• Basic Research •

Sensitized heat shock protein 27 induces retinal ganglion cells apoptosis in rat glaucoma model

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Abstract

● **AIM:** To investigate the relationships between the changes of heat shock protein 27 antibody (anti-HSP27) in serum/cerebrospinal fluid (CSF), intraocular pressure (IOP), retinal ganglion cell (RGC) apoptosis in a rat glaucoma model and disclose the underlying pathogenesis of glaucoma.

● **METHODS:** A total of 115 Wistar rats were randomly divided into 4 groups. Group 1 was the ocular hypertension group by condensing 3 episcleral & limbal veins or episcleral area of right eye (HP group, *n*=25) and sham operation group with conjunctiva incision without coagulation (*n*=25). Group 2: HSP27 or dose-matched PBS was injected into the vitreous (V-HSP27 group, *n*=15; V-PBS group, *n*=15). Group 3: HSP27 and complete Freund's adjuvant or dose-matched PBS was injected subcutaneously into the hind limb accompanied intraperitoneal injection of pertussis toxin [sensitized group (I-HSP27 group), *n*=15; I-PBS group, *n*=15)]. Group 4 was normal group without any treatment (*n*=5). IOPs of the rats were measured before, day 3, weeks 1, 2, 4, 6, and 8 after treatment. Paraffin-embedded sections were prepared for HE staining and RGCs apoptosis were detected by TUNEL. Anti-HSP27 level in serum and CSF were examined by ELISA.

● **RESULTS:** IOPs were elevated significantly in HP and V-HSP27, V-PBS groups (P<0.01) and positively related to anti-HSP27 levels in serum and CSFs. Anti-HSP27 levels in serum and CSF were elevated significantly in I-HSP27 group compared to other groups (P<0.05). However, the IOPs did not show any relationship with the high-level anti-HSP27 in serum and CSFs. RGC apoptosis were all elevated significantly in the HP, V-HSP27, V-PBS and I-HSP27 groups and also positively relative with anti-HSP27 level in serum and CSFs except that high-level of anti-HSP27 in the serum of I-HSP group.

● **CONCLUSION:** The increases of anti-HSP27 levels in serum and CSFs both promote IOP escalation and the increase of RGC apoptosis in retina when anti-HSP27 is at low level. The case of high-level anti-HSP27 is opposite and shows protective function in preventing IOP increase and RGC apoptosis.

● **KEYWORDS:** intraocular pressure; heat shock protein 27; retinal ganglion cells; autoimmune; apoptosis; glaucoma; rats

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INTRODUCTION

Eye is a meticulous organ with special immune environment which is established through the blood-aqueous barrier and the blood-retina barrier[1-2]. The immune system protects eyes from dangerous immune reactions, for example, it can wipe out activated cells that might invade the eye and destroy vision by doing damage to important structures such as the retina and local ocular defense system including vitreous cavity[3-4].

Heat shock proteins (HSP) belong to a family of proteins which are conservative in evolution and inducible by variety of stress in expression. Under physiological condition, one of many important functions is taken as molecular chaperones in protein translocation and folding[5-8].

HSPs are classified on the basis of molecular weight. HSP27 belongs to the member of small HSP family[1,9]. It was reported that the autoantibodies against HSP27 in the sera of patients with glaucoma, especially in patients with normal pressure glaucoma, were associated with the optic disease significantly[10-15].

Glaucoma is the second leading cause of vision loss in the world[16-19], which is characterized by gradual loss of retinal ganglion cells (RGCs) and progressive defects of visual field. Many mechanisms have been exploited to explain glaucoma, including disturbances of blood circulation, excitotoxicity and neurodegenration[20-23]. However, these mechanisms can only represent partial causes for glaucoma pathogenesis. In recent years, some studies indicated that autoimmune mechanism is also involved in the pathology of this disease. The antibodies in serum of glaucoma patients include HSPs[10,14,24-26], gamma-enolase[25], glutathione-s-transferase (GST)[27] and so on[28-31].

In our previous study[32-33], we built the rat model of chronic ocular hypertension model successfully. It was found that HSP27 level was up-regulated in retinal tissue and HSP27 antibody was also present in serum of rat. In addition, the concentration of HSP27 antibody showed proportional relation with hypertension. However, no antibody of HSP27 was detected in cerebrospinal fluid (CSF). Herein, the aim of the current study was to observe the change of HSP27 in serum and CSF with disease progress of rat glaucoma model.

METERIALS AND METHODS

Ethical Approval These animal studies adherence to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and have been also approved by the Ethics Committee of Kunming Medical University, Yunnan, China.

Animal Models Totally 115 Wistar rats (150-200 g) were obtained from the experimental animal center of Kunming Medical University. The rats were given free access to food and water in the quiet, light-free environment for one week.

Wistar rats were randomly divided into 4 groups. Group 1 was the ocular hypertension group by condensing 3 episcleral & limbal veins or episcleral area of right eye (HP group, *n*=25) and sham operation group with conjunctiva incision without coagulation (*n*=25). In details, rats were anesthetized with 846 mixture (0.1-0.15/kg, intramuscular injection). The cornea was anesthetized with 1 drop of oxybuprocaine hydrochloride (Santen Pharmaceutical Co.Ltd., Japan). The 3 vorticose veins and limbal veins were rinsed and condensed by using underwater bipolar electrocoagulation until the vascular tissue became white. Group 2: HSP27 (10 μL/50 μg; Victoria. BC Canada) or dose-matched PBS (10 μL) was injected into the vitreous (V-HSP27 group, *n*=15; V-PBS group, *n*=15). Group 3: HSP27 (100 μg) and complete Freund's adjuvant or dose-matched PBS (0.15 mL) was injected subcutaneously into the hind limb (0.1 mL in tail and 0.05 mL in each thigh) accompanied intaperitoneal injection of l μg pertussis toxin [Enzo Life Science, Inc., USA; sensitized group (I-HSP27 group), *n*=15; I-PBS group, *n*=15]. Group 4 was normal group without any treatment (*n*=5).

After treatment, rats were given erythromycin ointment and kept in room temperature. Then continually treated by ropping 0.25% chloramphenicol 3 times per day erythromycin ophthalmic at night for 5d. Intraocular pressure (IOP) of the rats were measured at day 3, weeks 1, 2, 4, 6, and 8 after treatment.

Sample Collection Rats were euthanized at the aforementioned time points. Paraffin-embedded sections were prepared for hematoxylin-eosin (HE) staining, and eyes were also harvested for RGCs apoptosis by TUNEL analysis. The level of anti-HSP27 in serum and CSFs were also measured by enzyme-linked immunosorbent assay (ELISA).

Enzyme-linked Immunosorbent Assay ELISA kit was obtained from Kirkegaard & Perry Laboratories, Inc. USA. The assay was performed following the manual. Briefly, HSP27 antigen (1 μg/mL, 100 μL) was added to wells followed by overnight incubation at 4°C. Then 100 μL of diluted serum/CSF was added to each well for 1.5h at 37°C. Secondary antibody incubated for 1h at 37°C. The plates were read at 405 nm with a plate reader.

TUNEL Assay The ApopTag® In Situ Apoptosis Detection Kits were obtained from Chemicon International Company. The TUNEL assay was performed as the manual described. In brief, the staining of apoptotic cells was performed on 8 μm frozen eye section. Cryosections were placed on gelatin-coated slides and fixed in 1% paraformaldehyde in PBS for 5min followed by post-fixation in precooled ethanol:acetic acid (2:1) solution for 5min at -20°C, quenching peroxidase by use of 3.0% hydrogen peroxide in PBS for 5min at room temperature. Working strength TdT enzyme 12 μL was added, incubating in a humidified chamber at 37°C for 1h, then incubating the slide in dH_2O for 5min. TUNEL-positive cells were viewed under the microscope Nikon 320 and photographed. The number of TUNEL-positive nuclei in the retinal ganglion cells layer (RGCL) was counted by light microscopy, and the data were expressed as number of TUNEL-positive nuclei per section.

Statistical Analysis All data are presented as the mean± standard deviation (SD). IOP and the level of anti-HSP27 data were performed using the variance analysis. The relationship between IOP, RGC apoptosis and anti-HSP27 were determined by regression analysis. The data from the TUNEL assay was analyzed with paired *t*-test using the SPSS 11.5 software. $P<0.05$ was considered to be statistically significant.

RESULTS

The Change of Intraocular Pressure IOPs increased significantly in right eyes of HP and vitreous cavity immunized group including V-HSP27 and V-PBS groups at all time points ($P<0.05$), compared to corresponding control left eyes (Table 1). However, the results of sham, I-HSP27, I-PBS and normal group were opposite (Table 1). In addition, IOPs of all left eyes

Table 1 The change of IOP mean±SD, mm Hg

Time	HP		Sham		V-HSP27		V-PBS		I-HSP27		I-PBS		Normal	
	R	L	R	L	R	L	R	L	R	L	R	L	R	L
Pre	14.60±0.89	14.30±1.10	14.20±1.30	14.70±0.80	15.20±0.84	14.20±1.20	14.00±1.00	13.86±1.00	13.93±1.33	14.23±1.00	14.53±1.02	13.90±1.40	14.40±1.14	13.90±1.10
3d	17.80±0.84[a]	14.00±1.40	15.00±1.00	15.00±1.10	17.00±0.71[a]	14.32±1.00	17.60±0.54[a]	13.70±1.31	13.40±0.91	15.32±0.64	14.20±0.31	15.00±0.80	14.00±0.71	14.00±0.90
1wk	19.80±0.84[a]	14.30±0.95	14.80±0.83	13.80±1.83	19.80±1.48[a]	15.10±0.73	19.60±0.89[a]	14.10±1.50	14.00±1.58	15.00±0.83	14.20±1.38	14.50±1.23	15.20±1.48	14.50±1.05
2wk	27.83±0.98[a]	14.97±0.70	15.17±0.75	15.04±0.65	26.67±2.87[a]	14.04±1.62	27.67±1.63[a]	14.29±0.75	14.33±0.65	14.14±1.32	13.93±0.65	14.60±1.00	13.83±0.75	14.87±0.40
4wk	31.00±1.58[a]	15.10±0.31	15.00±0.71	14.90±0.56	30.00±0.71[a]	14.90±0.43	31.20±1.64[a]	14.90±0.44	15.20±0.83	14.89±0.63	15.60±0.89	15.00±0.51	15.60±0.89	15.00±0.71
6wk	31.50±1.29[a]	14.50±1.57	15.50±0.57	15.20±0.59	29.75±0.95[a]	14.90±0.63	31.50±1.29[a]	14.98±0.63	13.98±0.93	14.00±1.13	14.90±1.23	15.20±0.57	15.00±1.15	14.63±1.07
8wk	32.80±0.84[a]	13.98±1.54	14.40±1.14	14.43±1.04	31.8±1.30[a]	14.73±0.72	32.2±0.83[a]	14.90±1.00	15.00±0.82	15.03±0.63	15.10±0.84	14.58±1.03	14.40±1.14	13.79±1.50

HP: Ocular hypertension group; V: Vitreous cavity immunized group; I: Sensitized group; R: Right eye; L: Left eye. [a]P<0.05.

did not show any statistically significant change in each group (Table 1).

The Change of anti-HSP27 Level in CSF/serum Table 2 showed that the level of anti-HSP27 in rat serum of I-HSP27 group increased significantly while any change of anti-HSP27 level was found in control I-PBS group. However, the higher concentration of anti-HSP27 in I-HSP27 group showed decrease as the time passed after injection of the antibody. Moreover, anti-HSP27 levels showed a gradual rise as time passed after surgery in HP, V-HSP27 and V-PBS groups compared to those in control sham group (Figure 1). Sham, I-PBS and normal group did not display the fluctuation of anti-HSP27 level in rat serum. The anti-HSP27 levels in CSF also showed a gradual rise as time passed after operations in HP, V-HSP27 and I-HSP27 group while did not in sham, V-PBS, I-PBS and normal groups (Table 3). Additionally, in each group, the anti-HSP27 levels in rat CSFs were obviously lower than those in rat serum.

The Correlations Between IOP and anti-HSP27 in Serum and CSF In HP group, anti-HSP27 levels in both rat serum and CSF were elevated along with the increase of IOP (Figure 1). There was positive correlation between IOP and anti-HSP27 levels in serum and CSF (R^2=0.74, P=0.06; R^2=0.80, P=0.019, respectively; Figure 1). IOP in control sham group were not associated with anti-HSP27 (R^2=0.00008, P=0.5; R^2=0.0196, P=0.5 repectively; Figure 2A-2C). There were also positive correlation between IOPs and anti-HSP27 levels in serum and CSF of V-HSP27 (R^2=0.94, P=0.0069; R^2=0.96, P=0.0039, respectively; Figure 2D-2F) and V-PBS group (R^2=0.91, P=0.01; R^2=0.89, P=0.015 respectively; Figure 2G-2I). However, in I-HSP27 group, IOP were not correlated with anti-HSP27 levels in rat serum and CSFs although their anti-HSP27 levels were significantly higher than those of other groups (Figure 2J-2L). There was no correlation between IOPs and anti-HSP27 levels in in I-PBS and normal groups (Figure 2M-2S).

The Apoptosis of Retinal Ganglion Cells in Rat Apoptotic cells with brown positive signals were observed in both RGCL and the inner nuclear layer (INL; Figure 3). The apoptotic cells were observed in a higher level in HP, V-HSP27, V-PBS and I-HSP27 groups than in normal group (Figures 3, 4). The positive rate of RGCs apoptosis was at a peak at week 8 in HP, V-HSP27 and V-PBS group while at week 6 in I-HSP27 group after subcutaneous injection of HSP27. The apoptotic RGCs of V-HSP27 group were higher than normal group and lower than HP group at all time points. Apoptosis of RGCs was not observed in sham, I-PBS, and normal group.

Anti-HSP27 levels in both rat serum and CSFs showed similar change with RGC apoptosis as time passed in HP group (Figure 5A). The anti-HSP27 level in rat serum and CSFs was

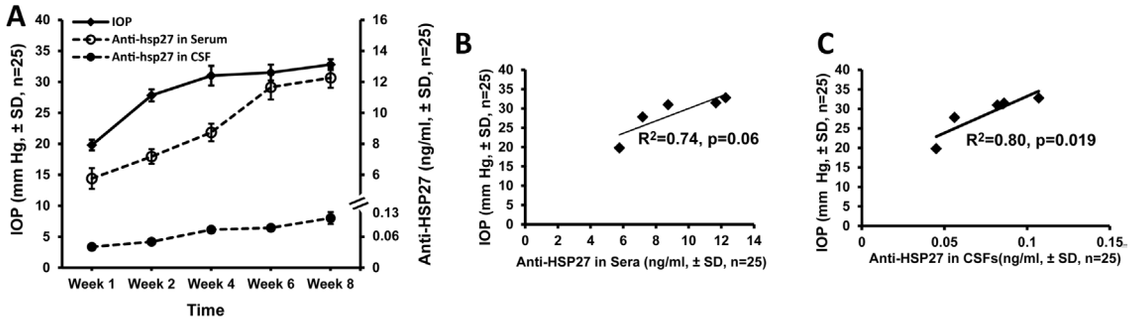

Figure 1 The relationship between IOP and anti-HSP27 in the HP group A: IOPs, anti-HSP27 levels in serum and anti-HSP27 levels in CSFs at weeks 1, 2, 4, 6, and 8 after treatment; B: The regression analysis of relationship between IOP and anti-HSP27 level in rat serum; C: The regression analysis of relationship between IOP and anti-HSP27 level in rat CSFs.

Table 2 The change of anti-HSP27 in serum mean±SD, ng/mL

Groups	Week 1	Week 2	Week 4	Week 6	Week 8
HP	5.76±0.67	7.18±0.47	8.74±0.57	11.66±0.80	12.26±0.64
Sham	5.20±0.79	5.54±0.55	5.75±0.47	5.51±0.32	5.62±0.34
V-HSP27	6.08±0.13	8.89±1.02	10.78±0.21	12.10±1.43	12.98±0.99
V-PBS	5.03±0.24	5.63±0.34	6.07±0.14	6.22±0.19	6.56±0.23
I-HSP27	74311.12±4017.26[b]	68103.32±3856.72[b]	64209.21±3257.25[b]	38389.91±2116.31[b]	29928.13±1157.30[b]
I-PBS	5.36±0.15	5.33±0.25	5.31±0.20	5.31±0.40	5.37±0.55
Nomal	5.33±0.20	5.33±0.20	5.33±0.20	5.33±0.20	5.33±0.20

HP: Ocular hypertension group; V: Vitreous cavity immunized group; I: Sensitized group. [b]P<0.01.

Table 3 The change of anti-HSP27 in CSF mean±SD, ng/mL

Groups	Week 1	Week 2	Week 4	Week 6	Week 8
HP	0.045±0.004	0.056±0.004	0.082±0.002	0.086±0.002	0.107±0.013
Sham	0.047±0.003	0.048±0.002	0.051±0.002	0.050±0.003	0.051±0.004
V-HSP27	0.040±0.004	0.070±0.002	0.082±0.003	0.085±0.003	0.104±0.015
V-PBS	0.046±0.003	0.053±0.002	0.055±0.002	0.059±0.002	0.061±0.002
I-HSP27	0.251±0.015[b]	0.168±0.027[b]	0.143±0.014[b]	0.331±0.026[b]	0.159±0.040[b]
I-PBS	0.050±0.002	0.050±0.003	0.050±0.003	0.051±0.002	0.050±0.003
Normal	0.052±0.004	0.052±0.004	0.052±0.004	0.052±0.004	0.052±0.004

HP: Ocular hypertension group; V: Vitreous cavity immunized group; I: Sensitized group. [b]P<0.01.

positively associated with RGC apoptosis R^2=0.92, P=0.009; R^2=0.97, P=0.002, respectively; Figure 5B, 5C) as well as in V-HSP27 and V-PBS groups (R^2=0.92; P=0.009; R^2=0.92, P=0.01, in V-HSP27 group; R^2=0.97, P=0.002; R^2=0.93, P=0.008 in V-PBS group Figure 5H, 5I, 5K, 5L). Anti-HSP27 level in both serum and CSFs showed similar fluctuation with RGC apoptosis in the two vitreous cavity damaged-associated groups (Figure 5G, 5J). In I-HSP27 group, the RGC apoptosis did not show any relationship with anti-HSP27 level in serum (R^2=0.36, P=0.28; Figure 5M, 5N) while there was positive relationship in CSFs (R^2=0.79, P=0.04; Figure 5O). Further statistical analysis demonstrated no relationship between anti-HSP27 and RGC apoptosis in other groups (Figure 5D-5F, 5P-5S).

DISCUSSION

Chronic hypertension model of rats induced by HSP27 showed that glaucoma is associated with autoimmune reaction. In our study, we successfully built the chronic hypertension glaucoma model. As is well known, there are two forms of the glaucomatous optic neuropathy: primary open-angle glaucoma (POAG) and normal-tension glaucoma (NTG). Furthermore, it has been well evidenced that glaucoma is strictly associated with IOP, however, the change of IOP is only one main cause of glaucoma in which many factors are involved. Here, we proved that anti-HSP27 should also be an important cause of glaucoma as a key for autoimmune reactions.

HSPs are widespread throughout the biosphere, from bacteria to human beings. They are conservative in evolution. They share similar structure in different species (80 percent similarity can be achieved). HSPs exist in variety of pathogenic microorganisms. They stand for dominant antigen in the immune system and are undoubtedly a big challenge to immune system[8,34]. Tezel *et al*[12] found the autoantibody of HSP27 in glaucoma patients' sera. HSP27 belongs to small

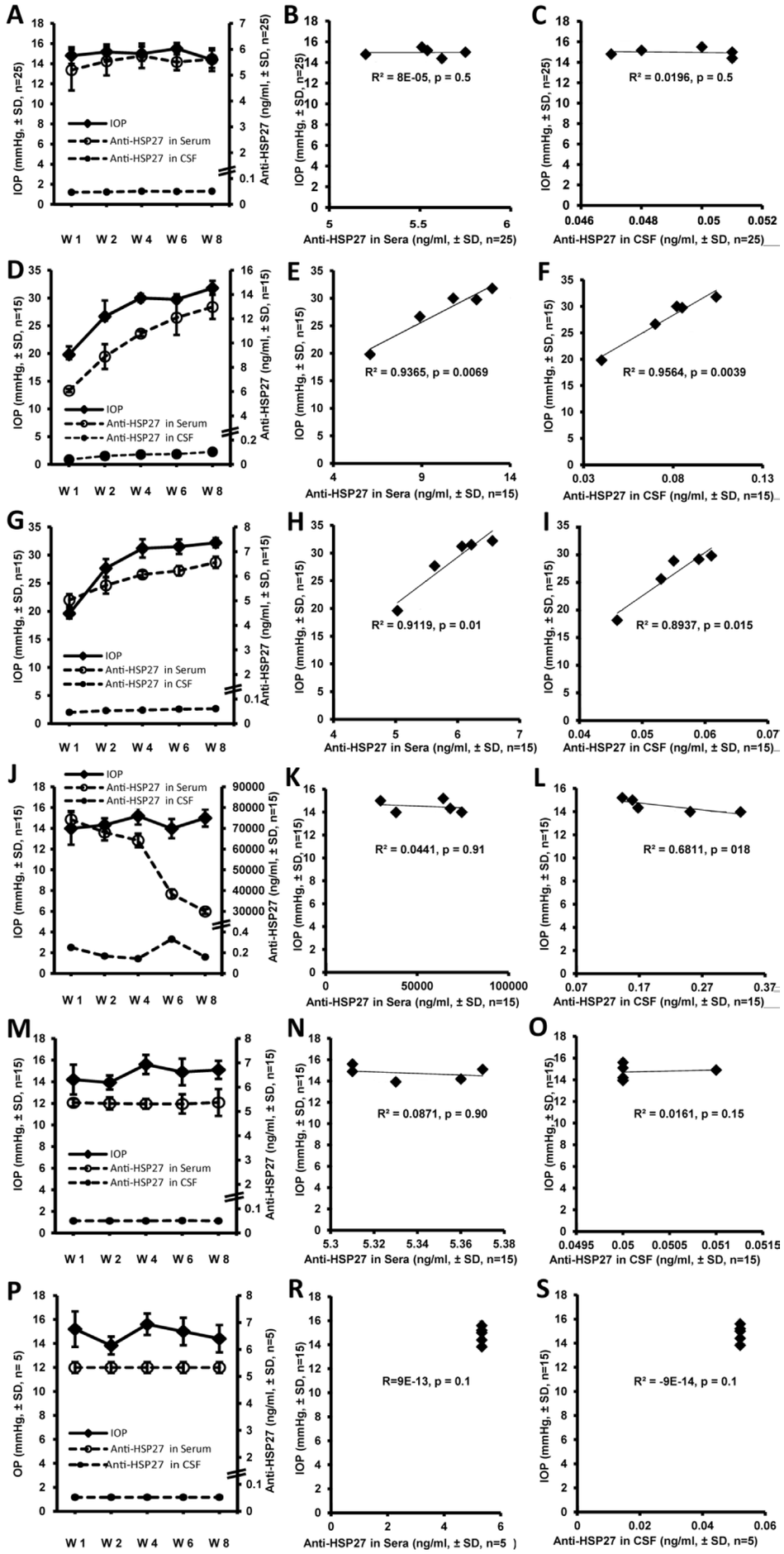

Figure 2 The relationship between IOP and anti-HSP27 in rat serum and CSFs.

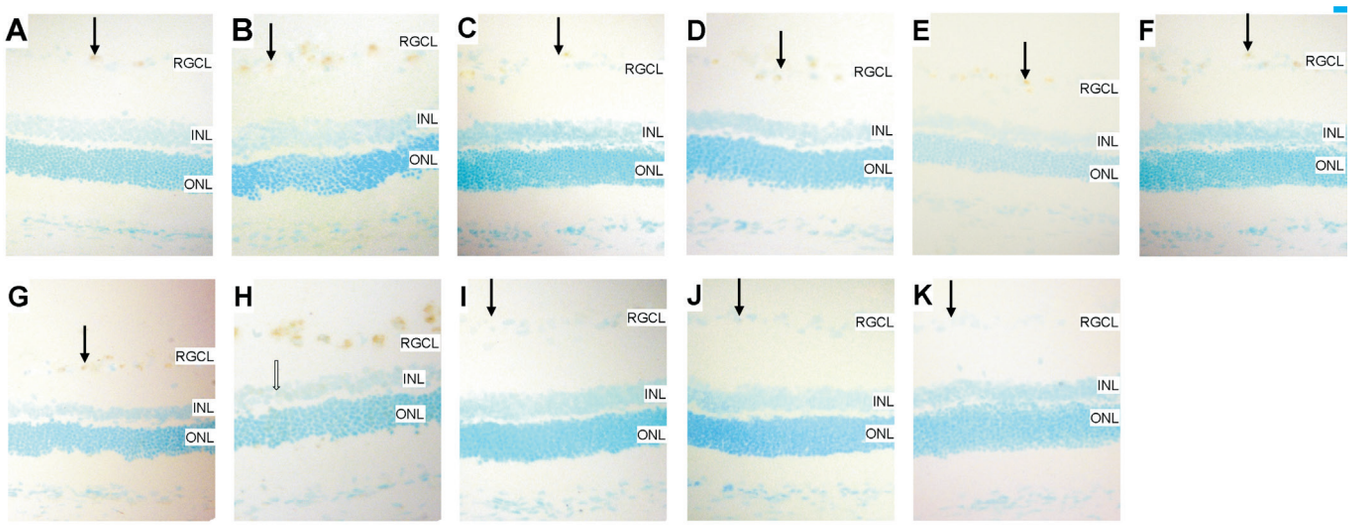

Figure 3 The representative images of RGCs apoptosis in the retinas of rat in different groups A, B: RGC apoptosis in HP rat at weeks 1 and 8; C, D: RGC apoptosis in V-HSP27 rat at weeks 4 and 8; E, F: RGC apoptosis in V-PBS rat at weeks 4 and 8; G, H: RGC apoptosis in I-HSP27 rat at weeks 4 and 6; I: RGC apoptosis in sham control rat; J: RGC apoptosis in I-PBS rat; K: RGC apoptosis in normal rat.

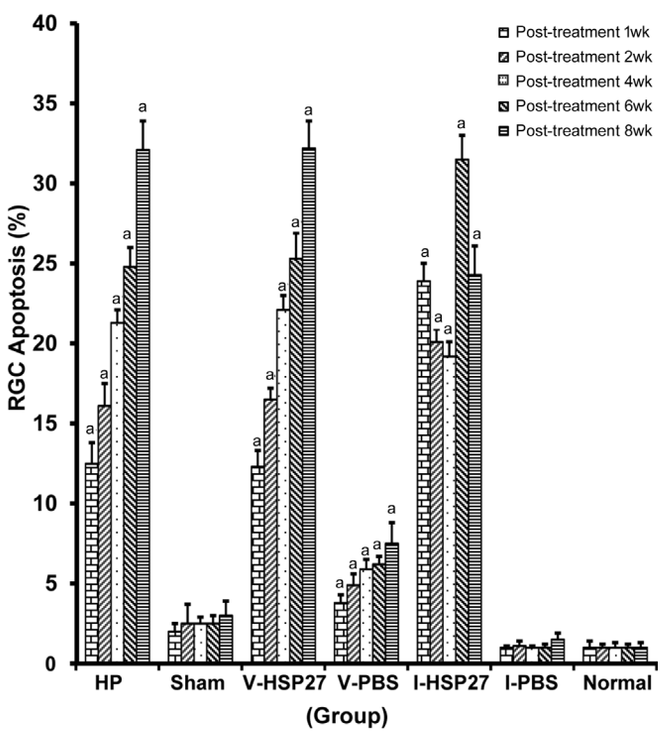

Figure 4 RGC apoptosis rate of different groups $^{a}P<0.05$ compared to normal group.

heat shock proteins (sHSP) family, sHSP protects cells and organisms from stress as molecular chaperone, and has anti-apoptotic activity in neurons[35-36]. However, sHSP does not show protective function in cardiomyocytes[37]. It was well evidenced that sHSPs were often upregulated both in neurodegenerative diseases[38] and in motor neuron cell injury[36]. In retinal ischemia preconditioning's model of rat, Li *et al*[39] found that HSP27 was induced and was further proved to be confined to the inner retinal layer. They thought that HSP27 should have a protective function in retina and RGCs. Yokoyama *et al*[40] also built the same rat glaucoma model, they injected HSP27 into rats' vitreous to investigate the change of RGC apoptosis. They found the same conclusion of the HSP27 function in the glaucomatous optic neuropathy. Several mechanisms of HSP27 protective activities have been proposed, including actin polymerization, chaperone activity, and regulation of reactive oxygen species[41-43]. However, as is demonstrated in our study, the overexpression of HSP27 did not show good protective effect on cell apoptosis in chronic hypertension model. The overexpression of HSP27 did not present beneficial effect on other chronic disease models including Huntington's disease model[44]. In our study, anti-HSP27 in serum promoted RGC apoptosis in immortalized retinal cells which were from one glaucoma patient (data not shown). The results proved that HSP27 autoantibodies could directly lead to neurodegenerative which was the same to the results of Tezel and Wax[45]. Moreover, we also built I-HSP27 of rat by subcutaneous immunization with HSP27, it was observed that the change of anti-HSP27 in serum was significant. The concentration of anti-HSP27 concentration in serum of I-HSP27 group was 2000 times to that of HP group and in CSF was 3 times to that of HP group. The evidences herein indicated that the level of anti-HSP27 in serum did not show strong influence on glaucoma development as the anti-HSP27 in CSFs and showed positive relationship with RGC apoptosis and promoted the progression of glaucoma. In addition, the optic nerve is the second of twelve paired cranial nerves but is considered to be part of the central nervous system (CNS) as it is derived from an outpouching of the diencephalon during embryonic development, unlike peripheral nerve which was protected by bone or by the blood-brain barrier. That was why the administration of HSP27 to

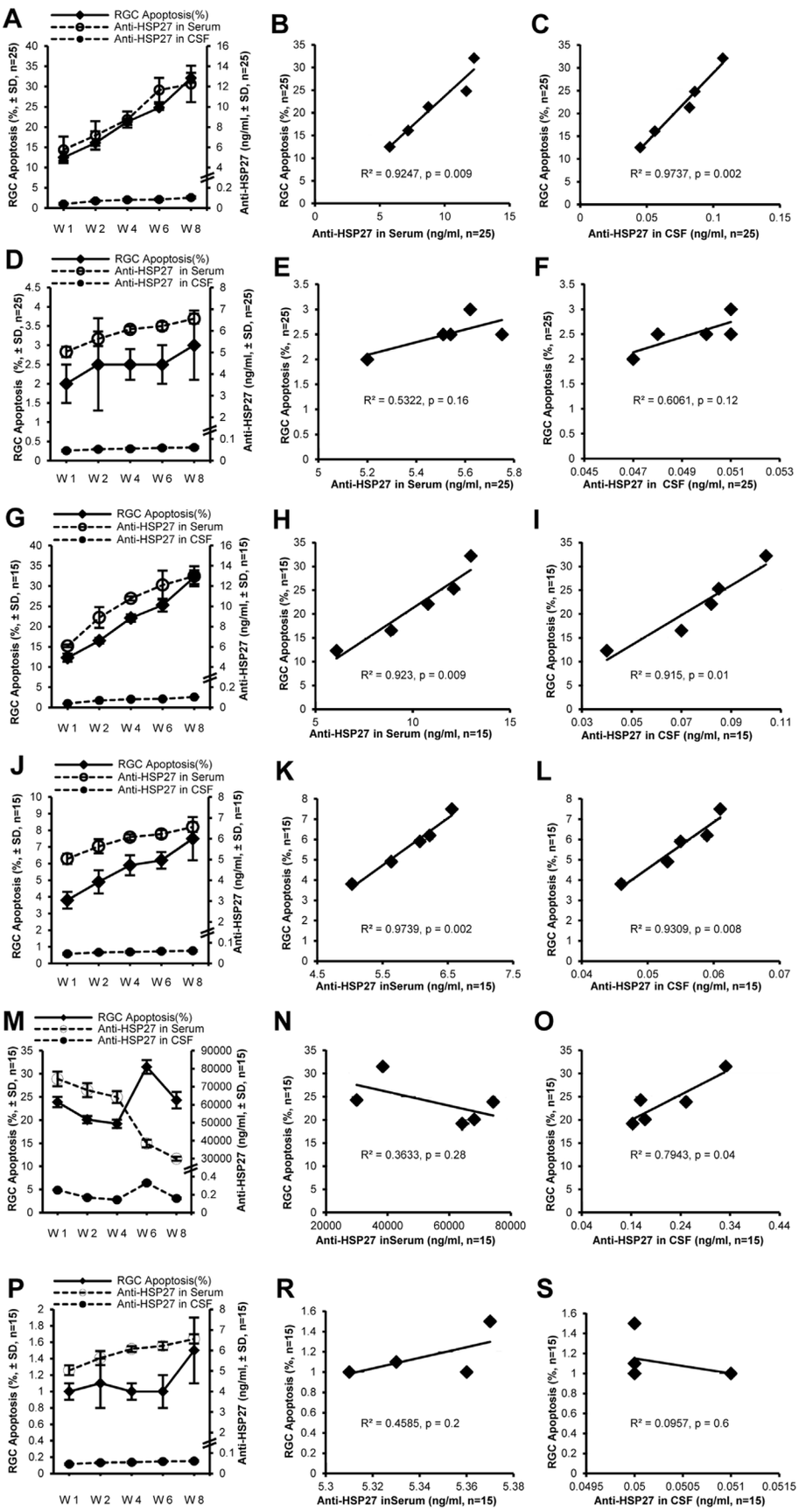

Figure 5 The relationship between RGC apoptosis and anti-HSP27 levels in experimental rat serum and CSFs A: RGC apoptosis rate and anti-HSP27 levels in HP group (A), sham group (D), V-HSP27 group (G), V-PBS group (J), I-HSP27 group (M), and I-PBS group (P) at weeks 1 (W1), 2 (W2), 4 (W4), 6 (W6) and 8 (W8) after treatment; B: The relationship between RGC apoptosis and anti-HSP27 levels in serum and CSFs in HP group (B, C), sham group (E, F), V-HSP27 group (H, I), V-PBS group (K, L), I-HSP27 group (N, O), and I-PBS group (R, S). *n*=25 in HP and sham groups, *n*=15 in other groups.

the peripheral body not only influenced anti-HSP27 level in serum, but also affected that in CSF and high level of anti-HSP27 in CSF would result in RGC apoptosis and glaucoma development.

It was also found that the level of anti-HSP27 in CSF arrived at a peak at week 6 while the percent of RGCs apoptosis also reached the top at the same time. However, the change of anti-HSP27 in serum was different, the concentration of anti-HSP27 in serum was highest at week 4. The optic never is unsheathed in meningeal layers, there is only some connections between optic nerve and CSF. It is different from peripheral nerve which is surrounded by CSF throughout its entire length. CSF is usually interrupted by some spaces, such as cisterns and the subarachnoid space (SAS), including the SAS of the optic nerves[46-49]. We proposed that CSF was taken as a window to understand the change of optic nerve. Some study found that some proteins such as idiopathic intracranial hypertension (IIH)[50], non-arteritic anterior ischaemic optic neuropathy (NAION)[50], papilloedema[51], just appear in SAS rather than in other space during the development of the diseases. The underlying mechanisms of these phenomena need to be elucidated. It will be more attractive that some difference in serum and CSF may be strictly associated with the characteristics of anti-HSP27. Tezel and Wax[45,52] found anti-HSP27 activated a proteolytic cascades including caspase-8 and caspase-3 which lead to dysfunction of HSP27. Therefore, in our study, we thought anti-HSP27 in serum perhaps triggered this switch. Although concentration of anti-HSP27 reached the peak at week 4, the most serious damage of influenced RGCs arrived at week 6. It was herein noted that the most serious damage of RGCs was described as highest apoptosis percentage.

Vitreous cavity injection of HSP27 in advance can avoid high IOP-induced HSP27 antibody. Lots of antigens were found in the anterior chamber which was able to induce the reduction of systemic cell-mediated immune response. More and more researches showed that the inoculation of the antibodies into the anterior chamber not only could evade the immune system response but also triggered an initiative suppression of delayed-type hypersensitivity with the possibility of retaining a normal catatonic T cell response and antibody response. In order to reflect its dynamic property, this special process was named after anterior chamber associated immune deviation (ACAID)[53-54]. Both anterior chamber and vitreous had been recognized as ocular immune privilege site[3,53]. Further studies have shown that almost all the soluble antigen are able to enter into the anterior chamber and induce ACAID[55]. It had been evidenced that vitreous cavity and sub retinal space also showed a similar immunological effect[56]. The studies in the different animals and different parts of maintain time of ACAID showed that vitreous-cavity-antigen-inoculation group in rat can maintain the time of ACAID for about 100d[57]. Data from I-HSP27 group which was built through the injection of HSP27 into the hind limb of rats accompanied by intaperitoneal injection of pertussis toxin at the same time, showed that serum HSP27 antibody level was significantly reduced as time passed, compared to that of the control group. There did not show statistical difference between the sham control group and normal group after the vitreous cavity presented ACAID successfully. Systemic immune response played a significant downstream adjustment in the role of blocking the expression of HSP27. Furthermore, some rats in vitreous-cavity-antigen-inoculation group still show ACAID after nine weeks.

The loss of the ability of the anterior chamber to support ACAID would lead to a process of retinal degeneration, and it was reported to be strictly associated with and dependent on age[58]. Here, we chose the young wistar rat which could keep this function in our experiments and observe this phenomenon successfully.

The injection of HSP27 into the vitreous cavity is beneficial to protect RGCs and immune. Although the pathogenesis of glaucoma remains ambiguous, the resulting RGC apoptosis is a common outcome. It has been evidenced that active or passive immunization of synthetic peptide copolymer compounds in the rats' cavity can significantly enhance RGC survival in biochemical insult-caused optic nerve injury or high IOP model[57]. This means it is possible that the injection of appropriate vaccines can enhance the body's physiological repair capacity and reduce glaucoma optic neuropathy. But the selection of antigen must be safe, that should exclude the occurrence of autoimmune diseases to the maximum extent possible. Therefore, it is important to balance the protective immune reaction and induced autoimmune diseases when an effective treatment method for optic neuropathy is deployed. In our experiment, the right eye RGCs apoptosis in I-HSP27 group decreased significantly at weeks 1, 2, and 3 when the anti-HSP27 level was maintained in a high level, compared to control and sham group. However, the injection of HSP27 in sensitized group also significantly resulted in more RGCs apoptosis compared to control and sham group. Simultaneously, the right eye RGCs apoptosis in vitrous cavity immunized group was higher than that in normal group. It indicated that high level of HSP27 should be able to inhibit RGCs apoptosis induced by high IOP effectively, however, it could not completely avoid apoptosis of RGCs. Early experiments also proved that high IOP was accompanied by a significant increase in HSP27 antigen in the rat model through retinal sweep[32]. Therefore, we can conclude from the contrary observation that retinal HSP27 antigens with autoimmune reaction lead to ganglion apoptosis, resulting

in optic nerve injury. It also offers a new immune theory to glaucoma pathogenesis. At the same time, an intraocular injection of HSP27 antibody paves a new way to protect RGCs and will be developed into new therapy for glaucoma. The key of anti-HSP27 therapy will depend on whether the occurrence of autoimmune diseases is induced or not which remains necessary to be elucidated by the further studies.

In summary, the laboratory tests of autoimmune diseases, especially the detection of autoantibodies, have been becoming an important and effective method in clinic. Analysis of autoantibodies can provide an objective basis for the differential diagnosis. Analysis of autoantibodies can also supply more and more proofs for studying the immune pathological mechanism and benefit new therapy for optic diseases in clinic.

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REFERENCES

1 Chiu MH, Shi C, Rosin M, Batulan Z, O'Brien ER. Biophysical analyses and functional implications of the interaction between Heat Shock Protein 27 and antibodies to HSP27. *Biochim Biophys Acta Gen Subj* 2019;1863(10):1536-1546.

2 Streilein JW. Immune privilege as the result of local tissue barriers and immunosuppressive microenvironments. *Curr Opin Immunol* 1993;5(3):428-432.

3 Jiang LQ, Streilein JW. Immune privilege extended to allogeneic tumor cells in the vitreous cavity. *Invest Ophthalmol Vis Sci* 1991;32(1):224-228.

4 Lin TC, Seiler MJ, Zhu D, Falabella P, Hinton DR, Clegg DO, Humayun MS, Thomas BB. Assessment of safety and functional efficacy of stem cell-based therapeutic approaches using retinal degenerative animal models. *Stem Cells Int* 2017;2017:9428176.

5 Grabska-Liberek I, Skonieczna K, Olesińska M, Terelak-Borys B, Kocięcki J, Sikora M, Jamrozy-Witkowska A, Tesla P, Czarnocka B. Levels of antibodies against human heat shock protein (HSP) 60 in patients with glaucoma in Poland. *Med Sci Monit* 2015;21:828-832.

6 Muranova LK, Ryzhavskaya AS, Sudnitsyna MV, Shatov VM, Gusev NB. Small heat shock proteins and human neurodegenerative diseases. *Biochemistry Moscow* 2019;84(11):1256-1267.

7 Scieglinska D, Krawczyk Z, Sojka DR, Gogler-Pigłowska A. Heat shock proteins in the physiology and pathophysiology of epidermal keratinocytes. *Cell Stress Chaperones* 2019;24(6):1027-1044.

8 Zininga T, Ramatsui L, Shonhai A. Heat shock proteins as immunomodulants. *Molecules* 2018;23(11):E2846.

9 Kim JY, Kim JW, Yenari MA. Heat shock protein signaling in brain ischemia and injury. *Neurosci Lett* 2020;715:134642.

10 Reinehr S, Kuehn S, Casola C, Koch D, Stute G, Grotegut P, Dick HB, Joachim SC. HSP27 immunization reinforces AII amacrine cell and synapse damage induced by S100 in an autoimmune glaucoma model. *Cell Tissue Res* 2018;371(2):237-249.

11 Anders F, Liu A, Mann C, Teister J, Lauzi J, Thanos S, Grus FH, Pfeiffer N, Prokosch V. The small heat shock protein α-crystallin B shows neuroprotective properties in a glaucoma animal model. *Int J Mol Sci* 2017;18(11):E2418.

12 Tezel G, Seigel GM, Wax MB. Autoantibodies to small heat shock proteins in glaucoma. *Invest Ophthalmol Vis Sci* 1998;39(12):2277-2287.

13 Chidlow G, Wood JP, Casson RJ. Expression of inducible heat shock proteins Hsp27 and Hsp70 in the visual pathway of rats subjected to various models of retinal ganglion cell injury. *PLoS One* 2014;9(12):e114838.

14 Lorenz K, Beck S, Keilani MM, Wasielica-Poslednik J, Pfeiffer N, Grus FH. Course of serum autoantibodies in patients after acute angle-closure glaucoma attack. *Clin Exp Ophthalmol* 2017;45(3):280-287.

15 Piri N, Kwong JM, Gu L, Caprioli J. Heat shock proteins in the retina: Focus on HSP70 and alpha crystallins in ganglion cell survival. *Prog Retin Eye Res* 2016;52:22-46.

16 Wang W, He M, Li Z, Huang W. Epidemiological variations and trends in health burden of glaucoma worldwide. *Acta Ophthalmol* 2019;97(3):e349-e355.

17 Song P, Wang J, Bucan K, Theodoratou E, Rudan I, Chan KY. National and subnational prevalence and burden of glaucoma in China: a systematic analysis. *J Glob Health* 2017;7(2):020705.

18 Bourne RR, Taylor HR, Flaxman SR, Keeffe J, Leasher J, Naidoo K, Pesudovs K, White RA, Wong TY, Resnikoff S, Jonas JB, Vision Loss Expert Group of the Global Burden of Disease Study. Number of people blind or visually impaired by glaucoma worldwide and in world regions 1990-2010: a meta-analysis. *PLoS One* 2016;11(10):e0162229.

19 Quigley HA. Number of people with glaucoma worldwide. *Br J Ophthalmol* 1996;80(5):389-393.

20 Faralli JA, Filla MS, Peters DM. Role of fibronectin in primary open angle glaucoma. *Cells* 2019;8(12):E1518.

21 Schwartz M, Yoles E. Self-destructive and self-protective processes in the damaged optic nerve: implications for glaucoma. *Invest Ophthalmol Vis Sci* 2000;41(2):349-351.

22 Tomita G. The optic nerve head in normal-tension glaucoma. *Curr Opin Ophthalmol* 2000;11(2):116-120.

23 Flammer J, Orgül S. Optic nerve blood-flow abnormalities in glaucoma. *Prog Retin Eye Res* 1998;17(2):267-289.

24 Tsai T, Grotegut P, Reinehr S, Joachim SC. Role of heat shock proteins in glaucoma. *Int J Mol Sci* 2019;20(20):E5160.

25 Maruyama I, Ohguro H, Ikeda Y. Retinal ganglion cells recognized by serum autoantibody against gamma-enolase found in glaucoma patients. *Invest Ophthalmol Vis Sci* 2000;41(7):1657-1665.

26 Tezel G, Hernandez R, Wax MB. Immunostaining of heat shock proteins in the retina and optic nerve head of normal and glaucomatous eyes. *Arch Ophthalmol* 2000;118(4):511-518.

27 Yang J, Tezel G, Patil RV, Romano C, Wax MB. Serum autoantibody against glutathione S-transferase in patients with glaucoma. *Invest Ophthalmol Vis Sci* 2001;42(6):1273-1276.

28 Rodriguez-Iturbe B, Pons H, Quiroz Y, Lanaspa MA, Johnson RJ. Autoimmunity in the pathogenesis of hypertension. *Nat Rev Nephrol* 2014;10(1):56-62.

29 Shirley Ding SL, Leow SN, Munisvaradass R, Koh EH, Bastion MC, Then KY, Kumar S, Mok PL. Revisiting the role of erythropoietin for treatment of ocular disorders. *Eye (Lond)* 2016;30(10):1293-1309.

30 Shoda H, Hanata N, Sumitomo S, Okamura T, Fujio K, Yamamoto K. Immune responses to Mycobacterial heat shock protein 70 accompany self-reactivity to human BiP in rheumatoid arthritis. *Sci Rep* 2016;6:22486.

31 Lee JY, Kim JM, Kim IT, Yoo CK, Won YS, Kim JH, Kwon HS, Park KH. Relationship between plasma homocysteine level and glaucomatous retinal nerve fiber layer defect. *Curr Eye Res* 2017;42(6):918-923.

32 Li Y, Yuan YS, Zeng Y, Chen ZF, Zhao W, Dai L. Expression of heat shock protein 27 induced in the experimental rat glaucoma model. *Ophthalmology in China* 2007;16(2):119-122.

33 Lü HB, Yuan YS, Li Y, Li J. The expression of heat shock protein 27 in retinal ganglion cells in the rat glaucoma model. *Zhonghua Yan Ke Za Zhi* 2005;41(6):533-539.

34 O'Reilly AM, Currie RW, Clarke DB. HspB1 (Hsp 27) expression and neuroprotection in the retina. *Mol Neurobiol* 2010;42(2):124-132.

35 Benn SC, Perrelet D, Kato AC, *et al*. Hsp27 upregulation and phosphorylation is required for injured sensory and motor neuron survival. *Neuron* 2002;36(1):45-56.

36 Wyttenbach A, Sauvageot O, Carmichael J, Diaz-Latoud C, Arrigo AP, Rubinsztein DC. Heat shock protein 27 prevents cellular polyglutamine toxicity and suppresses the increase of reactive oxygen species caused by huntingtin. *Hum Mol Genet* 2002;11(9):1137-1151.

37 Kamradt MC, Chen F, Sam S, Cryns VL. The small heat shock protein alpha B-crystallin negatively regulates apoptosis during myogenic differentiation by inhibiting caspase-3 activation. *J Biol Chem* 2002;277(41):38731-38736.

38 Dabir DV, Trojanowski JQ, Richter-Landsberg C, Lee VM, Forman MS. Expression of the small heat-shock protein alphaB-crystallin in tauopathies with glial pathology. *Am J Pathol* 2004;164(1):155-166.

39 Li Y, Roth S, Laser M, Ma JX, Crosson CE. Retinal preconditioning and the induction of heat-shock protein 27. *Invest Ophthalmol Vis Sci* 2003;44(3):1299-1304.

40 Yokoyama A, Oshitari T, Negishi H, Dezawa M, Mizota A, Adachi-Usami E. Protection of retinal ganglion cells from ischemia-reperfusion injury by electrically applied Hsp27. *Invest Ophthalmol Vis Sci* 2001;42(13):3283-3286.

41 Benndorf R, Hayess K, Ryazantsev S, Wieske M, Behlke J, Lutsch G. Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. *J Biol Chem* 1994;269(32):20780-20784.

42 Charette SJ, Lavoie JN, Lambert H, Landry J. Inhibition of Daxx-mediated apoptosis by heat shock protein 27. *Mol Cell Biol* 2000;20(20):7602-7612.

43 Dorion S, Landry J. Activation of the mitogen-activated protein kinase pathways by heat shock. *Cell Stress Chaperones* 2002;7(2):200-206.

44 Zourlidou A, Gidalevitz T, Kristiansen M, Landles C, Woodman B, Wells DJ, Latchman DS, de Belleroche J, Tabrizi SJ, Morimoto RI, Bates GP. Hsp27 overexpression in the R6/2 mouse model of Huntington's disease: chronic neurodegeneration does not induce Hsp27 activation. *Hum Mol Genet* 2007;16(9):1078-1090.

45 Tezel G, Wax MB. Inhibition of caspase activity in retinal cell apoptosis induced by various stimuli *in vitro*. *Invest Ophthalmol Vis Sci* 1999;40(11):2660-2667.

46 Bito LZ, Davson H. Local variations in cerebrospinal fluid composition and its relationship to the composition of the extracellular fluid of the cortex. *Exp Neurol* 1966;14(3):264-280.

47 Dichiro G. Movement of the cerebrospinal fluid in human beings. *Nature* 1964;204:290-291.

48 Milohart T. *Hydrocephalus and the cerebrospinal fluid.* Baltimore: Williams and Wilkins; 1972.

49 Wood JH. *Neurobiology of Cerebrospinal Fluid.* Plenum Press, New York:Springer US; 1982.

50 Killer HE, Jaggi GP, Flammer J, Miller NR, Huber AR. The optic nerve: a new window into cerebrospinal fluid composition? *Brain* 2006;129(Pt 4):1027-1030.

51 Killer HE, Laeng HR, Flammer J, Groscurth P. Architecture of arachnoid trabeculae, Pillars, and septa in the subarachnoid space of the human optic nerve: anatomy and clinical considerations. *Br J Ophthalmol* 2003;87(6):777-781.

52 Tezel G, Wax MB. The mechanisms of hsp27 antibody-mediated apoptosis in retinal neuronal cells. *J Neurosci* 2000;20(10):3552-3562.

53 Ksander BR, Streilein JW. Analysis of cytotoxic T cell responses to intracameral allogeneic tumors. *Invest Ophthalmol Vis Sci* 1989;30(2):323-329.

54 Streilein JW. Immune regulation and the eye: a dangerous compromise. *FASEB J* 1987;1(3):199-208.

55 Jiang LQ, Jorquera M, Streilein JW. Subretinal space and vitreous cavity as immunologically privileged sites for retinal allografts. *Invest Ophthalmol Vis Sci* 1993;34(12):3347-3354.

56 Peng GH, Li ZJ, Li C. Induction and maintenance of immune deviation in different intraocular compartment. *Chinese Journal of Ocular Fundus Disease* 1999;15(3):170-173.

57 Schori H, Kipnis J, Yoles E, WoldeMussie E, Ruiz G, Wheeler LA, Schwartz M. Vaccination for protection of retinal ganglion cells against death from glutamate cytotoxicity and ocular hypertension: implications for glaucoma. *Proc Natl Acad Sci U S A* 2001;98(6):3398-3403.

58 Welge-Lüssen U, Wilsch C, Neuhardt T, Wayne Streilein J, Lütjen-Drecoll E. Loss of anterior chamber-associated immune deviation (ACAID) in aged retinal degeneration (rd) mice. *Invest Ophthalmol Vis Sci* 1999;40(13):3209-3214.