

ASSOCIATION BETWEEN GLAUCOMA DAMAGE AND PLASMA CONCENTRATION OF HEAT SHOCK PROTEIN 70

Marija Trenkić^{1,2}, Tatjana Jevtović–Stoimenov³, Jelena Bašić³, Marija Radenković², Marija Cvetanović², Milan Trenkić³, Nevena Zlatanović⁴

Heat shock proteins (HSP) or stress proteins are induced in cells. They protect the cell and increase cell survival.

The aim of this research was to determine the plasma concentration of HSP 70 in patients with primary open-angle glaucoma with elevated intraocular pressure (POAG-HTG) and patients with pseudoexfoliative open-angle glaucoma (XFG), and to investigate the relationship between this biomarker and the structural and functional characteristics of glaucoma.

The study included 90 participants divided into three groups: 37 patients with primary open-angle glaucoma with increased intraocular pressure (hypertensive glaucoma, POAG-HTG), 24 patients with pseudoexfoliative open-angle glaucoma (XFG), and 29 participants without systemic diseases and glaucoma, matched by sex and age (control group of subjects, CONT). The concentration of circulating HSP 70 was measured in participants' plasma using the sandwich enzyme-linked immunosorbent assay (ELISA).

Plasma levels of HSP 70 were very similar in all three groups of participants, without any significant differences among the examined patients. A significant negative correlation of the plasma concentration of HSP 70 and RNFL Savg ($p < 0.05$) was found in POAG-HTG patients, whereas the negative correlations of HSP 70 with MD ($p = 0.0538$) and with RNFL Iavg ($p = 0.0584$) were very close to statistical significance.

There was no increase in the plasma concentration of HSP 70 in POAG-HTG and XFG patients. There was an interdependence between the plasma level of HSP 70 and the examined clinical parameters of POAG-HTG patients (MD, RNFL Avg, RNFL Savg and RNFL Iavg). HSP 70 can be a significant biomarker for glaucoma.

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Key words: heat shock protein 70, glaucoma, open-angle, hypertension glaucoma, pseudoexfoliation, plasma

¹University of Niš, Faculty of Medicine, Department of Ophthalmology, Niš, Serbia

²Clinic of Ophthalmology, University Clinical Centre Niš, Niš, Serbia

³University of Niš, Faculty of Medicine, Niš, Serbia

⁴Community Health Centre Niš, Niš, Serbia

Contact: Marija Trenkić
University of Niš, Faculty of Medicine, Department of Ophthalmology
Clinic of Ophthalmology, University Clinical Centre Niš, Serbia
81 Dr. Zorana Djindjića Blvd., 18000 Niš, Serbia
E-mail: marija.trenkic@medfak.ni.ac.rs

Introduction

Heat shock proteins (HSPs) are stress proteins and their production in cells protect the cell and increase cell survival. Their concentration

increases in physiologically or environmentally stressful conditions. HSPs function as chaperone molecules that prevent aggregation and facilitate the remodelling of deactivated proteins in the cell (1, 2).

HSPs act by the most diverse mechanisms including direct interaction with components of cellular signalling pathways, downstream or upstream regulation of caspase-dependent programmed cell death, or at the mitochondrial level. HSPs can also affect caspase-independent apoptosis, by interacting with apoptogenic factors such as Apoptosis-inducing factor (AIF) or acting at the level of lysosomes. HSP 70 is a guardian of the integrity of the lysosomal membrane. Due to oxidative stress, HSP carboxylation occurs, subsequently causing lysosome rupture. In addition, HSP 70 dysfunction activates nuclear factor- κ B (NF- κ B) signalling that may also promote neurodegeneration (1–6).

The heat shock protein 70 family (HSP 70 family) includes several members such as the constitutive form HSP 70, inducible HSP 72,

mitochondrial GRP 75, and endoplasmic reticulum GRP 78. These proteins exhibit distinctive neuroprotective effects and function in normal, developmental, and stressful conditions. Although the constitutive form exists under normal conditions, it is more pronounced under stressful conditions (7–13).

HSP 70 is the most structurally and functionally conserved protein in the HSP family. HSP 70 is a ubiquitous class of ATP-dependent chaperone proteins that exert cytoprotective effects. It plays a central role in the cellular control of protein quality. HSP 70 binds to the protein substrate facilitating its unfolding, degradation, transport, regulation, and preventing aggregation (1).

Elevated IOP is one type of mechanical stress that activates HSPs. Several studies have shown that HSPs and anti-HSP antibodies play an important role in glaucoma pathogenesis. Cellular stress and cell death are interrelated events, and HSPs, induced in response to stress, play a role in controlling apoptosis (11). HSPs include anti-apoptotic and proapoptotic proteins that interact with various cellular proteins involved in apoptosis. Their expression level can determine cell fate in response to a stimulus (14).

Increased immunohistochemical staining of HSPs in the glaucomatous retina reflects the function of these proteins in terms of cellular defense and stress response in glaucoma (15, 16).

Optic neuropathy and progression of glaucoma occur due to overexpression of HSP and activation of the auto-stimulatory response (17). Therefore, in patients with glaucoma, an elevated titer of anti-HSP antibodies occurs compared to healthy subjects, which reduces the protective ability of HSP (16). These data indicate that HSPs are essential for RGC survival as molecular chaperones and have pathogenic significance in glaucoma patients (18).

HSPs are involved in multiple stages of apoptosis and their function is to inhibit apoptosis (19). Overexpression of HSP 70 protects mitochondria from the harmful effects of reactive oxygen species (20). HSP 70 inhibits apoptosis by reducing the release of cytochrome-c and increasing the activity of caspase-3 (21). HSP 70 has also been described to inhibit protein kinase/c-Jun N-terminal kinase (SAPK/JNK) (22).

Kim et al. (8) demonstrated that HSP 70 is transferred from cell to cell. However, RGCs are not the sole target of HSP 70-induced neuroprotection. Glial cells also participate in HSP 70 transfer and contribute to neuroprotection.

The aim of our research was to determine the plasma concentration of HSP 70 in patients with primary open-angle glaucoma with elevated intraocular pressure (POAG-HTG) and patients with pseudoexfoliative open-angle glaucoma (XFG), and to investigate the relationship between this biomarker and the structural and functional characteristics of glaucoma disease.

Material and Methods

The research was carried out at the Clinic for Ophthalmology, University Clinical Centre Niš, Department of Biochemistry and Laboratory for Functional Genomics and Proteomics, and Scientific Research Centre for Biomedicine, University of Niš. All participants were informed about the objectives of the research and signed an informed consent to participate, according to the Declaration of Helsinki. The study was approved by the Ethical Committee of the Faculty of Medicine in Niš (Decision number 01-2625-18) and the Ethical Committee of the University Clinical Centre Niš (Decision number 338/43).

The study included 90 participants divided into three groups: 37 patients with primary open-angle glaucoma with increased intraocular pressure (hypertensive glaucoma, POAG-HTG), 24 patients with pseudoexfoliative open-angle glaucoma XFG, and 29 participants patients without systemic diseases and glaucoma, matched by sex and age (control group, CONT).

We performed complete ophthalmic examinations, including a review of the medical history, determination of visual acuity with refractions (Snellen tables), slit-lamp biomicroscopy of the anterior and the posterior segment, gonioscopy using a three-mirror Goldmann gonioscope, Goldmann applanation tonometry (GAT), indirect ophthalmoscopy and determination of the cup size of the optic nerve head (C/D ratio), using a 90 D lens, standard automatic perimetry (Humphrey Visual Field Analyzer, HFA, USA; Carl Zeiss Meditec, Inc., Threshold Test 24-2), and glaucoma OCT scan protocols (OCT, Stratus, Carl Zeiss Meditec, Inc., Dublin, CA) and determination of retinal peripapillary nerve fibre thickness (RNFL). We measured RNFL average (RNFL Avg), in the superior quadrant (RNFL Savg) and in the inferior quadrant (RNFL Iavg).

Inclusion criteria for patients with POAG-HTG were as follows: elevated IOP, characteristic Bjerrum's arcuate scotoma, and/or paracentral scotoma, and/or nasal, Rönne's step in Humphrey's computerized visual field, or other relevant defects in the visual field, optic disc cupping, and/or thinning of the nerve fibre layer on OCT, the finding of an open angle, and the absence of a secondary cause of glaucomatous optic neuropathy, such as previous trauma, previous corticosteroid administration, inflammation, or uveitis. Patients with POAG-HTG had intraocular pressure values greater than 21 mmHg upon daily measurement before diagnosis.

Inclusion criteria for patients with XFG were: elevated IOP, changes in the visual field, thinning of the RNFL on OCT, as for POAG, with the presence of pseudoexfoliations on the anterior capsule of the lens and/or along the pupillary margin.

Patients with systemic arterial hypertension, diabetes mellitus, systemic vasculopathies, retinal

disease, eye surgery, trauma and inflammation of the eye were excluded from the study. All control subjects were without glaucoma as confirmed by applying the same diagnostic criteria used for the diagnosis and with no family history of the condition, matched by sex and age. Patients with a history of congenital glaucoma or suspected normotensive (NTG) or hypertensive glaucoma (HTG) were also excluded from further examination.

Collected whole blood samples obtained from participants using EDTA as an anticoagulant, were centrifuged for 10 minutes at 3500 spins, at a temperature of 4 °C. The plasma was separated and frozen at a temperature of -80 °C afterward.

The concentration of circulating HSP 70 was measured by the sandwich enzyme linked immunosorbent assay (ELISA) method in participants' plasma, according to the manufacturer's instructions (Cusabio, CSB-E13463h, P.R. China). The concentration was determined using a standard curve and expressed in ng/ml. Minimum detectable dose (MDD) was 78 pg/ml. In accordance with the manufacturer's instructions, there was no significant cross-reactivity or interference with other proteins.

Statistical analysis

We used the methods of descriptive (absolute numbers, relative numbers, arithmetic mean, standard deviation, median, interval of variation (minimum and maximum values)) and analytical statistics (Mann-Whitney U test, Student's t-test of independent samples, Kruskal-Wallis test, ANOVA, χ^2 test or Fisher's test, Pearson's simple linear correlation coefficient, Spearman's rank correlation coefficient, univariate linear regression analysis). Statistical processing of the results was done with the SPSS 15.0 software package (SPSS Inc., Chicago, IL, USA). A value of $p < 0.05$ was used as the threshold for statistical significance.

Research results

Table 1 shows the demographic characteristics of 90 participants (37 POAG-HTG + 24 XFG + 29 CONTROL), basic clinical parameters of glaucoma (IOP, C/D ratio, MD, RNFL Avg, RNFL Savg, and RNFL Iavg), and plasma levels of HSP 70 (Figure 1).

We found no difference in age and sex between the examined groups (Kruskal—Wallis test and Mann—Whitney test). POAG-HTG and XFG were more prevalent in men (54.05%, i.e., 58.33%), whereas, in the control group, women were more prevalent (51.73%), without any significant difference between the groups. XFG patients had the highest IOP, and IOP values in both glaucoma groups were significantly higher compared to the control group ($p < 0.001$). There was no significant difference in the values of the C/D ratio between POAG-HTG and XFG. Although the absolute value of MD was higher in eyes with XFG, it was not significantly different from the value of this parameter in eyes with POAG-HTG. All POAG-HTG and XFG patients were in the second and third groups according to the Hadopp classification, without significant differences in distribution. Average RNFL thickness and RNFL thickness in the superior and inferior quadrants were higher in XFG patients, however, not significantly compared to POAG-HTG patients.

Plasma levels of HSP 70 were very similar in all three groups of subjects. Neither Kruskal—Wallis, nor Mann—Whitney, nor Student's t-test of independent samples, revealed any significant differences between the examined patients.

Spearman's rank correlation coefficient and Pearson's linear correlation coefficient were used to examine the connection between plasma concentration of HSP 70 and the intraocular pressure (IOP), C/D ratio, MD, RNFL Avg, RNFL Savg and RNFL Iavg of the same patient (Table 2). A significant negative correlation of the plasma concentration of HSP 70 and RNFL Savg ($p < 0.05$) was found in POAG-HTG patients, whereas the negative correlations of HSP 70 with MD ($p = 0.0538$) and with RNFL Iavg ($p = 0.0584$) were very close to statistical significance.

Table 1. Demographic and clinical characteristics, and plasma levels of HSP 70 of glaucoma patients and the control group of participants without glaucoma

	POAG-HTG (n = 37)	XFG (n = 24)	CONTROL (n = 29)	
Age (year) X ± SD (Me) Min–Max	70.95 ± 8.01 70.00 58–87	73.41 ± 6.25 76.00 59–84	71.77 ± 9.38 74.0 51–88	Kruskal–Wallis test Mann–Whitney test
Gender (M/F)	20 (54.05%) /17 (45.94%)	14 (58.33%) /10 (41.67%)	14 (48.27%) /15 (51.73%)	

IOP (mmHg) X ± SD (Me) Min–Max	21.86 ± 7.43 (20.00) 10–48	23.58 ± 11.31 (20.50) 10–56	14.76 ± 2.39 (14.00) 8–20	p < 0.001 Kruskal–Wallis and Mann–Whitney test
C/D ratio X ± SD (Me) Min–Max	0.64 ± 0.16 (0.60) 0.4–1	0.63 ± 0.18 (0.55) 0.4–1		Mann–Whitney test
MD (dB) X ± SD (Me) Min–Max	-11.73 ± 9.35 (-8.46) -0.38–-31.27	-12.72 ± 11.21 (-8.62) -0.07–-29.69		Mann–Whitney test
RNFL Avg (µm) X ± SD (Me) Min–Max	78.09 ± 24.48 (80.98) 24.46–143.71	78.62 ± 21.54 (81.63) 45.77–103.69		Mann–Whitney test
RNFL Savg (µm) X ± SD (Me) Min–Max	92.38 ± 34.87 (96.00) 26–180	100.64 ± 37.66 (104.00) 44–161		Mann–Whitney test
RNFL Iavg (µm) X ± SD (Me) Min–Max	94.41 ± 37.95 (101.00) 29–157	96.10 ± 31.11 (91.00) 57–144		Mann–Whitney test
HSP 70 (ng/ml) X ± SD (Me), Min–Max	2.27 ± 1,70 (1.97) 0.18–6.55	2.14 ± 0.96 (2.29) 0.29–3.70	2.20 ± 1.32 (1.93) 0.42–5.46	Kruskal–Wallis and Mann–Whitney test, Student's t-test

Legend: n—number of participants/eyes and examined plasma samples, POAG-HTG—primary open-angle glaucoma with elevated IOP, hypertensive glaucoma, XFG—pseudoexfoliative glaucoma, CONTROL—control group without glaucoma, IOP—intraocular pressure, C/D—cup/disk ratio, MD—mean deviation, RNFL Avg—average peripapillary retinal nerve fibre layer thickness, RNFL Savg—peripapillary retinal nerve fibre layer thickness in the superior quadrant, RNFL Iavg—peripapillary retinal nerve fibre layer thickness in the inferior quadrant, HSP 70—heat shock protein 70

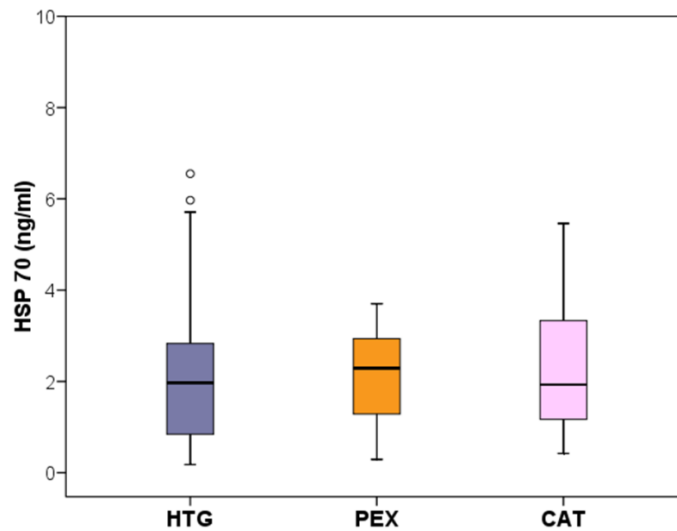


Figure 1. Median, minimum, maximum and 25th and 75th percentile values for HSP 70 in glaucoma and control group

Table 2. Spearman's and Pearson's correlation coefficients of HSP 70 and the examined parameters in glaucoma patients

ρ p (r) HSP 70 (ng/ml) and	IOP	C/D ratio	MD	RNFL Avg	RNFL Savg	RNFL Iavg
POAG-HTG	0.12	-0.12	-0.27	-0.26	*- 0.33	-0.30
XFG	0,07	0,12	-0,46	0,14	-0,05	-0,171

* $p < 0.05$, ρ —Spearman rank correlation coefficient
 r —Pearson coefficient of linear correlation (values in italics)

Univariate linear regression analysis did not find any effects of HSP 70 on the values of IOP, C/D ratio for POAG-HTG patients or XFG patients (Table 3). This analysis confirmed a significant effect of HSP 70 concentration on MD and RNFL Avg, RNFL Savg and RNFL Iavg of POAG-HTG patients. An increase in HSP 70 by one measurement unit caused a significant decrease in the MD value by 1.37 dB with a confidence

interval of 0.01–2.73, a significant decrease in the value of RNFL Avg by 4.78 with a confidence interval of 0.11–9.45, a significant decrease in the RNFL Savg value of 5.74 with a confidence interval of 0.29–11.20, a significant decrease in the RNFL Iavg value of 7.62 with a confidence interval of 0.89–14.36, in patients with POAG-HTG (Table 3).

Table 3. Results of univariate linear regression analysis and influence evaluation of the HSP 70 on the value of IOP, C/D ratio, MD, RNFL Avg, RNFL Savg and RNFL Iavg of POAG-HTG and XFG patients

Variable	POAG-HTG				XFG			
	t	p	B	95% CI for B	t	p	B	95% CI for B
IOP (mmHg)	0.50	0.6161	0.30	-0.87–1.47	0.07	0.9446	0.12	-3.28–3.52
C/D	-0.99	0.3255	-0.01	-0.04–0.01	0.51	0.6166	0.03	-0.10–0.16
MD (dB)	-2.02	0.0490*	-1.37	-2.73–0.01	-1.17	0.2678	-5.29	-15.27–4.69
RNFL Avg (μm)	-2.07	0.0450*	-4.78	-9.45–0.11	-2.07	0.0450	-4.78	-9.45–0.11
RNFL Sup	-2.13	0.0396*	-5.74	-11.20–0.29	-0.14	0.8932	-2.90	-50.36–44.56
RNFL Inf	-2.29	0.0276*	-7.62	-14.36–0.89	-0.52	0.6174	-8.42	-45.23–28.39

Discussion

Heat shock proteins (HSPs) are ubiquitous intracellular proteins. They are evolutionarily conserved molecules, with the role of chaperone and cytoprotective function (23).

HSP 70 plays an important role in protein metabolism, both under stress and under normal conditions. It facilitates the assembly of newly created proteins, translocation and degradation of damaged proteins, and participates in regulatory processes. In neurons exposed to extremely stressful conditions, such as ischemia and excitotoxicity, the expression of HSP occurs, and it is considered a neuroprotector. In addition, HSPs are highly antigenic, and HSPs or anti-HSP antibodies have a significant pathogenetic effect in glaucoma. The resulting immune response to HSP can have a protective or pathogenic effect. HSP production is up-regulated in RGCs of glaucoma patients. The amount of HSP, as well as HSP 70, is increased in monkeys with experimental glaucoma, and HSP 72 is increased in RGCs in a glaucoma rat model (24–27). However, elevated serum levels of HSP antibodies, detected in

glaucoma patients, may activate nerve cell death via apoptosis. Initially, increased HSP expression in the glaucomatous eye may be neuroprotective, protecting against further degeneration and inhibiting apoptosis. Since HSP can also act as an immunostimulatory signal, the breakdown of the immune tolerance and the loss of their protective effect can occur, which results in the loss of regulation of antiapoptotic processes and accelerates and facilitates apoptosis, and finally disease progression occurs. Thus, glaucomatous optic neuropathy may be a consequence of aberrant autoimmunity (28).

HSP expression determines cell fate. Reduced expressivity leads to an ineffective response to stress, and increased expression activates immunodestruction. In both cases, the outcome is cell death, however, through two mechanisms. The first involves oxidative free radicals, and the second involves cytokines as shown by Tezel et al. (16). The presence of HSP in RGCs and glial cells may be important for the stimulation of cytoprotective events at the beginning and then for neurodegenerative changes (16, 25).

Heat shock proteins 70 (HSP 70) are present in the peripheral circulation of healthy individuals (29).

Previously reported data indicate that HSPs are involved in the pathogenesis of glaucoma and the development of glaucomatous neuropathy due to increased IOP. The involvement of HSP in the development of glaucoma is twofold, it can be neuroprotective or degenerative related to the activation of the autoimmune response during the progression of the disease (16, 17, 30).

A study conducted by Lichtenaur et al. indicates that the serum concentration of HSP 70 in healthy subjects was $49 \text{ pg/mL} \pm 22$ (31). Another study showed that HSP 70 was detectable only in 77% of the analyzed samples, whereas the serum concentration of this protein decreases with age (400 ng/ml < 40 years; 20 ng/ml > or = 90 years), while the concentrations of anti-HSP 70 antibodies tend to increase with age, but without mutual dependence. These findings suggest that the stress response potential decreases with age (32).

This research found very similar plasma values of HSP 70 in all three groups of participants (POAG-HTG, XFG and CONT $2.20 \pm 1.32 \text{ ng/ml}$). Thus, it can be concluded that there is no increase in the plasma concentration of HSP 70 in POAG-HTG, however, not excluding the increase of this protein in the aqueous humour or the RGC nor the increase of antibodies to HSP 70. This partially supports the hypothesis that the decrease in HSP levels leads to a decrease in their protective role in the pathogenesis of glaucoma. Güler M. et al. showed that the level of HSP 70 in the aqueous humour was increased in patients with pseudoexfoliation without glaucoma compared to patients with cataract without pseudoexfoliation (33), thus supporting the neuroprotective role of HSP before the onset of glaucoma and the increase of anti-HSP antibodies.

A significant negative correlation between the serum concentration of HSP 70 and RNFL Sup ($p < 0.05$) was found in patients with POAG-HTG. Hence, plasma concentration of HSP 70 can be a measure of the development and progression of glaucomatous neuropathy in POAG-HTG. Our research has confirmed that HSP 70 concentration has a significant effect on MD and RNFL Avg, RNFL Savg and RNFL Iavg of POAG-HTG patients. However, it remains unclear why this is not the case with XFG.

Due to a limited number of studies investigating the concentration of HSP 70 in the plasma of glaucoma patients, and almost no research on the correlation between concentration and clinical parameters, we are unable to fully analyze the obtained results. Finally, further research, particularly larger and similarly designed studies should be conducted. Nevertheless, our research has a small contribution to the study of the role of HSP70 in glaucoma and the possibility of using this protein as a biomarker for glaucoma disease.

These results support a mechanism involving the immune response in glaucomatous damage, which may provide a new therapeutic approach to the neuroprotection of glaucomatous optic neuropathy. Understanding genetics and immunity in the pathogenesis of glaucoma is crucial for the development of new treatments.

Conclusion

There was no increase in the plasma concentration of HSP 70 in POAG-HTG and XFG patients. However, there was an interdependence between the plasma level of HSP 70 and the examined clinical parameters of POAG-HTG (MD, RNFL Avg, RNFL Savg and RNFL Iavg). This suggests that HSP 70 can be a significant biomarker for glaucoma.

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POVEZANOST OŠTEĆENJA IZAZVANIH GLAUKOMOM SA KONCENTRACIJOM PROTEINA TOPLOTNOG ŠOKA 70 U PLAZMI

Marija Trenkić^{1,2}, Tatjana Jevtović Stoimenov³, Jelena Bašić³, Marija Radenković², Marija Cvetanović², Milan Trenkić³, Nevena Zlatanović⁴

¹Univerzitet u Nišu, Medicinski fakultet, Katedra za oftalmologiju, Niš, Srbija

²Univerzitetski klinički centar Niš, Klinika za oftalmologiju, Niš, Srbija

³Univerzitet u Nišu, Medicinski fakultet, Niš, Srbija

⁴Dom zdravlja Niš, Niš, Srbija

Kontakt: Marija Trenkić
Bulevar dr Zorana Đinđića 81, 18000 Niš, Srbija
E-mail: marija.trenkic@medfak.ni.ac.rs

Proteini toplotnog šoka (engl. *heat shock proteins* – HSP) ili proteini stresa indukuju se u ćelijama. Štite ćeliju i produžavaju opstanak ćelije.

Cilj našeg istraživanja bio je da se utvrdi koncentracija HSP-a 70 u plazmi obolelih od primarnog glaukoma otvorenog ugla sa povišenim intraokularnim pritiskom (POAG-HTG) i obolelih od pseudoeksfolijativnog glaukoma otvorenog ugla (PEXG), kao i da se ispita veza između ovog biomarkera i strukturnih i funkcionalnih karakteristika glaukoma.

U ovu studiju uključili smo 90 ispitanika, koji su podeljeni u tri grupe: 37 obolelih od primarnog glaukoma otvorenog ugla sa povišenim intraokularnim pritiskom (hipertenzivni glaukom, POAG-HTG), 24 obolela od pseudoeksfolijativnog glaukoma otvorenog ugla (PEXG) i 29 ispitanika bez sistemskih bolesti i bez glaukoma, razvrstanih po polu i starosti (kontrolna grupa ispitanika; engl. *control group of subjects* – CONT). Koncentracija cirkulišućeg HSP-a 70 merena je metodom imunoapsorcionog enzimskog testa (engl. *sandwich enzyme-linked immunosorbent assay* – ELISA) u plazmi ispitanika.

Nivoi HSP-a 70 u plazmi bili su veoma slični kod ispitanika iz svih triju grupa, bez značajne razlike. Bolesnici sa POAG-HTG-om imali su značajnu negativnu korelaciju koncentracije HSP-a 70 i RNFL Savg-a u plazmi ($p < 0,05$), a veoma blizu statističke značajnosti bile su negativne korelacije HSP-a 70 i MD-a ($p = 0,0538$) i HSP-a 70 i RNFL Iavg-a ($p = 0,0584$).

Zaključili smo da nema povećanja koncentracije HSP-a 70 u plazmi bolesnika sa POAG-HTG-om i PEXG-om. Postoji međuzavisnost između nivoa HSP-a 70 u plazmi i ispitivanih kliničkih parametara POAG-HTG-a (MD, RNFL Avg, RNFL Savg i RNFL Iavg). HSP 70 može biti značajan biomarker glaukoma.

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Ključne reči: *protein toplotnog šoka 70, glaukom, otvoreni ugao, hipertenzivni glaukom, pseudoeksfolijacija, plazma*

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