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The Expression of PI3K/Akt Pathway and Related Apoptotic Proteins in Early Retinal Blast Injury in Mice

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Abstract: This research sought to dissect out the expression of the PI3K/Akt pathway- and retinal apoptosis-related proteins in response to early retinal blast injury in mice. Methods: C57BL/6 mice were randomly and equally arranged into a normal control and an experimental group. The control mice were fed conventionally, and the experimental mice were subjected to blast shock. After successful modeling, mice were assigned into a 6-h experimental group and a 48-h experimental group according to sampling time. The pathological changes of mouse retina, the number of retinal ganglion cells (RGCs)were assessed by hematoxylin-eosin staining, and the apoptosis of retinal tissue was measured by TUNEL assay. PI3K and Akt phosphorylation and the expression of Bax, Bcl-2, and Cytochrome C proteins were determined using Western blot analysis. Results: Relative to the control mice, the experimental mice had loose retinal structure, disordered cell arrangement, and decreased RGCs. The 48-h experimental group showed more obvious RGCs reduction than the 6-h experimental group. Moreover, the apoptosis of retinal cells was higher in the experimental mice than in the control mice, and the apoptosis positive cells diffused from the inner layer to the outer layer of the retina. In contrast to the control mice, PI3K and Akt phosphorylation and BAX, Bcl-2, and Cytochrome C protein expressions were potently elevated in the experimental mice, and they were higher in the 48-h experimental group than in the 6-h experimental group. BAX/ Bcl-2 expression was decreased in the experimental group relative to that in the control group. Conclusion: Conclusively, the PI3K/Akt pathway was involved in early retinal blast injury in mice, and its effect was related to the upregulation of PI3K and Akt phosphorylation and Bcl-2 expression.

Keywords: Retinal blast injury; PI3K/Akt pathway, Retinal cell apoptosis, Retinal ganglion cells, BAX, Bcl-2.

1. Instruction

Ocular blast injury is a prevalent disease among victims injured in blasts, and the extensively used explosive weapons in military conflicts and terrorist attacks contribute to significant increased incidence of bomb-related blast injury [1]. The retina, as an extension of the central nervous system, is more vulnerable to injuries and can be utilized as a more sensitive indicator of blast injuries [2]. Therefore, it is of great value to understand the retinal injury, inflammation, and neurodegeneration caused by blast waves for diagnosis and treatment. As a critical pathway for cell survival, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is involved in oxidative stress, inflammation, and apoptosis in the organism [[3][4]. The function of this pathway has been extensively researched in diabetic retinopathy and age-related macular degeneration. However, little is known about the mechanism of the PI3K/Akt pathway in early retinal blast injury. Hence, this research was intended to preliminarily measure the PI3K/Akt pathway-related protein expressions in mouse retina following blast injuries and to lay a foundation for further research on this pathway.

2. Materials and Methods

Ethics statement

This research was performed under ratification of animal ethics committee of our hospital. Extensive efforts were made to ensure minimal suffering of the animals included.

Experimental animals and groups

Sixty healthy male C57BL/6 mice (clean grade; 20-22 g) were supplied by the animal experimental center of General Hospital of Northern Theater Command of Chinese People's Liberation Army (Shenyang, China), all of which were housed in clean experimental animal rooms and acclimated for 1 week. The mice were randomly assigned into the control group and experimental group, and mice in the experimental group were grouped into a 6-h experimental group and a 48-h experimental group based on the sampling time.

Experimental reagents and instruments

The reagents and instruments utilized in our research consisted of a protein extraction kit (FUDE Biological Technology Co., Ltd., Hangzhou, China), a bicinchoninic acid (BCA) protein quantification kit (FUDE Biological Technology Co., Ltd.), a TdT-mediated dUTP-biotin nick end-labeling (TUNEL) kit, antibodies (Abcam, Cambridge, UK) to PI3K, Akt, phosphorylation (p)-Akt, p-PI3K, Bcl-2-Associated X (Bax), B-cell lymphoma-2 (Bcl-2), Cytochrome C, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a Constant temperature shaking table (YXQ-LS-50A, Changsha Yingtai Instrument Co., Ltd., Changsha, China), electrogenerated chemiluminescence (ECL) western blotting luminescent reagents, a microplate reader (iMark, Bio-Rad, Hercules, CA, USA), a gel imaging system (Dophin-1D), an embedding machine (4715, Nihonseiki Co., Ltd., Osaka, Japan), a microtome (RM2145, Leica , Nussloch, Germany), a paraffin section cooling table (Zh-3, Leica).

Mouse model establishment

C57BL/6 mice aged 6-8 weeks were anesthetized intraperitoneally using pentobarbital sodium and placed in the mesh area of the high-simulated blast injury model device [[3]. The mouse head was exposed, and the other parts of the mice were protected and adequately fixed. An aluminum slice with

a thickness of about 0.8 mm was placed in the middle of high-simulated blast injury model device, and an air compression device was placed below. After the device was powered on, when the pressure reached the critical level, it could lead to the rupture of the aluminum film, and its blast wave could cause retinal injuries in mice (the air compression device showed the pressure of about 0. 11-0.14 MPa.

hematoxylin-eosin (HE) staining

The mouse eyeballs were completely enucleated and placed in modified formaldehyde-acetic acid (FAA) fixative for 30-60 min. The eyeballs were opened at the limbus and soaked in FAA for 24 h. Then, the eyeballs were routinely dehydrated, waxed, and sectioned. The paraffin sections were baked in a 60℃ oven for 1-2 h, conventionally dewaxed with xylene and ethanol, and stained with hematoxylin for 10 min. The sections were rinsed with tap water to remove the residual color, followed by differentiation with 0.7% hydrochloric alcohol for 3 s and water washing. After eosin staining for 90 s, the sections were immersed in I 95% ethanol for 30 s, in II 95% ethanol for 3 min, in I absolute ethanol for 3 min, in II absolute ethanol for 5 min, in I xylene for 5 min, and in II xylene for 5 min, and mounted using neutral gum. After the microscopical observation of retinal pathological changes , the number of retinal ganglion cells (RGCs) were calculated.

TUNEL staining

The paraffin sections were routinely deparaffinized, dropped with 3% Triton X-100 for 10-min water bath, and rinsed twice in phosphate buffer saline (PBS, 5 min for each time). The TUNEL reaction solution was added dropwise into the section for 1-h incubation in the dark at 37°C. Following 3 PBS washes, the sections were mounted with 4',6-Diamidino-2-Phenylindole, photographed, and observed. The Image J software was employed for counting apoptotic cell numbers and the apoptotic rate (AI) was calculated.(AI = apoptotic cell number/total cell number × 100%)

Western blot analysis

Mouse retinal tissues were cut into pieces, and lysed thoroughly with lysis solution. Then, the tissues were broken using an ultrasonic pulverize, homogenized, and centrifuged (12000 r/min, 4°C, 15 min), followed by removal of the precipitate. The protein concentration of the tissue fluid was quantified by the BCA method and balanced, and then the samples were placed at -80°C for use. Subsequent to the preparation of 10% polyacrylamide gels, proteins were electrophoresed, and then electro-blotted into polyvinylidene fluoride membranes. The membranes were cut according to the molecular weight of the target proteins, and hybridized (overnight, 4°C) with primary antibodies against GAPDH, PI3K, Akt, p-PI3K, p-Akt, Bcl-2, Bax, and Cytochrome C, followed by 2-h incubation with secondary antibodies and then 3 washes with phosphate-buffered saline with Tween 20 (5 min each). A liquid and B liquid from the ECL kit were mixed at the ratio of 1:1 for luminescence detection. Western blot images were analyzed with the Image J software. The relative expressions of target proteins and internal reference were calculated.

Statistical analysis

SPSS 25.0 was applied to analyze statistical data, with $p < 0.05$ expressing statistically significant difference. The data were represented as mean ± standard deviation. All experiments were done repeatedly at least 3 times. Data from each group were checked for homogeneity of variance by the Levene's method. Comparison between two groups was analyzed using the t-test.

3. Results

The retinal injury was facilitated in mice after modeling over time

As reflected by HE staining results, in the control group, the retinal structure was complete and layered with close cell arrangement. Relative to the control group, the experimental groups had notably reduced number of RGCs ($p < 0.05$) and potently increased retinal NFL thickness ($p < 0.05$), with condensed retinal nuclei and disordered cell arrangement. The number of RGCs was strikingly reduced in the 48-h experimental group, as compared to that in the 6-h experimental group ($p < 0.05$) (Figure 1A-C; Table 1).

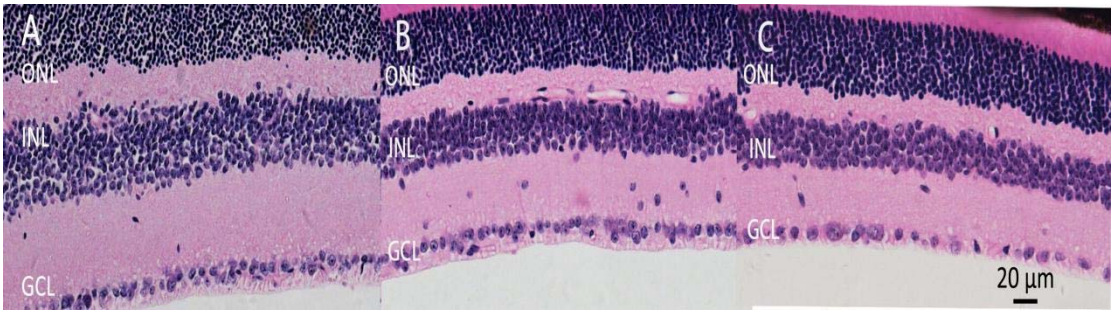

Figure 1. The results of HE staining of the control group (A), the 6-h experimental group (B), and the 48-h experimental group (C) (× 400).

Table 1. The number of retinal ganglion cell layer (RGCL) cells

Groups	The number of RGCL cells
The control group	46.66 ± 1.52
The 6-h experimental group	38.33 ± 2.05*
The 48-h experimental group	26 ± 3*

Note: * $p < 0.05$ vs. the control group.

Retinal apoptosis was accelerated in mice after modeling in a time-dependent manner

As manifested in Figure 2A-C, TUNEL positive cells (indicated by white arrows) were mainly distributed in retinal ganglion cell layer (RGCL) and inner nuclear layer (INL). Apoptotic cell numbers were counted using the Image J

software, which was converted to apoptotic rate. Retinal cells showed dramatically augmented apoptosis rate in the experimental groups than the control group ($p < 0.05$), and the apoptosis-positive cells in the experimental groups spread from the inner to the outer retinal layers. The apoptosis rate of retinal cells in the 48-h group was 4-fold more than that of the control group, showing a remarkable increase relative to that in the control group ($P<0.05$).

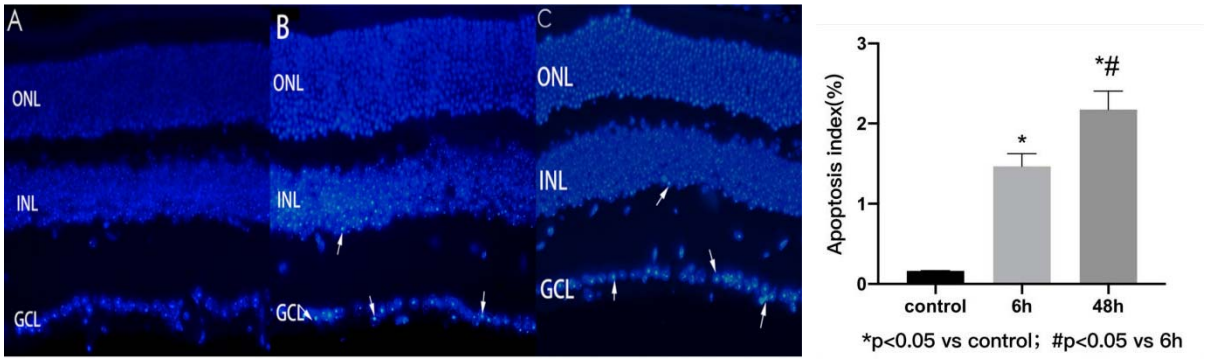

Figure 2. TUNEL staining results of the control group (A), the 6-h experimental group (B), and the 48-h experimental group (C) (TUNEL positive cells are indicated by arrows).
Note: * $p < 0.05$ vs. the control group; # $p < 0.05$ vs. the 6-h experimental group.

PI3K and Akt phosphorylation and Bax, Bcl-2, and Cytochrome C protein expressions were elevated in mice

p-Akt/Akt phosphorylation was considerably higher in the experimental groups than that in the control group ($p < 0.05$, Figure 3). Relative to the control group, p-Akt/Akt levels increased by 70% in 6-h group and p-Akt/Akt increased by almost 90% in 48-h group. The 48-h experimental group showed conspicuously enhanced p-PI3K phosphorylation compared to the 6-h experimental group ($p < 0.05$, Figure 3). Relative to the control group, the experimental groups exhibited elevated Bax, Bcl-2, and Cytochrome C protein expressions ($p < 0.05$). The same trends were observed in the 48-h experimental group versus the 6-h experimental group ($p < 0.05$, Figure 3). From all above, the elevated pro-apoptotic factors in the retina aggravates the retinal injury over time after detonation shock injury, which is consistent with TUNEL results, and the PI3K/AKT pathway is activated. It has been demonstrated that the PI3K/AKT pathway is crucial in resisting apoptosis. Bax/Bcl-2 is particularly important when the bcl-2 and Bax trends are consistent. In this study, Bax/Bcl-2 showed a decreasing trend at 6-h and 48-h, suggesting that early retinal knock injury contributed to the PI3K pathway activation to enhance cell resistance to apoptosis.

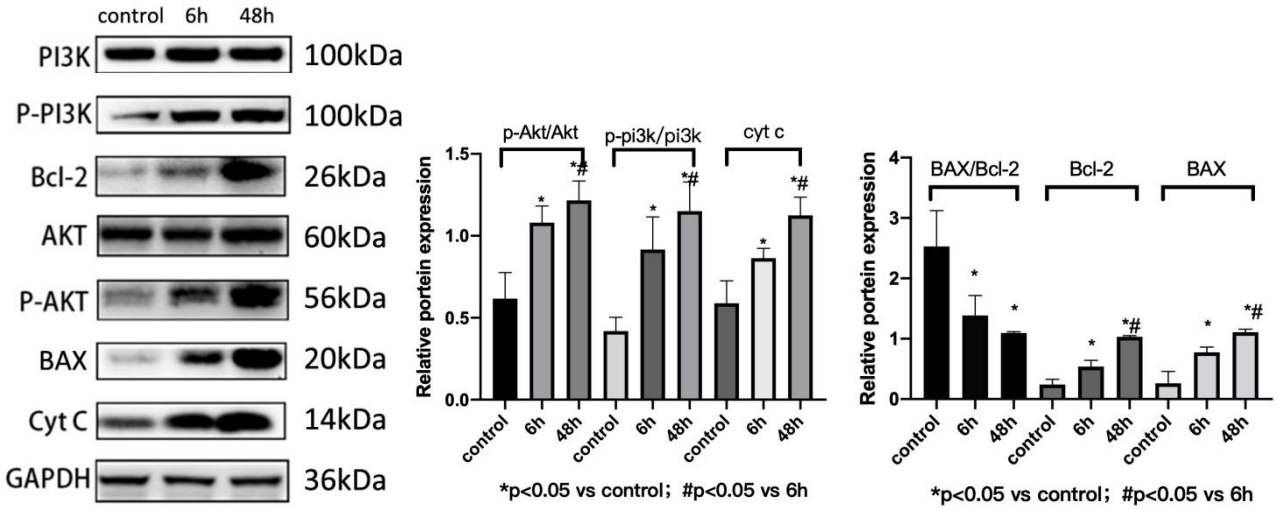

Figure 3. Western blot analysis results. A, Western blot analysis of phosphorylation of p-Akt/Akt, p-PI3K/PI3K and Cytochrome C in mice B, Western blot analysis of the expression of Bax, Bcl-2, and Bax/Bcl-2 proteins in mice.C
Note: * $p < 0.05$ vs. the control group; # $p < 0.05$ vs. the 6-h experimental group.

4. Discussion

In recent years, explosive devices have become the major weapons of terrorist attacks, which are relatively easy to manufacture and carry, making them the first choice for terrorists and insurgents. Blast brain injury has also become the most common war injury in current warfare [1] [5]. As has been pointed out, the shock force of the blast wave can trigger transient compression and ischemia, leading to axonal damage and death of RGCs [7]. The researches on blast injury models at home and abroad have mainly focused on the closed ocular injury induced by high compressed air [5]. In this research, a special military air compression device was adopted to fully expose the brains of mice, protect other parts of mice, and simulate primary craniocerebral blast injury [4]. Our research found that the structure of the tissue was loose and the cells were arranged in a disordered manner with reduced RGCs in the retina after blast shock, which was

consistent with previous studies. It has shown that levels of inflammatory and apoptotic markers are evidently increased 4 h subsequent to ocular exposure to blast waves [6]. In their report on retinal changes in a closed eye injury model, Blanch RJ et al. observed the enhancement of TUNEL labeling and apoptosis in retinal tissue after injury was induced by firing air gun bullets to the mouse eyes [7]. TUNEL-positive cells in the GCL and INL in the retina were observed as soon as 6h after the blast injury, and there was a substantial elevation in TUNEL-positive cells in the GCL and INL at 48 h, concurrent with previous findings. Cranial exposure to blast waves can contribute to retinal cell apoptosis, and the most sensitive layer to injury is GCL and INL, the injury of which will have a profound effect on visual function.

PI3K/Akt pathway is a classical pathway for cell survival [10]. Akt, also known as protein kinase B (PKB), has been confirmed to be one of the key downstream proteins of PI3K and is able to manipulate various biological processes, including cell survival, development, and glycogen metabolism [9]. The PI3K/Akt pathway is tightly involved in numerous ocular tumor development and progression. In a prior work, the PI3K/Akt/mTOR pathway activation/repression is tightly related to ocular abnormalities, like oxidative stress-induced RPE damage, RGC survival and axonal regeneration, and choroidal neovascularization [13]. Zhou et al. reported that in vitro, high glucose-induced retinal ischemia and hypoxia could activate the intracellular PI3K/Akt pathway expression and increase phosphorylation of Akt, a key protein in this pathway, which in turn led to retinal angiogenesis [14]. It has been documented that acute intraocular pressure elevation can mediate RGC survival through the PI3K/Akt pathway activation in the ganglion cell layer and inner nuclear layer [20]. Our investigation exhibited that phosphorylated PI3K and Akt expressions in mouse retina augmented conspicuously 48 h after blast shock. Activated Akt could phosphorylate Ser126/Ser112 residues of the pro-apoptotic protein Bad. Moreover, phosphorylated Bad depolymerized with Bcl-2 or Bcl-xl, and free Bcl-2 protein restrains apoptosis. In this experiment, the relative expression of Bax protein and the release of mitochondrial Cytochrome C protein increased 48 h following blast injury, confirming the process of retinal apoptosis, which was consistent with the relevant research results， but also activated the PI3K signaling pathway to enhance cell resistance to apoptosis.

In conclusion, the PI3K/Akt pathway is tightly associated with retinal injury and apoptosis following early blast injury. Therefore, it can be inferred that alteration of this pathway can provide reference for the treatment and prevention of retinal blast injury.

References

[1] Notghi B , Bhardwaj R , Nguyen T D . Biomechanical evaluations of injury risk for blast loading[J]. Journal of Biomechanical Engineering, 2017, 139(8).

[2] Mammadova N, Ghaisas S, Zenitsky G, et al. Lasting Retinal Injury in a Mouse Model of Blast-Induced Trauma. Am J Pathol. 2017;187(7):1459-1472.

[3] Wang H, Shen L, Ji X. Effects of DHA vitrectomy on photoreceptor apoptosis and PI3K/ Akt pathway in age-related macular degeneration rats [J]. International Eye Science,2019,19(12):2012-2016.

[4] Shi XY, Liu YE, Zhang YB, Tong CC, Shi L, Cong PF, Liu Y, Jin HX, Hou MX. Expression of PI3K/Akt pathway and related apoptotic proteins in mouse brain induced by traumatic shock [J]. Trauma and Critical Care Medicine,2017,5(04):215-219.

[5] Geisert, Eldon, E, et al. A mouse model of ocular blast injury that induces closed globe anterior and posterior pole damage (vol 99, pg 63, 2012)[J]. Experimental Eye Research, 2014.

[6] Jiang Y, Liu L, Pagadala J, Miller DD, Steinle JJ. Compound 49b protects against blast-induced retinal injury. J Neuroinflammation. 2013;10:96. Published 2013 Jul 30.

[7] Blanch RJ, Ahmed Z, Sik A, et al. Neuroretinal cell death in a murine model of closed globe injury: pathological and functional characterization. Invest Ophthalmol Vis Sci. 2012;53(11):7220-7226. Published 2012 Oct 17. doi:10.1167/iovs.12-9887

[8] Phillips BN, Chun DW, Colyer M. Closed globe macular injuries after blasts in combat. Retina. 2013;33(2):371-379. doi:10.1097/IAE.0b013e318261a726

[9] Huang JX, Bao CY, Li J. Research progress of PI3K/Akt pathway in diabetic cardiomyopathy [J]. Chinese Pharmacological Bulletin,2019,35(09):1202-1205

[10] Ge H, Zhou XW, Wang XB. Design,Synthesis and Antitumor Activity Evaluation of Novel PI3Kα Inhibitors [J]. Journal of Hubei University of Science and Technology (Medical Sciences),2019,33(04):281-286.

[11] Bricker-Anthony C, Hines-Beard J, D'Surney L, Rex TS. Exacerbation of blast-induced ocular trauma by an immune response [published correction appears in J Neuroinflammation. 2016;13(1):220]. J Neuroinflammation. 2014;11(1):192. Published 2014 Nov 29. doi:10.1186/s12974-014-0192-5

[12] Yang J, Tang YJ, Li WD, Li HJ, Kang S. Effect of Osthole in Inducing Apoptosis of Retinoblastoma Y79 Cells and Inhibiting Tumor Formation in Nude Mice by Inhibiting Activation of PI3K/AKT/mTOR [J]. Medical & Pharmaceutical Journal of Chinese People's Liberation Army,2020,32(08):15-19

[13] Jun, Zeng, Han, et al. DJ-1/PARK7 inhibits high glucose-induced oxidative stress to prevent retinal pericyte apoptosis via the PI3K/AKT/mTOR signaling pathway - ScienceDirect[J]. Experimental Eye Research, 189:107830-107830.

[14] Zhou SJ, Yu P, Yu DM. The effect of high glucose on PI3K-Akt signal pathway in RF/6A cells [J]. Chinese Journal of Clinicians (Electronic Edition),2012,6(04) :928-931.