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Caspase-3 inhibitor Z-DEVD-FMK enhances retinal ganglion cell survival and vision restoration after rabbit traumatic optic nerve injury

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Abstract.
Purpose: Vision loss after traumatic optic nerve injury is considered irreversible because of the retrograde loss of retinal ganglion cells (RGCs) which undergo apoptosis. Because the second messenger caspase-3 plays a major role in apoptosis, we now evaluated the efficacy of the specific caspase-3 inhibitor, Z-DEVD-FMK, in a rabbit model of fluid percussion injury (FPI) which mimics traumatic optic nerve injury in humans to enhance cell survival and improve vision.
Methods: Survival of RGCs and recovery of vision were studied using retinal morphological markers and visual evoked potentials (VEP), respectively. The FPI traumatized animals were treated in their right eye with a single intravitreal or peribulbar injection of Z-DEVD-FMK 30 min post-injury compared to 2% DMSO control injections in their left eye.
Results: Intravitreal Z-DEVD-FMK, but not control injections, led to down-regulation of capase-3 and reduced, in a dose-dependent manner, RGCs apoptosis from 7 to 21 days post-injury. These morphological improvements were accompanied by vision restoration as documented by VEP. The neuroprotection after intravitreal injection of Z-DEVD-FMK was more effective than the peribulbar application.
Conclusions: The caspase-3 inhibitor Z-DEVD-FMK is neuroprotective by inhibiting RGCs apoptosis when injected 30 min after optic nerve damage and significantly promotes restoration of vision. A controlled clinical trial is now needed to evaluate the efficacy and safety of Z-DEVD-FMK in humans.

Keywords: Caspase-3 inhibitor, optic nerve injury, apoptosis, neuroprotection, vision restoration

1. Introduction

The Canadian adult trauma center has documented the epidemiology of traumatic nerve injury (TON) from 1986 to 2007 and found that 0.4% of all trauma patients suffered from traumatic optic nerve damage (Pirouzmand et al., 2012). Approximately 50% of these patients suffer permanent vision loss (Singer et al., 2002) and 40% have vision loss to different degrees (Ansari et al., 2005). To date different strategies to improve vision after optic nerve injury have been tried, including glucocorticoid therapy, surgical decompression, endoscopic optic nerve decompression, neurotrophic factors and combinations thereof (Li et al., 2008; Kermer et al., 1999). However, the therapeutic efficiency of those strategies is still controversial because of the rather variable clinical effects

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(Yang et al., 2012). And there are some limitations such as unwanted side effects of glucocorticoids, unpredictable complications of surgical optic nerve decompression (Zhilin et al., 2011) or failure to support long-term neuroprotection by neurotrophic factors (Kermer et al., 1999). Recent therapeutic strategies in animal experiments included transcorneal electrical stimulation (Henrich-Noacket al., 2013) and the application of calcium inhibitors (Koch et al., 2010).

Apoptosis, a programmed cell death mediated through the activation of the caspase family - in which caspase-3 acts as the key mediator for cell death - has been widely acknowledged to play a role in non-visual traumatic brain and spinal cord injury (Lingor et al., 2012). After damage to the visual system the massive degeneration of retinal ganglion cells (RGCs) is attributed to this apoptosis (Heiduschka et al., 2004; Bien et al., 1999), especially in early stages of optic nerve injury (Vuojola et al., 2012). It has been suggested that Z-DEVD-FMK (Z-Asp-Glu-Val-Asp-FMK), a specific and irreversible caspase-3 inhibitor, can reduce the apoptosis of RGCs following optic nerve transaction when administrated intraocularly (Chaudhary et al., 1999) and has neuroprotective effects in rat traumatic spinal cord injury when injected intraspinally (Barut et al., 2005). Besides having an anti-apoptosis effect, Z-DEVD-FMK can protect the effects of focal cerebral ischemia by anti-inflammatory action (Li et al., 2000) and it may act as a potent calpain inhibitor with protective effects after brain injury (Knoblach et al., 2004).

Because we previously established a new traumatic optic nerve injury animal model using fluid percussion injury (FPI), which simulates clinical optic nerve injury (Yan et al., 2012), the present study was designed to evaluate the therapeutic window and the morphological and functional effects of Z-DEVD-FMK for neuroprotection. Specifically, we wished to establish effective doses and injection routes to achieve protection of RGC from apoptosis to enhance recovery of visual functions as monitored by visual evoked potentials (VEP).

2. Materials and methods

2.1. Animals and experimental protocol

Adult Chinese rabbits ($n = 248$) were bred at Tianjin Medical University (496 eyes, female or male, body weight of $1.8 \sim 2.2$ kg). The study protocol was approved by the institutional review board according to national guidelines for the use of experimental animals.

Before including the rabbits in the experiment, we first ascertained that each eye had clear refractive media, a normal fundus and good light reaction as evident by iris contraction. Bilateral trauma by a fluid percussion brain injury device (FPI) was applied to the optic nerves of 240 rabbits (experimental group) as described by Yan and colleagues (Yan et al., 2012). The remaining 8 rabbits (normal group) served as normal controls without trauma. For the experimental group both eyes of each rabbit were injured, but only the right (experimental) eye was treated with a caspase-3 inhibitor, Z-DEVD-FMK, while the left (injury control) eye was injected only with a vehicle to allow intra-individual comparisons. The 240 traumatized rabbits were then divided randomly into two groups: one group (group A, $n = 144$) was further randomly subdivided into three subgroups receiving different doses of the capase-3 inhibitor: a low (1.5 μg/kg, $n = 48$), medium (2.5 μg/kg, $n = 48$), or high dose (3.5 μg/kg, $n = 48$). The drug was injected 30 min. after injury into the vitreous of the right (experimental) eyes. Caspase-3 inhibitor, Z-DEVD-FMK, was dissolved in a 2% DMSO phosphate buffer solution (PBS). All left (control) eyes received 2% DMSO in PBS as control (injection volume: 5 ul). Rabbits in group B were randomized to an intravitreal injection ($n = 48$) or peribulbar injection subgroup ($n = 48$). Here, the injection dose of Z-DEVD-FMK was 2.5 μg/kg for all animals which was given 30 min after injury. Again, the right eyes received injections of Z-DEVD-FMK and the left eye an equal volume of 2% DMSO/PBS as control to Z-DEVD-FMK. Animals were subsequently sacrificed at 1, 4, 7, 10, 14, and 21 days after drug injections ($n = 8$ rabbits at each time point) by injecting 10 ml air into the ear vein following anesthesia with 10% chloral hydrate which was injected intraperitoneally.

2.2. Reagents

The following reagents used were: caspase-3 inhibitor Z-DEVD-FMK (Biovision Company, USA); caspase-3 antibody (Santa Cruz, USA), a TUNEL kit (Zhongshan Goldenbridge Biotechnology Co) and an immunohistochemical kit (Haoyang Biotechnology Co). The protocols of the tissue staining method were

described by Yan et al. (2012), Duan et al. (2003) and Cheng et al. (1998)

2.3. Rabbit model of traumatic optic nerve injury using fluid percussion injury (FPI)

FPI is a standard rabbit models of traumatic optic nerve injury based on a FPI design described by Yan and colleagues (2012). Briefly, a general anaesthetic agent (10% chloral hydrate) was injected intraperitoneally. Then the conjunctiva was dissected from the 10–2 o'clock position around the limbus. The rabbit was fixed to the FPI table and a self-made blow tube filled with saline was inserted about 2 mm deep through the conjunctival incision along the outer wall of the sclera. The hammer position was 25° which produced a pressure of 699 ± 70 Kpa upon impact. Following the injury, the conjunctival incision was then sutured and ofloxacin eye drops were applied topically to each eye three times a day for 1 week to prevent infection.

2.4. Drug injection

Z-DEVD-FMK was dissolved in 2% DMSO in phosphate buffered saline (PBS). Thirty min. after injury, the experimental (right) eyes received either intravitreal or peribulbar injections of capase-3 inhibitor by micro-injector after routine disinfection, while the left eyes received equal volume of 2% DMSO as control (5 ul injection volume in each eye).

2.5. Visual-Evoked Potential (VEP)

The American Nicolet electrophysiological diagnostic equipment was used to test bilateral VEP of all rabbits according to International Clinical Visual Evoked Potential levels. To this end, the same general anaesthetic (10% chloral hydrate) was first injected intraperitoneally. Silver needle electrodes (impedance <15 kΩ) were then applied to record the VEP, with an active electrode inserted subcutaneously into the occipital tuberosity (OZ position). The reference electrode was inserted into the nose (Fpz position), and a ground electrode was inserted subcutaneously into the mastoid processes post aurem. Flashes of light were then delivered from a light-emitting diode (LED) at a frequency of 1.6 Hz, transmission band width of 30–1,000 Hz, wave width of 0.2 ms, applied for 500 ms. Each eye was stimulated more than 3 times continuously at 10 min intervals. While one eye was being tested, the fellow eye was covered by a piece of opaque cloth. The amplitude was determined by measuring the distance from the peak of the dominant wave in reference to baseline. Latency was defined as the time from stimulus onset to the peak of the dominant wave (P100). The amplitude and latency accompanied with two stable waves of each eye was recorded and averaged.

2.6. HE staining and RGC counts

Rabbits of group A were sacrificed to enucleate eyes and optic nerves after VEP monitoring. All of the optic nerve specimens were cut into two parts. One part was fixed in 10% formalin for 1 week, and paraffin sectioned and stained with routine hematoxylin (HE). The other part was used for western blotting. After fixation in formalin, the specimen were dehydrated through a series of graded alcohols (70/80/90/95%) for a total of 48 hrs and were then immersed in absolute alcohol and dimethylbenzene solution for 20 min. The samples were embedded in paraffin and 4 μm serial sections were cut along the longitudinal axis of the optic nerve and HE and eosin stained for light microscopy examination. The total number of cells was counted in the RGC layer in 10 randomly selected regions of one single slide at 40× magnification. The average number of cells in the RGC layer was counted in three serial slides to analyze the variation of RGC number.

2.7. TUNEL assay

Rabbits of group B were sacrificed after VEP monitoring. Eyes were enucleated to evaluate RGC apoptosis by TUNEL assay using a reagent kit in accordance with the manufacturer's specifications. Briefly, sections were deparaffinized, rehydrated, and finally inserted in 3% H2O2. Tissues were digested by protease and terminal deoxynucleotidyl transferase and dUTP-digoxigenin added to the sections and incubated in a humidified room at 37° for 1 hr. Sections were then washed 3 times and incubated with anti-digoxigenin-peroxidase solution, colorized with DAB, and stained with haematoxylin again. The slices were then covered by general mounting solution and studied with fluorescence microscopy (Olympus 71DP, Japan). Cells were considered positive if they showed brownish color or brown-yellow granules. The rate of apoptosis was then calculated by the percentage of apoptotic cells of all cells in the same sampling field. The number of cells was counted in 5 fields at 400x magnification.

2.8. Immunohistochemistry

Immunohistochemistry was used to evaluate the capase-3 expression in the retina of rabbits in the medium dose group only. To this end, sections were deparaffinized, rehydrated, and washed 3 times by PBS, water bath heated for 15 min. to recover, and blocked with bovine albumin in PBS for 10 min. Sections were then incubated with primary antibody (mouse anti-rat capase-3) for one hour at 37°; the avidin–biotin-peroxidase complex was added for 40 min. at 37° and colorized with DAB to visualize both positive and negative cells. Sections were then double distilled water washed, stained with haematoxylin again, and cover-slipped with general clarity resin.

The signal was quantified across all layers of the retina, especially the RGC layer, inner nuclear layer (INL) and outer nuclear layer (ONL). An Olympus 71 DP image analyzer with IPP software was applied to test the integral optical density of caspase-3 in immunohistochemical slides. Four sampling fields with clear tissues of one slide were analyzed by IPP and the mean integral optic density was recorded.

2.9. Western blotting

Optic nerves of 8 rabbits which received the same dose and sacrificed at the same time point in group A were pooled, so protein could be extracted from this small volume of tissue. The extracted tissue was frozen immediately in liquid nitrogen, lysed in RIPA lysis buffer consisting of Tris, NaCl, Triton X-100, leupetin, aprotinin, sodium deoxycholate, sodium orthovanadate, and EDTA. Tissues were ground in liquid nitrogen and then protein was collected and extracted on ice for one hour. The protein was separated with 12% SDS-PAGE, after which the target protein was transferred onto the PVDF membrane, blocked with 5% skimmed milk powder overnight. The primary antibody (anti-capase-3 antibody) was incubated for one hour and visualized with chemiluminescence to identify bound antibody. Beta-actin antibody was used as a reference value to quantify the grey values.

2.10. Statistical analyses

Statistical analyses were performed using SPSS17.0. Descriptive data are presented as mean ± standard deviation except as otherwise noted. Student's two-tailed *t*-test with Bonferroni correction for multiple comparisons was used to analyze the data including dominant wave latency, amplitude, and number of RGCs, RGCs apoptotic rate, integral optical density of caspase-3 between the experimental and injury control eyes of each group at each observational time point respectively. Three factors including time, dose and time-dose effect of VEP, RGC counts in three dose groups were performed by three-way analysis of variance (ANOVA) and posthoc Student-Newman-Keuls test to reveal the statistical differences between groups. One-way ANOVA was performed to compare the dominant wave latency, amplitude, and RGCs apoptotic rate among the intravitreal and peribulbar group at different time points. Student's *t* test was performed to compare the VEP and RGCs apoptotic rate between the intravitreal and peribulbar experimental group at each time points separately. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Rabbit model of optic nerve injury

The successful application of our rabbit optic nerve injury model was documented by the function of optic nerves with VEP which was in line with values previously reported (Yan et al., 2012)

3.2. Visual evoked potentials (VEP)

The normal VEP reference of the dominant latency of the N-P1-N wave (42.72 ± 1.74 ms) and amplitude (8.39 ± 2.12 μv) were established by examining 8 normal rabbits prior to injury. At 1d after injury the dominant wave latency prolonged to 86.94 ± 7.72 ms and the amplitude declined significantly from 10.08 ± 1.17 μv to 5.34 ± 0.78 μv ($p < 0.05$). Compared to the normal control eyes, both the experimental and injury control eyes VEP of group A and B were statistically different at each time point.

Group A: Compared to the injury control eyes, the functional decline of VEP seen in experimental eyes of the three dose groups were similar at 1d and 4d post-injury ($p > 0.05$). This was followed by recovery starting from post-injury day 7 ($p < 0.05$). Specifically, with the passage of time, the dominant wave latency of the experimental eyes showed

Table 1
VEP latency (Mean ± SD) dose response in group A (ms)

Groups		Days post injury					
		1	4	7	10	14	21
Low-dose	Experimental	85.72 ± 4.06	104.53 ± 4.90	100.06 ± 6.92■	94.53 ± 6.86■	84.53 ± 6.65■	80.72 ± 6.62■
	Control	86.11 ± 5.47	107.13 ± 6.30	114.22 ± 8.41	121.72 ± 8.20	125.28 ± 8.99	136.34 ± 8.49
Medium-dose	Experimental	85.75 ± 8.28	99.06 ± 7.04	90.50 ± 7.61■	83.91 ± 5.10■	70.59 ± 7.65■	67.97 ± 7.93■
	Control	86.11 ± 5.47	107.13 ± 6.30	114.22 ± 8.41	121.72 ± 8.20	125.28 ± 10.01	134.22 ± 8.51
High-dose	Experimental	84.56 ± 7.47	96.25 ± 6.19	88.09 ± 6.82■	80.60 ± 7.09■	67.19 ± 5.64■	62.88 ± 10.36■
	Control	87.16 ± 5.03	104.15 ± 5.69	112.34 ± 10.71	119.41 ± 8.72	126.56 ± 8.25	132.88 ± 7.95
Normal control		43.27 ± 5.16	44.29 ± 4.75	42.25 ± 5.54	41.97 ± 4.76	43.25 ± 6.59	44.21 ± 6.19

For the experimental eye of each dose group, statistical differences.(■ = $P < 0.05$) were observed at each time point,when compared with the injury control eye, respectively. Statistical difference was observed among the three dose group ($p = 0.000$). Evident differences were observed between the low vs. medium and the low vs. high dose group ($p < 0.05$). There was no statistical difference between the medium vs. high dose group ($p > 0.05$). SD = standard deviation.

Table 2
VEP amplitude (Mean ± SD) dose response in group A (ms)

Groups		Day post injury					
		1	4	7	10	14	21
Low-dose	Experimental	5.24 ± 0.68	4.63 ± 0.65	4.60 ± 0.72	4.80 ± 0.89	5.32 ± 0.67	5.64 ± 0.91
	Control	5.41 ± 0.66	4.72 ± 0.64	4.31 ± 0.64	4.08 ± 0.87	3.73 ± 0.68■	3.60 ± 0.74■
Medium-dose	Experimental						
		5.29 ± 0.55	4.82 ± 0.69	5.24 ± 0.54	5.50 ± 0.50	6.19 ± 0.65	6.64 ± 0.85
	Control	5.35 ± 0.78	4.68 ± 1.02	4.37 ± 0.72■	3.94 ± 1.14■	3.80 ± 0.43■	3.66 ± 0.63■
High-dose	Experimental	5.23 ± 0.86	4.77 ± 0.65	5.38 ± 0.37	5.59 ± 0.40	6.30 ± 0.56	6.72 ± 0.56
	Control	5.32 ± 0.67	4.62 ± 0.43	4.44 ± 0.52■	3.87 ± 0.57■	3.82 ± 0.51■	3.74 ± 0.36■
Normal control		8.23 ± 3.47	7.97 ± 2.91	8.45 ± 2.29	7.98 ± 3.15	8.17 ± 3.52	8.19 ± 3.87

For the experimental eye of each dose group, statistical differences (■ = $P > 0.05$) were observed at each time point when compared with the injury control eye, respectively. Statistical difference was observed among the three dose group ($p = 0.000$). Evident differences were observed between the low vs. medium and the low vs. high dose group ($p < 0.05$). There was no statistical difference between the medium vs. high dose group($p < 0.05$). SD = standard deviation.

Table 3
VEP latency (Mean ± SD) of three dose groups in group B (ms)

Groups	Days post injury					
	1	4	7	10	14	21
Intravitreal experimental	85.75 ± 7.43	99.06 ± 8.71■	90.51 ± 7.67■	83.90 ± 6.95■	70.59 ± 8.14■	65.46 ± 6.97■•
Intravitreal control	86.93 ± 8.13	106.18 ± 9.23	113.59 ± 8.79	120.37 ± 7.44	125.81 ± 9.71	129.21 ± 10.13
Peribulbar experimental	86.16 ± 7.95	98.43 ± 8.53▼	91.25 ± 8.21▼	85.62 ± 9.17▼	74.94 ± 7.11▼	72.06 ± 6.57▼
Peribulbar control	85.74 ± 9.11	107.57 ± 7.19	115.17 ± 9.34	122.28 ± 7.42	127.12 ± 9.52	130.41 ± 11.12
Normal control	43.27 ± 5.16	44.29 ± 4.75	42.25 ± 5.54	41.97 ± 4.76	43.25 ± 6.59	44.21 ± 6.19

For the the intravitreal experimental group, ■ = $P < 0.05$ stands for the statistical difference when compared with the peribulbar control group; For the the peribulbar experimental group, ▼ = $P < 0.05$ stands for the statistical difference when compared with the peribulbar control group; For the the intravitreal experimental group, • = $P < 0.05$ stands for the statistical difference when compared with the peribulbar experimental group. SD = standard deviation.

a prolonged-recovery with the latency being shorter and the amplitude being higher, which was observed in all 3 dose groups (Fig. 2), although the recovery rate was different among the 3 dose group (linear test $F = 16.425$, $p < 0.05$). This result demonstrates that the visual function recovered gradually. There was no statistical difference in the interaction of time and dose ($F = 1.855$, $p > 0.05$). Similarly, there was a significant difference among the 3 dose groups ($F = 37.753$, $p < 0.05$), which is in line with what we have seen in the RGC counts, i.e. a significant difference was seen between the low vs. medium and the low vs. high dose

Table 4
VEP amplitude (Mean ± SD) of three dose groups in group B(ms)

Groups	Days post injury					
	1	4	7	10	14	21
Intravitreal experimental	5.29 ± 2.13	4.82 ± 1.21	5.23 ± 1.87■	5.51 ± 2.06■	6.28 ± 2.39■	6.75 ± 2.75■●
Intravitreal control	5.34 ± 1.87	4.68 ± 1.25	4.37 ± 1.42	3.93 ± 0.77	3.79 ± 0.85	3.66 ± 0.71
Peribulbar experimental	5.41 ± 2.24	4.91 ± 2.13	5.29 ± 2.09▼	5.35 ± 2.38▼	5.97 ± 2.39▼	6.02 ± 1.98▼
Peribulbar control	5.47 ± 1.69	4.77 ± 1.18	4.19 ± 1.45	3.71 ± 0.82	3.69 ± 0.65	3.53 ± 0.47
Normal control	0.975	0.885	0.190	0.211	0.194	0.068

For the the intravitreal experimental group, ■ = $P < 0.05$ stands for the statistical difference when compared with the peribulbar control group; For the peribulbar experimental group, ▼ = $P < 0.05$ stands for the statistical difference when compared with the peribulbar control group; For the intravitreal experimental group, ● = $P < 0.05$ stands for the statistical difference when compared with the peribulbar experimental group. SD = standard deviation.

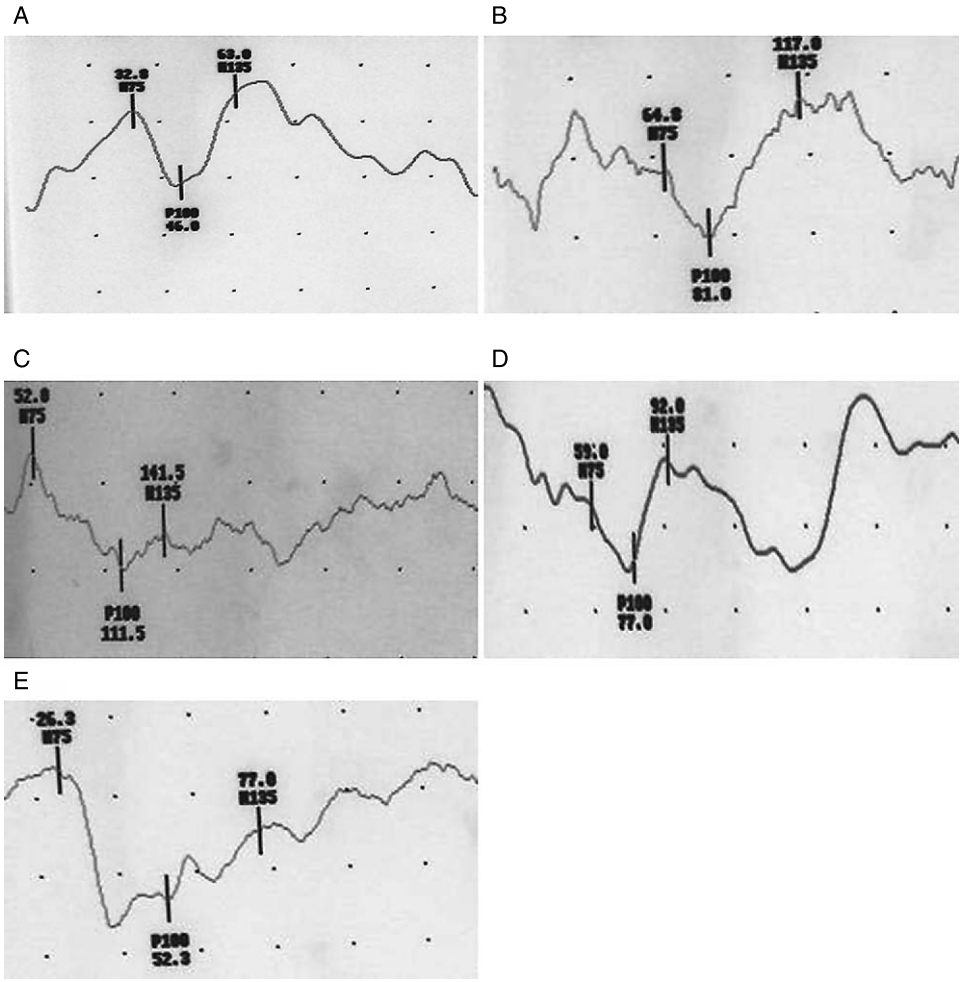

Fig. 1. VEP of group B at different time points. The value under the "p100" represents latency, and the longitudinal separation between the N75 and p100 represents amplitude. Panel A: VEP of normal rabbit, the latency was 46.0 ms and the amplitude was 7.58 μv; Panel B: VEP of eye injected with Z-DEVD-FMK intravitreally at 1d postinjury; here the latency was distinctly prolonged as 81 ms and the amplitude was shortened as 5.19 μv; Panel C: VEP of eye injected with DMSO intravitreally at 7d postinjury; here the latency was distinctly prolonged as 111.5 ms and the amplitude was shortened as 4.37 μv; Panel D: VEP of eye injected with Z-DEVD-FMK peribulbarly at 7d postinjury; here the latency was distinctly shortened at 77 ms and the amplitude was 5.19 μv; Panel E: VEP of eye injected with Z-DEVD-FMK intravitreally at 14d post-injury; here the latency was distinctly shortened to 52.3 ms and the amplitude was 6.19 μv;

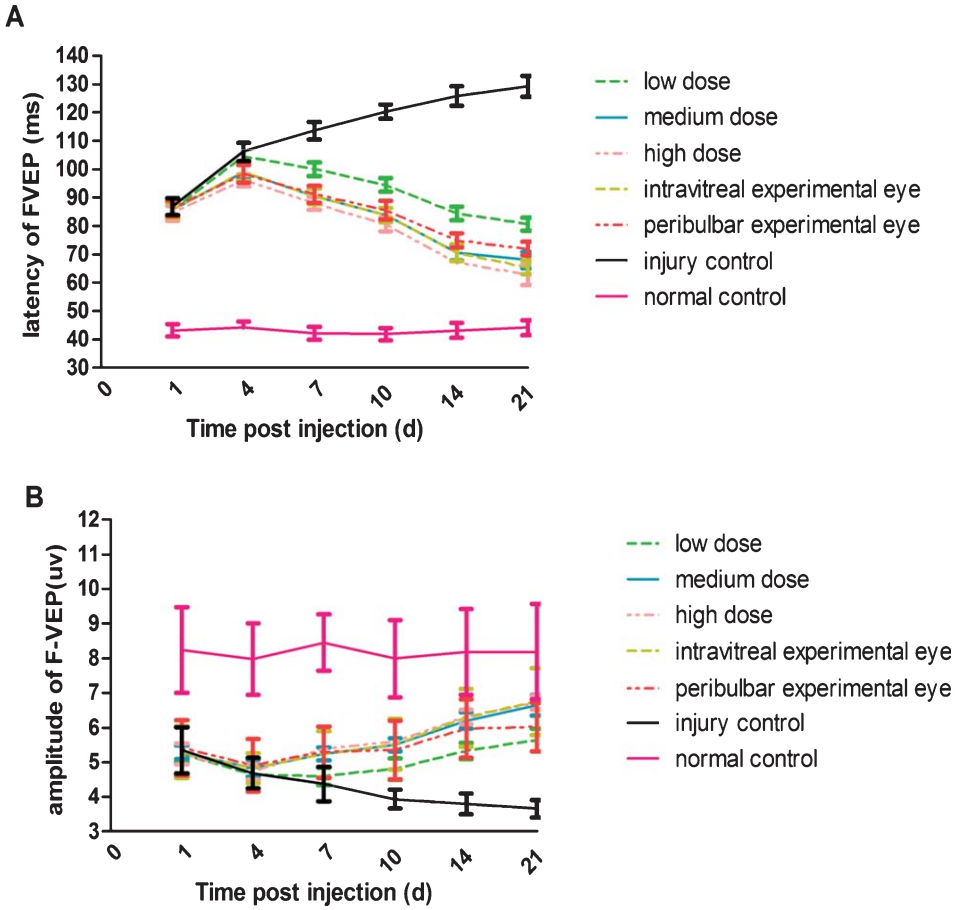

Fig. 2. Latency and amplitude of VEP of all groups. The graph displays the mean and standard error bars of the VEP recordings. Panel A: Latency of VEP of all groups. The latency of the injury control groups is prolonged over time and the decrease stabilizes at days 14 and 21. Latency of VEP of the experimental eyes treated with Z-DEVD-FMK are all prolonged at days 1 and 4 with a recovery trend from 7 d onward, stabilizing at 21d post-injury. Panel B: Amplitude of VEP of all groups. The amplitudes of injury control groups decreased over time and the decrease was stable at days 14 and 21. Amplitudes of VEP of all experimental groups treated with Z-DEVD-FMK decreased at days 1 and 4, with a recovery trend starting at 7d post-injury, stabilizing at 28d.

group ($p < 0.05$) and no difference between the medium vs. high dose group. However, as far the low dose group is concerned, a significant difference was observed in the latency and amplitude of VEP. The changes of VEP of group A is shown in Fig. 2 and Tables 1 and 2.

Group B: For the intravitreal injection group, the dominant wave latency of the experimental eyes showed a prolonged-recovery trend with the latency being shorter from day 7 while the injury control eyes showed a delayed recovery. The dominant wave latencies of the experimental eyes were gradually shorter at 7, 10, and 14d, and stabilized at 21d. Here, significant differences ($p < 0.05$) were observed between days 10 and 14, but no difference between 14 and 21d ($p < 0.05$). Compared to the injury control eyes there were evident differences ($p < 0.05$) at various times except at 1d post injury. Likewise, the results of the peribulbar injection group were comparable to the intravitreal one. In addition, significant differences ($t = 7.823$, $p < 0.05$) were observed between experimental eyes injected with Z-DEVD-FMK intravitreally and peribulbarly at 21d post-injury (Table 3). The amplitude changes were similar to those of the latency except that no difference was observed in experimental and injury control eyes at 4 day post injury ($p > 0.05$) (Table 4). The images of VEP of group B are shown in Figs.1 and 2.

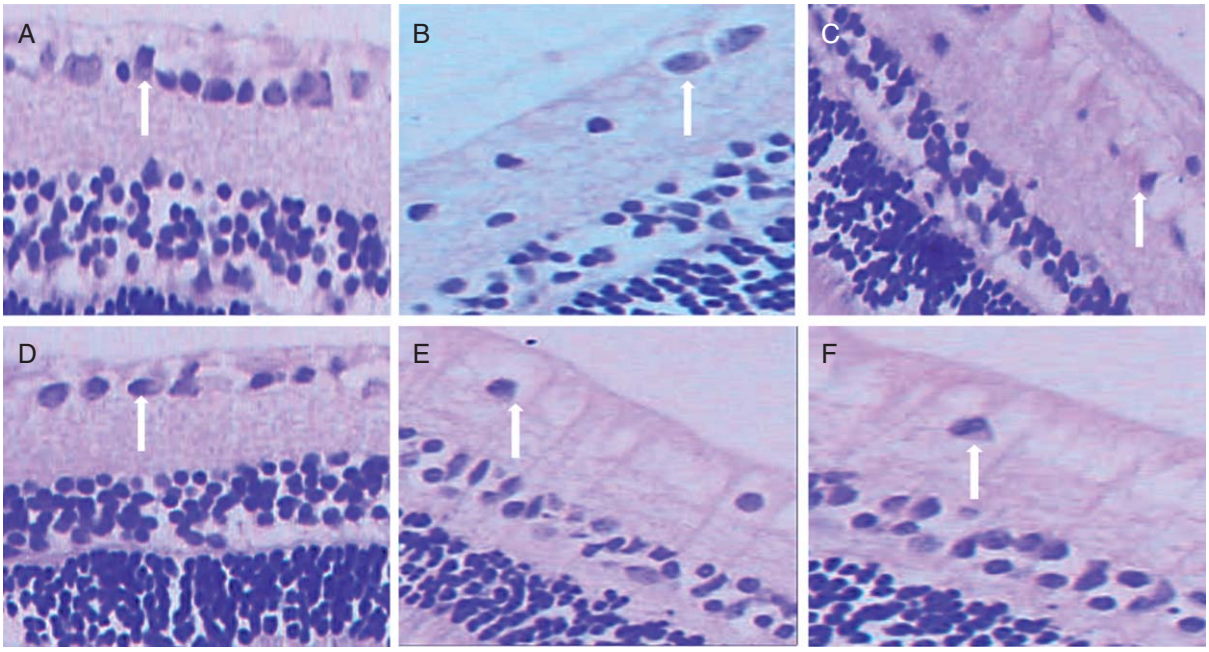

Fig. 3. Morphology of RGCs of rabbits in the middle dose group. Panel A: slight loss of the mesenchymal of RGCs in the experimental eyes at 4d post-injury shown by the white arrow; Panel D: slight loss of mesenchymal of RGCs in the injury control eyes at 4d post-injury shown by the white arrow, which was similar to the experimental eyes. Panel B: vacuolization of RGCs and margination of nuclear chromatin were noted in the experimental eyes at 14 post-injury. Panel E: decrease of RGCs, vacuolization of RGCs and margination of nuclear chromatin were more obvious in the injury control eyes at 14 post-injury than the experimental eyes. Panel C: the morphology of RGCs in the experimental eyes at 21d were the same to those at 14d post-injury. Panel F: the morphology of RGCs in the injury control eyes at 21d were the same to those at 14d post-injury.

3.3. Apoptosis of RGC detected by RGC counts and TUNEL

In the light microscope we observed a decrease of RGCs after injury, and injured RGCs showed vacuolization, loss of mesenchymal and margination of nuclear chromatin (Fig. 3). When compared to normal control eyes, RGC counts of injury control eyes declined slightly at 1d, and the decline increased from 4d on, with the percentage values of decline of 29% at 7d, 43% at 14d, and 47% at 21d. However, when compared to injury control eyes, in eyes treated with Z-DEVD-FMK the RGC counts showed no statistical difference ($p > 0.05$) in the low dose group; but at later time points the RGC counts showed an increased difference ($p < 0.05$) from 7d post injection in the medium dose group; a statistical increase ($p < 0.05$) was observed from 4 d post injection in the high dose group. RGCs were protected from apoptosis until the maximum study time of 21d post injection. The changes of RGC counts in the three dose groups were statistically different ($F = 16.425$, $p < 0.05$) as shown in Fig. 4. There was no statistical difference in the interaction of time and dose ($F = 1.304$, $p > 0.05$). Moreover, significant differences were observed among the three dose groups ($F = 59.798$, $p < 0.05$), and significant differences were seen between the low vs. medium and the low vs. high dose group ($p < 0.05$), with no difference between the medium vs. high dose group (Table 5). This contrasted what we have seen in the VEP examination, i.e. a recovery of visual functions. This means that there was a structural-functional mismatch (see discussion): the RGC number declined while the function improved.

In injury control animals, TUNEL-positive cells (Figs. 5 and 6) were observed at day 1, the number of which increased by day 4 and peaked at 7 day post-injury. Then they diminished gradually at 14d and 21d in the injury control eyes. However, in eyes injected with Z-DEVD-FMK at 2.5 μg/kg intravitreally or peribulbarly in group B few TUNEL-positive cells were detected at 1d, the number of which increased more slightly when compared to the injury control group. There was a statistically significant difference

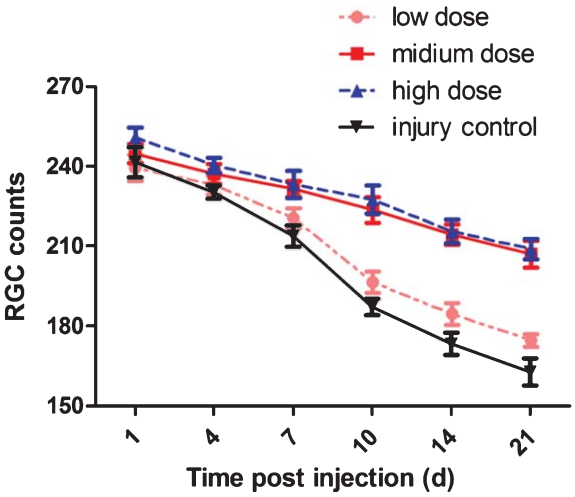

Fig. 4. RGC counts of the three dose groups. The graph displays the mean and standard error bars of the RGC counts. RGC counts of injury control eye declined slightly at 1d, and the decline exacerbated from 4d, and persisted to 21d. However, compared to the injury control eye, in the eye treated with Z-DEVD-FMK the RGC counts showed the same changing trend in the low dose group; the RGC counts increased from 7d post injection in the medium dose group, and persisted to 21d; the RGC counts increased from 4d post injection in the high dose group, and persisted to 21d. RGCs were protected from apoptosis until the maximum study time of 21d post injection, and the changing trend of RGC counts in the three dose groups are different.

of RGC apoptotic rate between the experimental and injury control eyes at all time points except for 1 day in both intravitreal and peribulbar groups. Furthermore, statistical differences ($p < 0.05$) were found between 1d/4d, 4d/7d, 7d/10d post-injury in experimental eyes of intravitreal and peribulbar group. However, no statistical difference ($p > 0.05$) was seen between 10d and 14d, and between 14d and 21d. Hence the RGC protection of Z-DEVD-FMK happened at 4d, peaking at 7d to 10d, and then persisted to 21d. The differences of RGC apoptotic rate between experimental eyes of intravitreal and peribulbar injection groups are evident at days 14 and 21 post-injury ($p < 0.05$, Table 6).

3.4. *Expression and activation of caspase-3 in retina tested with Western Blotting and Immunohistochemistry*

Caspase-3 precursor (32kDa) and its fragment (12kDa) was detected in retinae of both experimental and control eyes of group A (Fig. 8). The two isoforms of caspase-3 were up-regulated in the retina of the injury control eyes, while those of the experimental eyes were lower including the ratio of fragment/precursor. The expression of caspase-3 precursor and its fragment did not change over time. The

Table 5
RGCs count (Mean ± SD) of low (L), medium (M) and high (H) dose groups

Groups		Day post injury					
		1	4	7	10	14	21
group L	Experimental	239.52 ± 13.99	233.11 ± 10.18	220.70 ± 10.74	196.64 ± 8.42	184.77 ± 11.59	174.80 ± 6.97
	control	241.59 ± 15.95	230.28 ± 6.99	213.82 ± 11.43	187.2 ± 8.69	173.39 ± 11.83	162.79 ± 14.42
group M	Experimental	244.84 ± 10.26	237.25 ± 10.50	231.62 ± 8.30*	223.81 ± 13.74*	214.54 ± 10.72*	207.14 ± 14.30*
	control	240.15 ± 12.29	228.5 ± 8.41	209.98 ± 12.23	184.09 ± 9.69	170.81 ± 9.77	161.53 ± 8.5
group H	Experimental	250.95 ± 10.48	240.50 ± 7.94*	233.33 ± 14.38*	227.63 ± 14.90*	215.69 ± 12.58*	209.12 ± 10.97*
	control	244.71 ± 10.22	229.34 ± 14.63	209.21 ± 9.72	186.4 ± 7.51	168.51 ± 12.58	157.89 ± 8.21

Compared with the control group in the three dose group respectively, * = P < 0.05. Statistical difference was observed among the three dose group ($p = 0.000$). Evident differences were observed between the low vs. medium and the low vs. high dose group ($p < 0.05$). There was no statistical difference between the medium vs. high dose group ($p > 0.05$). SD = standard deviation.

Table 6
RGCs apoptotic rate (%) (Mean ± SD) of group B

Groups	Day post injury						
	1	4	7	10	14	21	*P*-value
Intravitreal experimental group	2.73 ± 0.85	5.54 ± 2.23■	10.14 ± 3.12■	15.61 ± 4.23■	17.86 ± 5.21■●	18.65 ± 5.43■●	0.000
Intravitreal control group	3.71 ± 1.05	10.32 ± 3.35	28.93 ± 7.87	35.76 ± 9.06	39.12 ± 10.14	44.11 ± 11.14	
Peribulbar experimental group	2.53 ± 0.96	4.95 ± 2.12▼	9.91 ± 3.73▼	14.86 ± 5.14▼	20.49 ± 6.83▼	21.91 ± 7.65▼	0.000
Peribulbar control group	3.73 ± 1.32	11.71 ± 14.28	29.53 ± 8.43	34.56 ± 9.45	39.54 ± 10.34	43.14 ± 12.27	

Compared with the intravitreal control group, ■ = P < 0.05, compared with the peribulbar control group, ▼ = P < 0.05, compared with the peribulbar experimental group, ● = P < 0.05. SD = standard deviation.

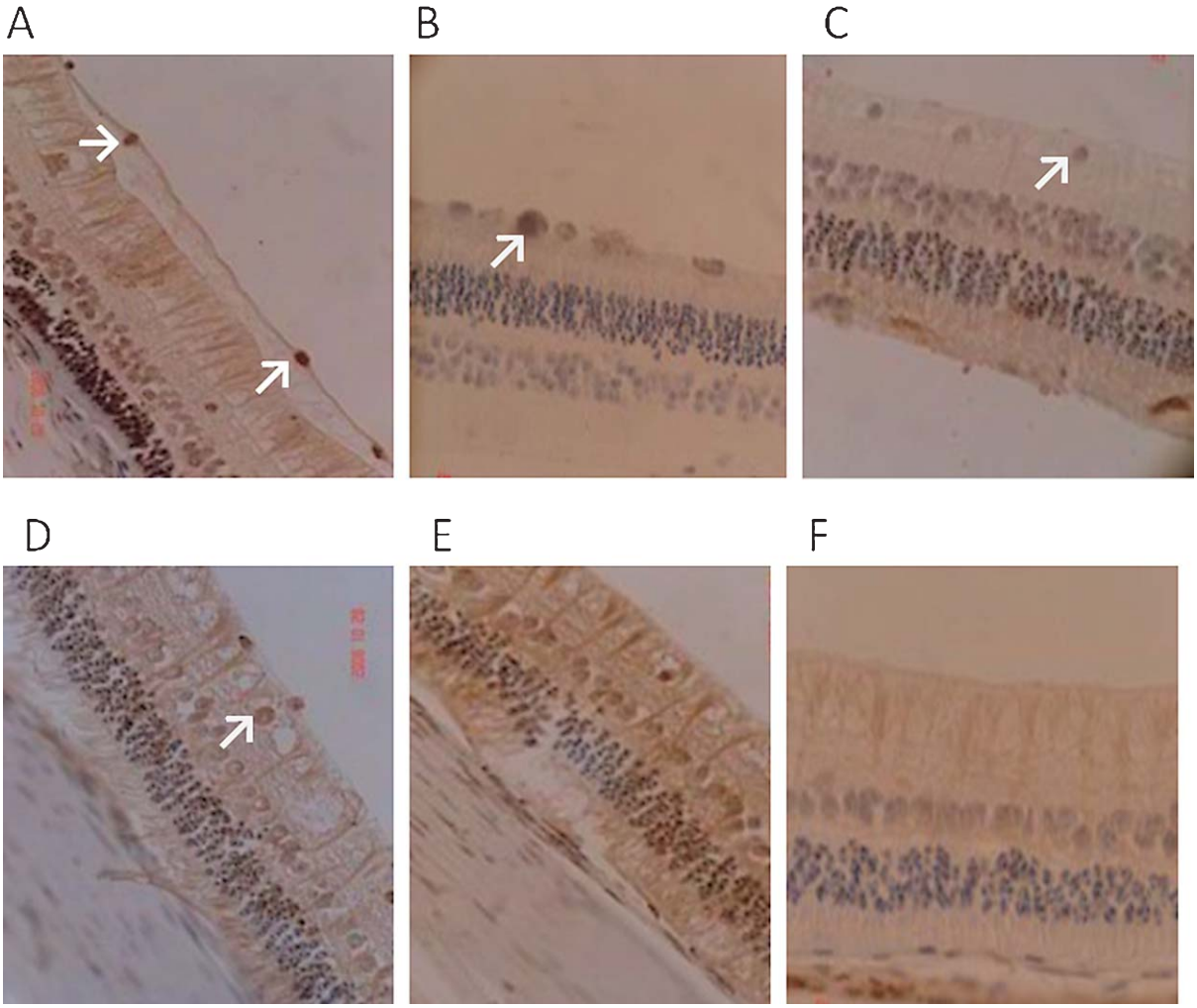

Fig. 5. TUNEL staining of RGCs of group B (TEM x400). TUNEL staining of RGCs of group B (TEM x400) (A) shows control eyes at 4d post-injury; a small number of TUNEL positive cells and apoptotic bodies (white arrow) were observed. (B) Injury control eyes at 7d post-injury; the number of apoptotic cells increased notably. (C) Injury control eyes at 21d post-injury. (D and E) Z-DEVD-FMK treated eyes at 4d and 7d post-injury: only a few TUNEL positive cells were observed. (F) TUNEL staining of treated eyes at 14d post-injury showing no apoptotic cells.

results indicated that Z-DEVD-FMK could efficiently block the expression and activation of caspase-3 which resulted in the inhibition of apoptosis.

In immunohistochemical slides, caspase-3 positive cells were detected as brown yellow nuclei and cytoplasm. They were mainly located in the ganglion cell layer, inner nuclear layer and outer nuclear layer after optic nerve injury. Caspase-3 was measured in retinae of both experimental and injury control eyes with damage. Caspase-3 positive cells (Fig. 7) were detected post-injury, the number and staining intensity of which increased from 1 to 14 days and were similar at 14 and 21 days post injury. A significant decrease was detected in experimental eyes compared to the injured controls from 7 to 21 days post-injury, especially at 10 day, but the differences were nearly identical to those at 14 and 21 days. Olympus 71 DP imaging analyzer with IPP software was applied to test the integral optical density of caspase-3 in immunohistochemical slides (Table 7). There was no obvious difference at 1 and 4 days post-injury ($p > 0.05$), while statistical differences ($p < 0.05$) were observed after 7 days especially at day 10, and this persisted to day 21. These results demonstrated that Z-DEVD-FMK inhibited the expression of caspase-3 starting from 7 day to 21 day post-injury, with the greatest effect was seen at day 10.

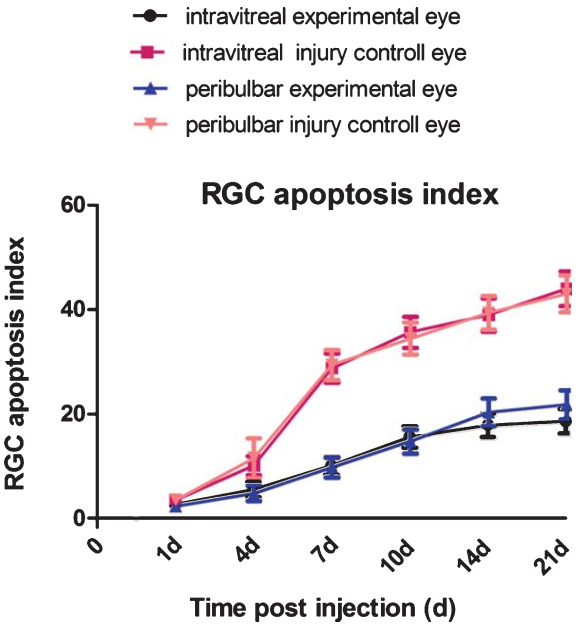

Fig. 6. Apoptosis index of RGCs of group B. The graph displays the mean and standard error bars of apoptosis index of RGCs. In injury control animals, the apoptosis index of RGCs increased from 1d post injury, peaked at 7 day post-injury, and persisted to 21d. However, in eyes injected with Z-DEVD-FMK at 2.5 μg/kg intravitreally or peribulbarly in group B the apoptosis index of RGCs increased from 1d post injury, but it increased more slightly, remaining stable from 14d to 21d post injection. The apoptosis index of RGCs in the intravitreally injected eyes is lower than the peribulbar group at 21d post injection.

4. Discussion

We investigated the anti-apoptotic and neuroprotective effect of Z-DEVD-FMK in a rabbit model of traumatic optic nerve injury. Z-DEVD-FMK decreased apoptotic rate of RGCs detected by RGC counts and TUNEL, and improved visual functions as shown by VEP. The neuroprotection of Z-DEVD-FMK revealed a time- and dose-dependent effect, i.e a typical threshold dosing effect (discussed below). The intravitreal injection had the most pronounced benefit: at a dose of >1.5 μg/kg, Z-DEVD-FMK protected the RGC and improved visual function from 7d ∼ 10d, and this persisted to 21d.

Further, we confirmed the validity of the new rabbit model of optic nerve injury with FPI. To date, two major animal models of optic nerve injury including direct and indirect model have been studied. Our rabbit model is "indirect" as it avoids direct damage to the optic nerve which has several advantages. Unlike another indirect model of traumatizing the rat brain (Wang et al., 2011; Levkovitch, 2004) our model is well reproducible and it better simulates the clinical traumatic optic nerve injury. In the "direct" model, on the other hand, the optic nerve is exposed and subsequently crushed or cut directly (Timothy et al., 2012; Levkovitch, 2004), which does not simulate the clinical situation where optic nerve injury is indirect. With our rabbit model, we can control the severity of injury rather well by adjusting the height of the hammer. Furthermore, as Yan et al. (2012) reported, the FPI induced optic nerve model has advantages of a low rate of failure, good quantification, maintaining the integrity of optic nerve, and little concomitant injury to the animal as it avoids vascular damage.

Although rodents are widely used for traumatic optic nerve injury animal models, the rabbit model first described by Solomon et al. (1996) has several advantages: First of all, FPI is feasible to induce traumatic optic nerve injury in an animal model. Secondly, the RGC numbers and anatomic structure of the rabbit optic nerve are closer to humans than mice or rats (Albrecht, 2008). Thirdly, owing to the relatively large ocular structures, the rabbit allows ease of surgical manipulation including intravitreal injection and provides more tissues for molecular studies. Lastly, rabbit eyes make it easier to observe light reflections, optic disc, ocular fundus, and pupil diameter and this helps to evaluate the degree of optic nerve injury. Consequently, the rabbit model is a well-suited model of traumatic optic nerve injury.

Using the rabbit model, we studied neuroprotection and recovery of visual function after optic nerve damage following Z-DEVD-FMK treatment by counting RGCs, apoptotic rate and VEP. Apoptosis of RGC was observable mostly in the first 2 weeks post-injury, especially at 7d, which is in line with the findings by the Sabel lab (Bien et al., 1999). This provides a long time window for possible anti-apoptotic therapeutic interventions for optic nerve injury. The RGC counts and apoptotic rate revealed that the protection of RGC induced by Z-DEVD-FMK was observable at 4d and more clearly at 10d, indicating that the effect is time-dependent. One reason accounting for this time-dependent effect might be that local inflammation during the early 10 days after injury may be reduced by Z-DEVD-FMK, as the treatment might influence various inflammatory factors responsible for RGC apoptosis by way of reducing caspase-3.

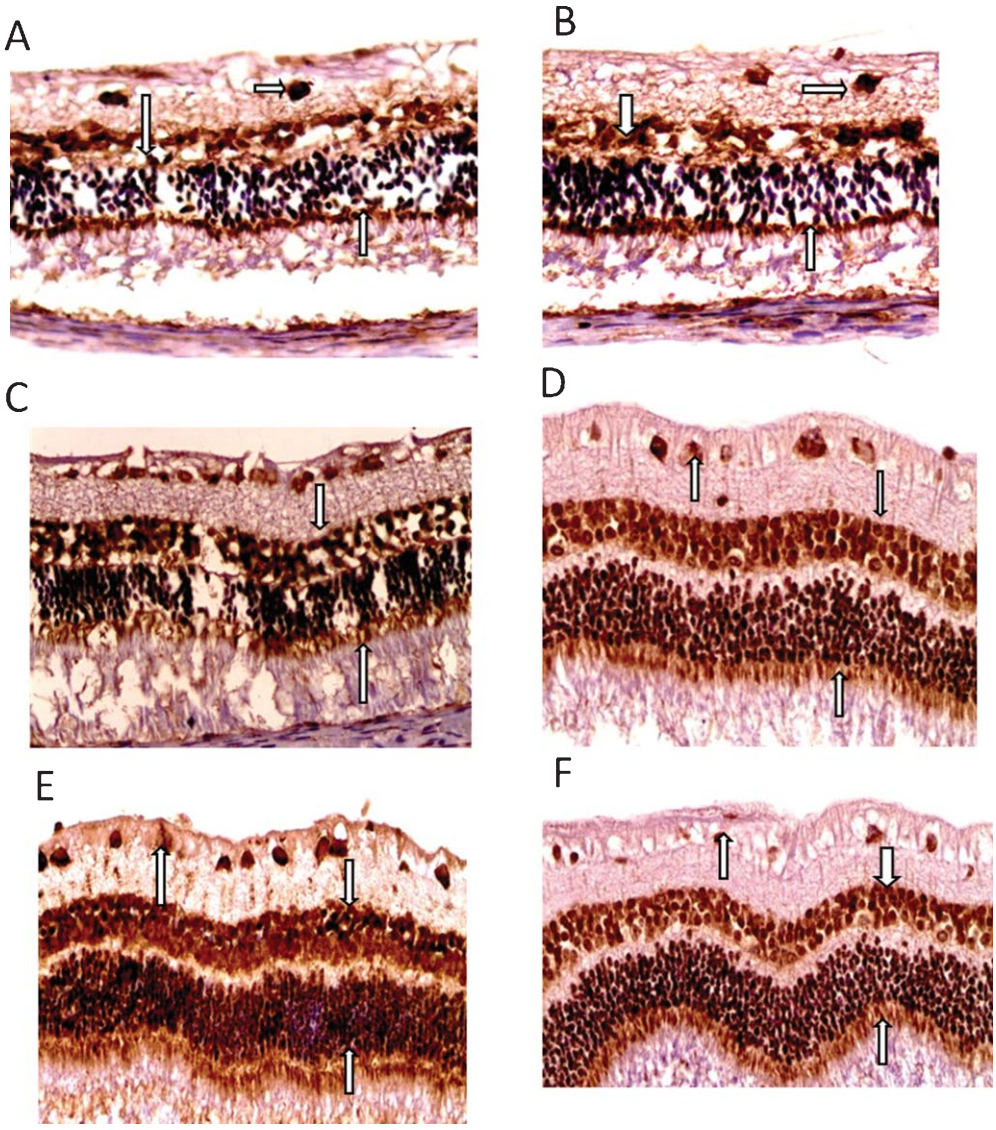

Fig. 7. Immunohistochemical staining of caspase-3 expression. Left panels: injury control; right panels: Z-DEVD-FMK treatment conditions. Parts A and B show typical staining results at 4d post-injury (x400). The white arrows show positive cells expressing caspase-3 detected in the RGC layer, inner nuclear layer and outer nuclear layer respectively with weak labeling; Parts C and D are taken from 10d post-injury. Here, white arrows show more positive cells in the RGC layer, inner nuclear layer and outer nuclear layer respectively with strong labeling, and the intensity of labeling and number of positive cells under Z-DEVD-FMK treatment conditions were lower than control(400x). Parts E and F were observed at 21d post-injury, positive cells with strong labeling were shown by white arrows (x400), the intensity and number of which were similar to those at 14d.

VEP is an important functional index to quantify optic nerve function clinically. It reflects the integrity of the visual system and the degree of injury after optic nerve damage in a sensitive manner (Bode et al., 2003). In the injured control eyes, the dominant wave latency was prolonged and the amplitude decreased gradually, so optic nerve conduction velocity was slowed down and the intensity of visual processing clearly weakened. The latency and amplitude of experimental (treated) eyes showed a recovery trend from day 7 onward. Several events may contribute to this situation: (i) reduction of edema of surrounding tissue affecting optic nerve conduction as indicated by our MRI monitoring (data not shown); (ii) the reduction of

Table 7
Integral optical density of caspase-3 (Mean ± SD) expressed in retina of medium dose group

Groups	Days post injury					
	1	4	7	10	14	21
Injury control	0.278 ± 0.014	0.288 ± 0.032	0.458 ± 0.024	0.403 ± 0.025	0.396 ± 0.032	0.394 ± 0.030
Experimental	0.264 ± 0.011	0.268 ± 0.029	0.396 ± 0.023	0.295 ± 0.012	0.303 ± 0.015	0.314 ± 0.016
P-value	0.845	0.432	0.014	0.000	0.002	0.006

SD = standard deviation.

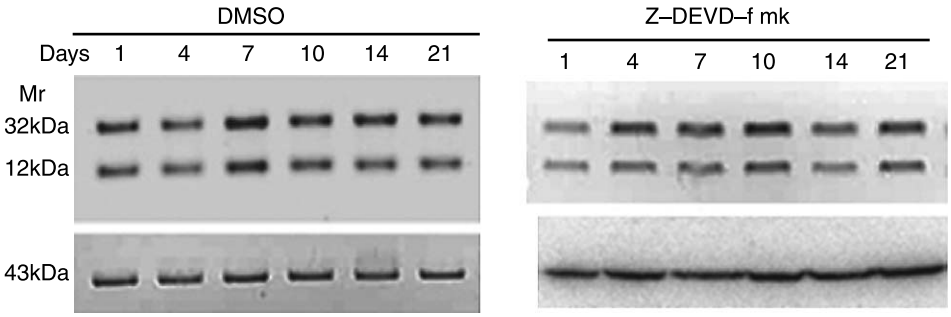

Fig. 8. Western blot analysis of retinal caspase-3 precursor (32kDa) and its fragment (12kDa). Western blot analysis of retinal caspase-3 precursor (32kDa) and its fragment (12kDa) expression after intravitreal DMSO and Z-DEVD-FMK administration at medium does of group A. In eyes injected with DMSO, expression of caspase-3 precursor and fragment increased on 1d, 4d, 7d post-injury, and presented a decreased trend from 10th day. Compared with the control eyes, expression of caspase-3 precursor and fragment presented at a lower level post-injury of Z-DEVD-FMK.

local inflammation, in which the anti-apoptotic effect of Z-DEVD-FMK takes some time to impact cell survival; or (iii) various inflammatory factors enhance the cell apoptosis, which aggravates secondary damage to the optic nerve at early stages after optic injury. The recovery trend peaked at 10-14d post-injury, which is in line with the natural course of recovery in rats (Sautter and Sabel, 1993). However, functional recovery was observed in all three dose groups. Thus, the caspase-3 inhibitor Z-DEVD-FMK can enhance RGC survival at 10d ∼ 14d post-injury and this protection persists at least till 21d post-injury.

We suspected that the VEP might be insensitive to the different degrees of injury in our model, but it should also be recognized that the number of surviving RGCs may not correlate well with functional parameters (Sabel et al., 2011a) which was also the case in the present study.

One explanation of this "structural-functional mismatch" is the brain's plasticity that attempts to compensate for the cell loss (Sabel, 2008; Sabel et al., 2011) and a possible involvement of top-down brain network influences on vision restoration (Bola et al. 2014). Sabel et al. (Rousseau et al., 2001) showed that after optic nerve injury, approximately 70% RGCs died after fast and massive soma swelling, while the rest undergo moderate soma swelling which contributed to RGCs survival and subsequent visual recovery. Meanwhile, VEP reflects the function of the whole retina, and so we suspect that the surviving RGCs partially compensate for the loss of apoptotic RGCs. Recovery of visual functions depends on the number of surviving RGCs at the early stage of injury but the minimum number of RGCs required for vision recovery may be very small: when as little as an estimated 10–20% RGCs survive and maintain a viable connection with their brain targets, visual functions could recover to some extent within 2 weeks after injury (Sabel, 1999). Thus, there is no one-to-one relationship between structure and function due to the dynamic changes in the damaged visual system, possibly because of brain plasticity. Clearly, more sensitive tests (such as behavioral performance measures) will help to establish the efficacy of this or any other drug treatment (Sabel et al., 2011b)

We also explored the intervention time, dose and best route of administration of Z-DEVD-FMK, aiming to provide insights for the treatment of traumatic optic nerve injury in the clinical context. The results of RGC counts suggest that Z-DEVD-FMK shows no obvious neuroprotection to RGCs at doses lower than 1.5 μg/kg Z-DEVD-FMK in rabbits, while intense anti-apoptotic effects were found with a dose higher than 1.5 μg/kg.

Hence, efficacy of Z-DEVD-FMK on RGC protection after traumatic optic nerve injury is dose-dependent. We conclude that a low dose inhibitor can't block the pro-apoptotic effect produced by inflammatory factors. Rather, the anti-apoptotic effect gradually increases with the dose, showing the character of a typical "dosing threshold". Despite this neuroprotection effect an increased dose did not lead to further improvements in VEP as seen Fig. 6 and Tables 5 and 6.

Likewise, a certain number of RGCs surviving the damage is the foundation of optic nerve recovery after injury. Furthermore, intravitreal injections had more persistent effects than peribulbar injections. It is possible that drugs injected peribulbarly diffuse more easily, which may limit the maintenance of a sufficiently high drug concentrations locally. In addition, much research has been conducted on the intervention time of caspase-3 inhibitors, and it was observed that apoptosis appears early following injury, at which pointed caspase-3 inhibitor must be administrated to reverse apoptosis. In fact, drug therapy given before rather than after injury may be more efficacy in the animal model of apoptosis (Sapieha et al., 2003; Martin et al., 2003, 2004). But selecting an administration time point of 30 min after injury simulates the clinical situation of traumatic optic nerve injury much better. Considering that the minimal time to obtain medical care in China is about 30 minutes, any clinical treatment needs to still be effective at least at this time point.

In addition, the changing levels of caspase-3 in the retina as shown immunohistochemically are similar to that of RGCs cell death. But the specific time course in both was not identical. The small difference might be ascribed to the autophagy pathway, some changes in specific extracellular matrix (ECM) components, a variety of genetic products induced by apoptotic genes including p53 and bax, and/or the elevation of calcium during apoptosis that does not rely on the caspase-3 inducing apoptotic pathway (Park et al., 2012; Li et al., 2005; Nikells, 1999; Prilloff et al., 2007, Koch et al., 2010).

When taken together, we demonstrated that the caspase-3 inhibitor Z-DEVD-FMK revealed clear neuroprotection accompanied by the down-regulation of caspase-3 expression, inhibition of RGC apoptosis and recovery of the optic nerve as shown by VEP, RGC morphology and counts, and RGC apoptosis. These converging results are compatible with the hypothesis that Z-DEVD-FMK improves recovery of optic nerve and visual function in the rabbit model of traumatic optic nerve injury. Moreover, the neuroprotection induced by Z-DEVD-FMK revealed a time- and does-dependent effect, and the intravitreal injection had the most pronounced benefit. Further studies are now needed to determine the time course of caspase-3 inhibitors and to determine the long-term survival of any RGCs rescued by Z-DEVD-FMK. Because of the positive results in our animal model we believe that the evaluation of efficacy and safety of the caspase-3 inhibitor Z-DEVD-FMK in first clinical trials may be feasible to determine if this may be a therapeutic option for the clinical management of acute traumatic optic nerve damage to enhance vision recovery and restoration.

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