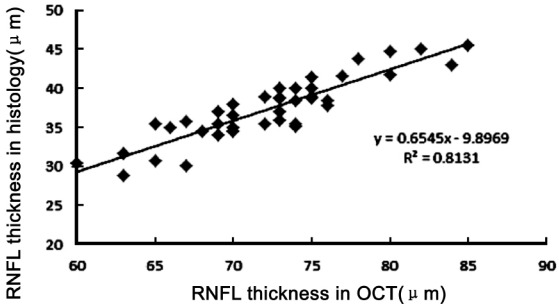


**Figure 5 Correlation analysis of the rat RNFL thickness in OCT and histology.**

**Rat RNFL Thickness Values on the Calculation Circle of All Quadrants by OCT and the Temporal-Superior-Nasal-Inferior-Temporal (TSNIT) Profile** The data of optic calculation circle by OCT showed that all of the RNFL thickness values were closed (Figure 6A), and TSNIT pattern was smooth (Figure 6B), with minimal variation among the different quadrants. This pattern is quite different from the human one. Figure 6C-D shows a typical human TSNIT pattern with the characteristic bimodal pattern, with thicker inferior and superior quadrants. This pattern is seen in all normal human eyes.

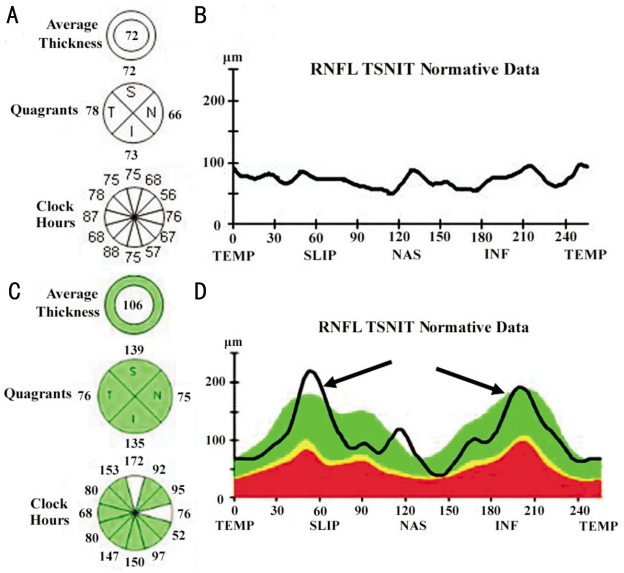


**Figure 6 Average RNFL thickness in OCT image and TSNIT profile corresponded to average RNFL thickness of rat and human** A,B: Rat; C,D: Human; Arrows indicated bimodal feature.

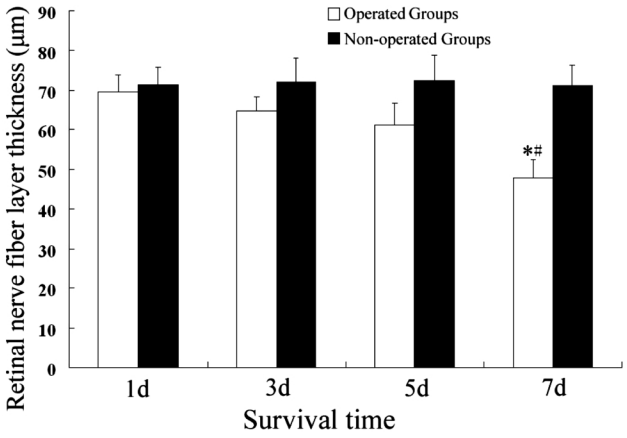


**Figure 7 Statistical analysis of RNFL thickness around the OD using OCT in rat after ON transaction** *: *versus* non-operated 7-day group: $P$=0.000; #: *versus* operated 1-day group: $P$=0.000; operated 3-day group: $P$=0.000; operated 5-day group: $P$=0.006.

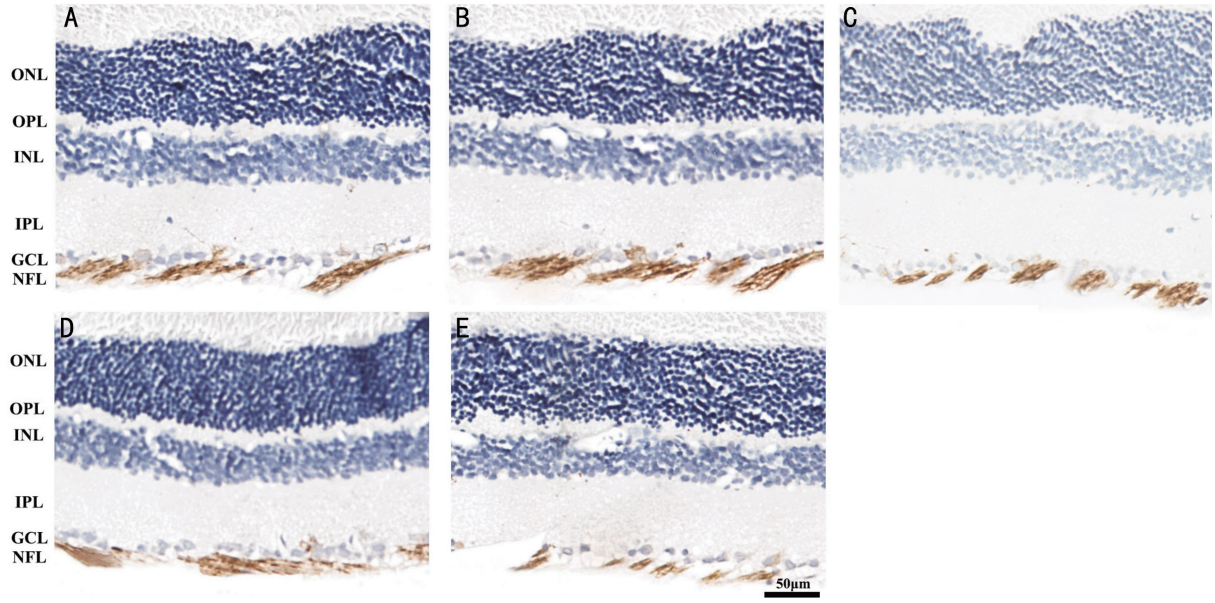


**Figure 8 Immunohistochemistry staining of NF-200 of rat retina after ON transection** A: Non-operated 1-day group; B-E: 1 day, 3, 5 and 7 days after ON transection. NFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer. Scale bar=50μm.

**OCT Measurements** The RNFL thickness by OCT of the transected ON groups was 69.56±4.28μm at 1 day, 64.78±3.56μm at 3 day, 61.19±5.40μm at 5 day and 47.94±4.50μm at 7 day, which is time-dependent manner (see Figure 7). This reduction was statistically significant when compared with non-operated group at 7 days ($P$=0.000).

**Histological Measurements** The haematoxylin staining of transected ON groups and non-operated groups showed clear structure of retina and the amount of the positive staining cells decreased along with the survival time. Whereas the retinal thickness of different survival times could not be observed and markedly decreased under the light microscope (Figure 8). The thickness by histological measurements of transected ON rats was 35.28±4.10μm at 1 day, 32.52±4.91μm at 3 day, 31.32±6.07μm at 5 day and 25.74±3.66μm at 7 day, which is time-dependent manner (Figure 9). Compared with non-operated 7 day group, 7 day group had statistical significance ($P$=0.001).

**Positive Correlation of RNFL Thickness between the OCT and Histological Measurements Following ON Transection** The RNFL thickness measured by OCT or by histological method showed a good correlation ($R^2$=0.8265, $n$=20) with the linear regression analysis (Figure 10). It should be noted that the RNFL thickness by OCT was thicker than that of histological measurement following ON transection.

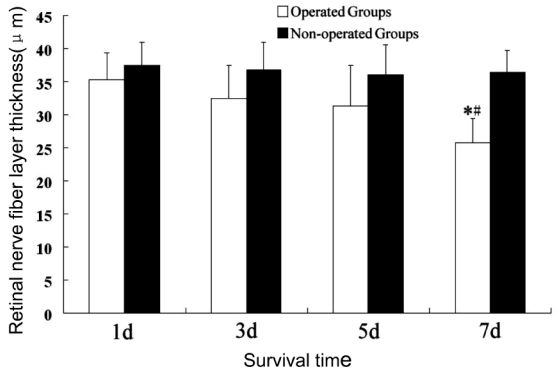


**Figure 9 Statistical analysis of RNFL thickness around OD using histology in rat after ON transaction** *: *versus* nonoperated 7-day group: $P$ =0.001#: *versus* operated 1-day group: $P$=0.000; operated 3-day group: $P$=0.000; operated 5-day group: $P$=0.000.

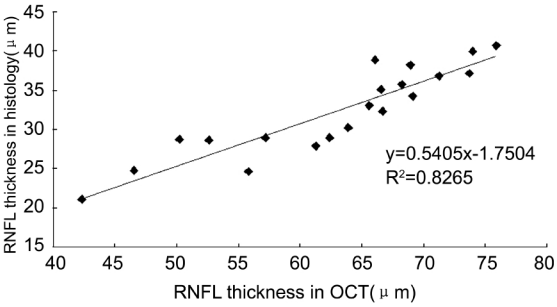


**Figure 10 Correlation analysis of rat RNFL thickness by OCT and histology after ON transection.**

## DISCUSSION

**Application Prospect of Evaluating Rat RNFL Thickness Alteration *in vivo* by OCT** So far, some specialists had already measured the thickness of the entire retina of many experimental animals using OCT. When compared with histological method, they suggested there was a good correlation between these two methods ($R^2$=0.995) [19]. Besides, Ji and his colleagues [8] had already measured the entire light damaged rat retina thickness values at different time points by OCT as well as by histological measurement. Their result also showed a good correlation. Actually, OCT could be used to measure the alteration of the RNFL thickness under optical crash, fatty disease in rats and a HIOP injured retina, even in multiple sclerosis patients, which was more important than the entire retinal thickness in clinical, and especially to those people who have glaucoma or ON contusion [12,20-26]. However, when speaking of the correlation between OCT and histological methods to measure the RNFL thickness alteration following TON, the studies were scarce. In this experiment, the right RNFL thickness of normal rats was 72.35±5.71μm, and the left was 72.65 ±5.88μm, which showed no statistical significance between the bilateral eyes and was consistent with Liu's result (80.26μm of contralateral CTL eye of chronic HIOP rats) [14]. Our present data of the RNFL measurements following ON transection suggested the thickness around optic disc became thinner along with the survival time, and there was statistical significant difference between 7 day group and normal group. In brief, our data suggested the RNFL thickness had the same alteration pattern following ON transection by two methods, which indicated OCT measurement values could represent the RNFL thickness variation following ON damage *in vivo*.

It should be noted that the rat RNFL thickness around OD which was measured by histological method of the right eye was 37.54 ±4.05μm and the left was 37.38 ±4.23μm. The value was obviously lower than the OCT measured value (right eye: 72.35 ±5.71μm; left eye: 72.65 ±5.88μm). We speculated that OCT measurement might be closer to reality than histological measurement, as histological measurements were done in "dead" tissue. Dead tissue loses its water content and fixation caused quick reduction in the thickness [1,2,6]. Besides, the optical systems involved in capturing the images and measurements have their own systematic errors within two methods that need to be considered. Another explanation we considered was that the OCT machine and its program were for human beings, not specific for rats. In our own opinion, it's reasonable for the difference between values of OCT scanning and histological measurement. Based on the realistic values of OCT scanning, the OCT scanning method has many advantages such as authentic and précised. Therefore, we thought that the OCT measurement was suitable for describing the dynamic RNFL thickness change in research. It is worth noting that it was quicker and easier to get OCT measurements than the histological measurements. To be precise, OCT measurements could also be achieved *in vivo*. In summary, we believe that the OCT scanning could replace the histological measurements and become a latent research method for RNFL thickness alteration *in vivo*.

**TSNIT Profiles Did not Follow "Bimodal" Pattern in Rat Retina** Zhao [18] pointed out that the retinal thickness of rats around the optic disc was the same in all four quadrants within 1 500μm from optic disc edge. And they speculated that they might have some differences in the distribution of RNFL thickness between rats and humans. Our results also indicated that the average RNFL thickness was closed and did not fluctuate in all clock hour direction. The curve which could represent 360° thickness from TSNIT profiles was also smooth without the bimodal feathers as that of humans. The rat RNFL thickness in superior and inferior quadrants was thicker than other quadrants. Therefore, we agreed with Zhao's speculation that there might possibly be some differences in the RNFL thickness between rats and humans. The RNFL thickness of rat had no significant difference within a certain range from the optic disc. This part of work needs more solid evidences to confirm.

In conclusion, the RNFL thickness measured by OCT has a strong correlation with that measured by histological method. Through OCT scanning, we found the thickness of RNFL becomes gradually thinner in a time-dependent manner.

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