

Thermal Injury Induces Heat Shock Protein in the Optic Nerve Head In Vivo

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PURPOSE. To investigate the induction of heat shock protein (Hsp)70 in the optic nerve head by localized laser application in transpupillary thermotherapy (TTT).

METHODS. TTT was performed on the right eye of Norwegian brown rats with an 810-nm diode laser installed on a slit lamp biomicroscope. The laser was aimed at the center of the optic nerve head with a 50- μ m spot size. Various exposures (range, 60–200 mW) were used with an exposure duration of 60 seconds, and the various exposure durations (range, 1–5 minutes) were used with a power of 100 mW. Twenty hours after laser irradiation, immunohistochemical staining and Western blot analyses were performed. For morphologic analysis of the optic nerve head, confocal scanning laser ophthalmoscopy and scanning electron microscopy were performed.

RESULTS. In the control eyes, Hsp70 was detected minimally in the optic nerve tissues by immunohistochemistry. After TTT, Hsp70 in the optic nerve tissue was induced more than in the control eyes. By Western blot, Hsp70 expression was found to increase progressively after TTT as the power was increased, but it also decreased slightly at powers >140 mW. The optimal setting of TTT without tissue damage was determined to be 100 mW for 60 seconds.

CONCLUSIONS. Transpupillary laser irradiation of the optic nerve head induces Hsp70 expression. This result can be applied to the neuroprotective experiments in glaucoma by enhancement of a natural cytoprotective stress response. (*Invest Ophthalmol Vis Sci.* 2006;47:4888–4894) DOI:10.1167/iovs.05-1328

Heat shock protein (Hsp) is present in every organism and in almost all living cells.¹ It is rapidly induced by a variety of environmental stresses, and its role is to protect cells against stress.^{2,3} Induced Hsp is known to have neuroprotective effects against ischemic injury of the central nervous system in mammals.^{4–6} The Hsp70 family comprises several members, which are classified according to the molecular mass. The constitutive form is Hsc70 and the inducible form is Hsp72. Although the constitutive form exists under normal conditions, it is expressed more under the stressful conditions. In contrast, the inducible form is expressed only in stressed cells. More-

over, the synthesis of both forms increases after hyperthermia.⁷ The induction of Hsp72 in the mammalian central nervous system by hyperthermia has been associated with neuronal tolerance against ischemic insults and neuroprotective effects against light-induced injury in the rat retina.^{8–10}

Glaucoma is a disease that causes progressive optic nerve damage. At the same time, the disease accompanies a selective loss of retinal ganglion cells due to mechanical injury that produces high intraocular pressure or ischemic injury.^{11,12} It has been widely accepted that apoptosis is the final common pathway for RGC death.¹³ The current treatment of glaucoma mainly involves reducing intraocular pressure and diurnal fluctuation. Recently, the importance of neuroprotection in the treatment of glaucoma has become a worldwide issue. From the animal models of chronic intraocular pressure elevation by whole-body hyperthermia, with a rectal temperature of 40°C to 42°C, both intraperitoneal zinc injection and intraperitoneal geranylgeranylacetone induces Hsp72 in RGCs.^{14,15} It has also been found that Hsp70 overexpression in these experiments increases the survival rates of RGCs.^{14,15} These methods of Hsp induction in RGCs are proceeded through the systemic therapy that may have a chance to accompany systemic effects. The most ideal way to induce Hsp in RGC for a neuroprotective purpose is to develop a safe and simple method focused on the selective target.

Transpupillary thermotherapy (TTT), which is currently being applied to retinal diseases such as subfoveal occult choroidal neovessels in age-related macular degeneration (AMD), slowly increases tissue temperatures to as much as approximately 10°C above baseline levels.^{16,17} An experimental study showed heat shock protein (Hsp70) hyperexpression in chorioretinal layers after TTT.¹⁸ However, no study on Hsp expression induced by laser irradiation of the optic nerve head has been presented. To apply the expression of Hsp to the neuroprotective treatment of glaucoma, a method of inducing Hsp safely and selectively in the optic nerve tissue is necessary. The optic nerve head is known as a primary site of glaucomatous optic nerve damage where the primary and secondary degeneration of RGC begins. So we considered the optic nerve head as a target of TTT rather than RGC bodies in the whole retina. The purpose of this study was to develop a simple, safe, and standardized method of Hsp70 induction in the optic nerve tissue using TTT without causing tissue damage.

MATERIALS AND METHODS

All experimental procedures were designed to conform to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and our own institution's guidelines.

One hundred twenty-six, 8-week-old Norwegian brown rats weighing between 200 and 250 g were used in the experiments. Preoperative ocular examinations were performed with rats under deep anesthesia with intramuscular injections of a cocktail solution of ketamine (100 mg/kg) and xylazine (10 mg/kg). Pupil dilation was achieved with 0.5% tropicamide and 10% phenylephrine eye drops. The coverslip was placed with methylcellulose (Methocel; Novartis Ophthalmics AG, Hettlingen, Switzerland) on the cornea, to examine the retina. Slit lamp

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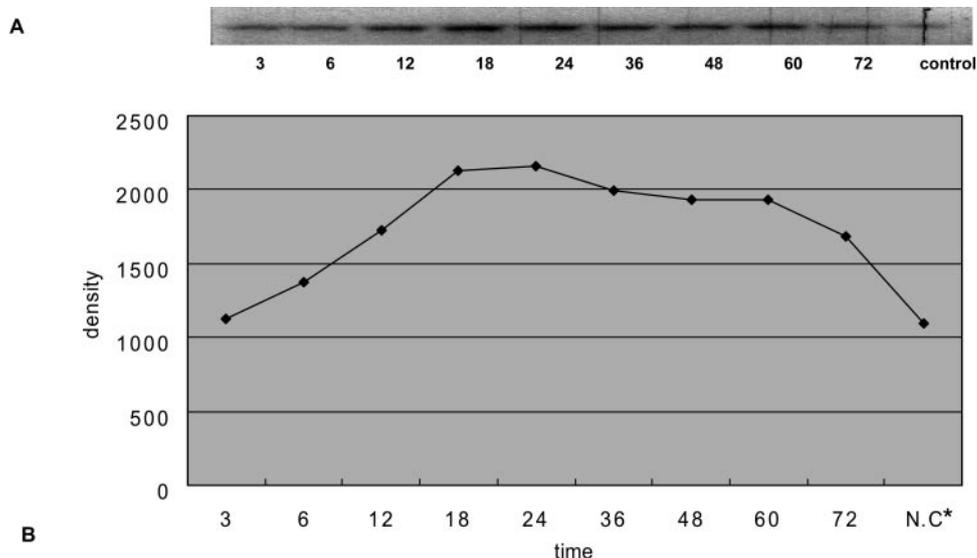


FIGURE 1. Hsp70 expression versus time after TTT. **(A)** Western blot findings of Hsp70 induction after TTT at 100 mW for 60 seconds; **(B)** Western blot densitometry results on the blot in **(A)**. Hsp70 induction was increased after TTT and was induced maximally between 18 and 24 hours after TTT and then decreased slowly. *Normal control.

(model 900; Haag-Streit, Bern, Switzerland) biomicroscopic examinations were performed before TTT to exclude any ocular abnormalities of both anterior and posterior segments.

Transpupillary Thermotherapy

An 810-nm diode laser (Iridex Corporation, Mountain View, CA) with a TTT adaptor installed on a slit lamp (model 900; Haag-Streit) was used in a continuous mode. Rats were anesthetized with the same cocktail solution and the coverslip was placed on the cornea with methylcellulose. After that, TTT was performed on the right eyes. A laser beam was focused to the center of the optic nerve head with a spot diameter of 0.5 mm. The gross examination of the optic nerve head was performed before, during, and after the treatment with a slit lamp biomicroscope and a coverslip mounted on the cornea with methylcellulose.

First, various exposure powers (60, 80, 100, 120, 140, 160, 180, and 200 mW) were used with the same exposure duration of 60 seconds. This first step was performed to determine the optimal laser power necessary to induce Hsp70 without tissue damage. The left eyes were used as the control. Immunohistochemical staining was performed to determine Hsp70 expression. In addition, confocal scanning laser ophthalmoscopy (Heidelberg Retina Tomograph; Heidelberg Engineering, Heidelberg, Germany) and scanning electron microscopy (SEM) were performed to evaluate the optic nerve head structure. These two experiments were performed in the selected power group (100, 120, and 140 mW), in which Hsp70 was induced without gross damage of the optic nerve head tissue. Based on the optimal power level determined by the first experiment, various exposure durations of 1, 2, 3, and 5 minutes, respectively, were examined in the second step. Furthermore, immunohistochemical staining and Western blot were performed during the second step of the experiment.

Immunohistochemical Study

One eye was used in each rat in each group. Twenty hours after TTT, the time point for maximum expression of Hsp70 (Fig. 1), the induction and localization of Hsp70 were examined by immunohistochemical staining. Immunohistochemical staining for glial fibrillary acidic protein (GFAP) was also performed on the optic nerve head tissue, to investigate whether the glial cells express Hsp70. At 20 hours after TTT, the enucleated 14 eyes, 2 in the control groups, 8 in the different power groups, and 4 in the different exposure time groups, were perfused with 10% NBF (neutral buffered formalin) solutions for 24 hours. After fixation, the eyes were cut equatorially behind the ora serrata and immersed overnight in the same fixative. The eyes were

embedded in paraffin and sectioned at 4 μ m thickness along the vertical meridian through the optic nerve head. Paraffin sections were prepared, including the optic nerve head, and placed on the slides. After deparaffinization and hydration, the endogenous peroxidase was inactivated by incubation with 0.3% hydrogen peroxidase in PBS. After they were washed with PBS, the slides were incubated with blocking solution for 5 minutes, followed by incubation with mouse monoclonal antibodies to either Hsp72 (SPA-810, 1:150; StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) or rabbit polyclonal antibodies to GFAP (1:500; DakoCytomation, Inc., Carpinteria, CA) for 3 hours. Antigen-antibody complexes were detected by an avidin-biotin-peroxidase technique (LSAB kit; DakoCytomation). As a substrate to the peroxidase, diaminobenzidine (DAB) was used to produce a brown color in the target tissue, and the nuclei were counterstained with Mayer's hematoxylin. The stained tissues were examined with a light microscope. For negative control of the immunohistochemical staining, the sections were incubated with blocking solution by replacing primary antibody.

To study colocalization of Hsp, we performed a double-labeling procedure. For the double-labeling procedure, sections were incubated for 3 hours at room temperature with each primary antibody. After they were washed with PBS, the slides were incubated for 1 hour in the dark with Alexa Fluor 488 anti-mouse IgG (1:1000, Invitrogen-Molecular Probes, Eugene, OR) and Alexa Fluor 555 anti-rabbit IgG (1:5000, Invitrogen-Molecular Probes). Stained sections were examined by fluorescence microscope (BX-61; Olympus, Tokyo, Japan) with the use of an FITC filter and a rhodamine filter.

Western Blot Analysis

Four eyes were used for each Western blot group. To quantify optic nerve head Hsp expression, Western blot analysis was performed using Hsp-specific antibodies. After the protocol described above was used for TTT, the eyeballs were enucleated, and optic nerve heads were dissected, 20 hours after TTT based on the data in Figure 1. The proteins from optic nerve head extracts (20 μ g) were separated on an SDS-polyacrylamide gel and were transferred to nitrocellulose membranes. The membrane was blocked by incubation in 0.1% Tween-20 in Tris-buffered saline containing 5% nonfat dried milk for 1 hour. The membrane was incubated with mouse monoclonal antibodies to Hsp72 (1:1000; StressGen) for 3 hours and then with goat anti-mouse IgG (1:5000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 hour. The immunoreactive bands were detected by enhanced chemiluminescence (GE Healthcare, Piscataway, NJ).

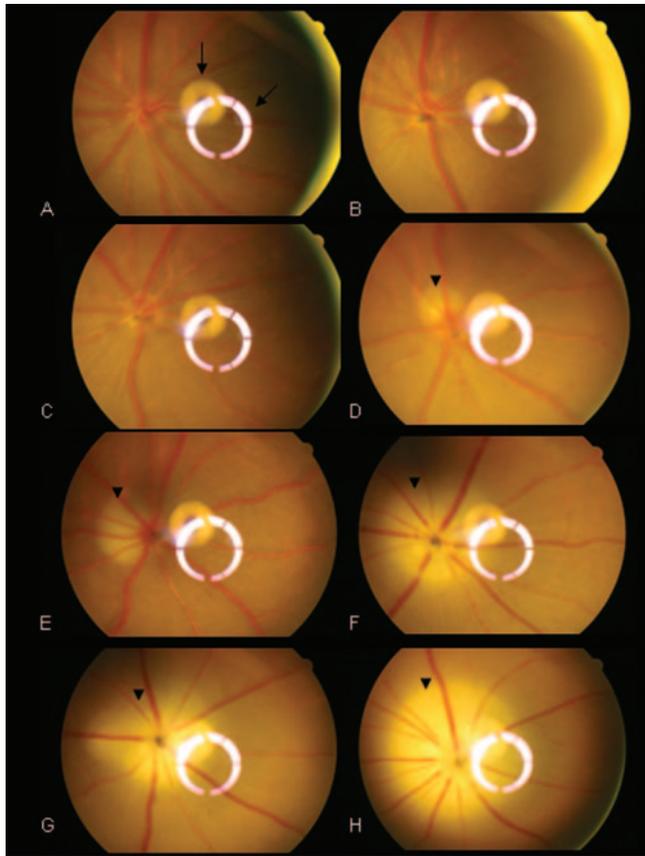


FIGURE 2. Fundus photographs after laser irradiation with different powers: 60 (A), 80 (B), 100 (C), 120 (D), 140 (E), 160 (F), 180 (G), and 200 mW (H) for 60 seconds, respectively. After lower-power laser treatment (A–C ≤ 100 mW), optic nerve heads showed no changes in color or shapes. With the power of 120 mW one fundus of four treated showed a peripapillary change (D). With higher-power (E–H ≥ 120 mW), peripapillary whitening was observed in all treated eyes with a dose-dependent pattern. (A, *arrows*) Artifacts made by corneal and retinal reflex. (D–H, *arrowheads*) Margin of peripapillary whitening after laser treatment.

Confocal Scanning Laser Ophthalmoscopy

Experimental rats were divided into three groups on the basis of applied laser power at 100, 120, and 140 mW. One eye was used in each rat in each group. In this case, none of the powers damaged the optic nerve head grossly. In addition, optic disc topographies were analyzed by using a confocal scanning laser ophthalmoscope at 1 day, 3 days, and 1 week after TTT. The optic nerve head image was taken three times in each eye. Mean topographic images were obtained from three images at a fixed-reference-plane height. The images were derived from 32 optical sections at consecutive focal depth planes. Each image consisted of 256×256 pixels at each pixel location that corresponded to the retinal height.

Scanning Electron Microscopy

The experimental rats were killed at 1 week after TTT. Three pars plana sclerotomies were made with a no. 11 blade, to ensure rapid fixative penetration. Globes were soaked in cups filled with 2.5% glutaraldehyde for 24 hours. The optic nerve heads were then dissected from the globes. Next, the vitreous was removed with an absorbent sponge (MEROCEL; Medtronic Solar, Jacksonville, FL), and the optic nerve heads were prepared for SEM. Specimens were postfixed in 1% osmium tetroxide, dehydrated in increasing grades of ethanol, and washed with isoamyl acetate. After they were coated by an ion coater, optic nerve heads were observed and photographed

with the scanning electron microscope (model JSM 300; JEOL, Tokyo, Japan).

RESULTS

According to our histologic findings, Hsp70 was induced by TTT at a power of 100 mW for 60 seconds in the optic nerve head. When we examined the fundus photograph before and after treating TTT laser by the change of TTT laser power, the change in the optic nerve head was grossly observed at a power of ≥ 140 mW (Fig 2). When the power was changed, Hsp70 was also induced at 60, 80, 100, 120, 140, 160, 180, and 200 mW. However, at a power of ≥ 140 mW, Hsp70 induction was accompanied by photocoagulation of the optic nerve tissue (Fig. 3). At a power of ≤ 100 mW, Hsp induction was minimal. Compared with GFAP staining, Hsp70 induced in the optic nerve tissue seemed to have come mainly from the astrocytes (Fig 4). To study the colocalization of Hsp, we performed a double-labeling procedure. Double-label staining (Fig. 4I) showed the astrocytes to be positive for Hsp70.

No significant morphologic change was found by confocal scanning laser ophthalmoscopy at 1 or 3 days or 1 week after irradiation with 100 or 120 mW for 1 minute (Fig. 5). On the contrary, definite morphologic changes were observed at 1 and 3 days, after 140 mW for 1 minute. One week after irradiation,

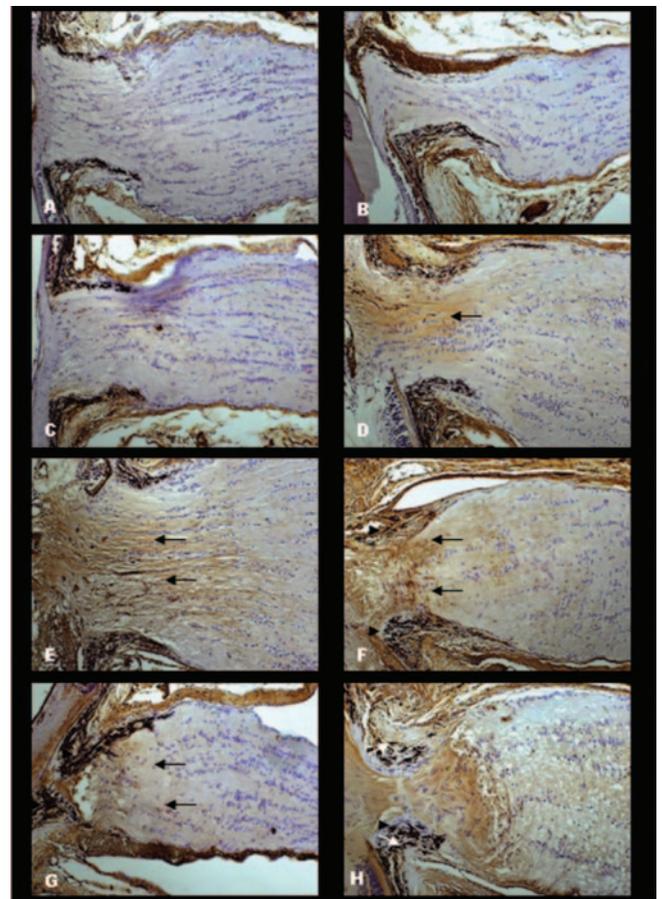


FIGURE 3. Hsp70 immunohistochemical staining of the optic nerve. Negative Hsp70 staining in the control optic nerve head (A) and positive staining (*arrows*) in the optic nerve head of rat eyes irradiated at 60 (B), 80 (C), 100 (D), 120 (E), 140 (F), 160 (G), or 180 (H) mW with an 810-nm laser for 60 seconds. (F–H) Coagulation of optic nerve tissue (*arrowheads*) and coagulation combined with scleral tissue contracture (*white arrows*) were observed at ≥ 140 mW. Magnification, $\times 200$.

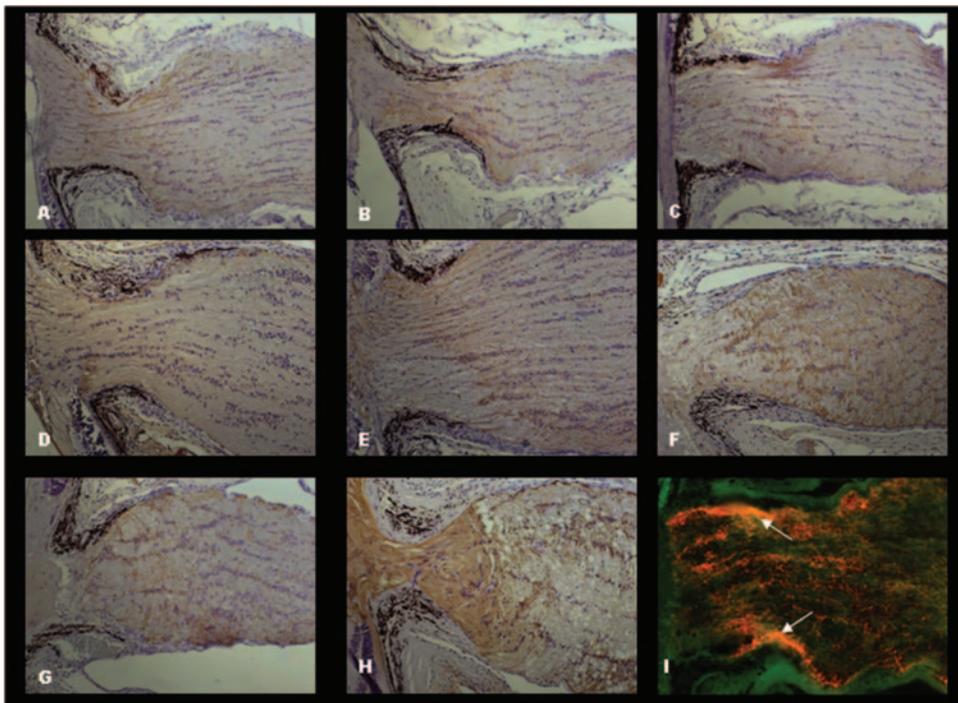


FIGURE 4. GFAP immunohistochemical staining and double immunofluorescence labeling in the optic nerve tissue. Weak positive Hsp70 staining of astrocytes in the control optic nerve head (A) and more strongly positive staining in the optic nerve head of rat eyes irradiated at 60 (B), 80 (C), 100 (D), 120 (E), 140 (F), 160 (G), or 180 (H) mW with an 810-nm laser for 60 seconds. The higher power induced more Hsp in the astrocytes. Double immunofluorescence labeling (I) with irradiation at 80 mW for 60 seconds also showed astrocytes to be positive (*white arrows*) for Hsp70. Magnification, $\times 200$.

the topographic image appeared to show recovery after 140 mW for 1 minute (Fig. 5). However, pseudo-3-dimensional (3-D) images revealed that the overall retina including the optic nerve head surface was depressed at 1 week (Fig. 6), which gave the false impression of recovery in the topographic image (Fig. 5).

After 1 week of laser irradiation, the SEM findings showed a smooth and normal contoured optic nerve head at 100 mW for 1 minute, whereas a membrane-like attachment of vitreous was observed after 120 mW for 1 minute, and an irregular coarse attachment of vitreous was observed at 140 mW (Fig. 7). In

particular, lymphocyte-like inflammatory cells were found to accumulate on retinal surfaces near the optic nerve head in magnified views (Fig. 8).

When the exposure duration was altered (i.e., 1, 2, 3, or 5 minutes), and the output power was held at 100 mW, all durations, except for 1 minute, showed photocoagulation of the optic disc and peripapillary retina. At 100 mW for 1 minute, no visible change was observed during the experiments, and no modifications of irradiated areas were detected. For those durations longer than 1 minute, peripapillary whitening was observed (Fig. 9).

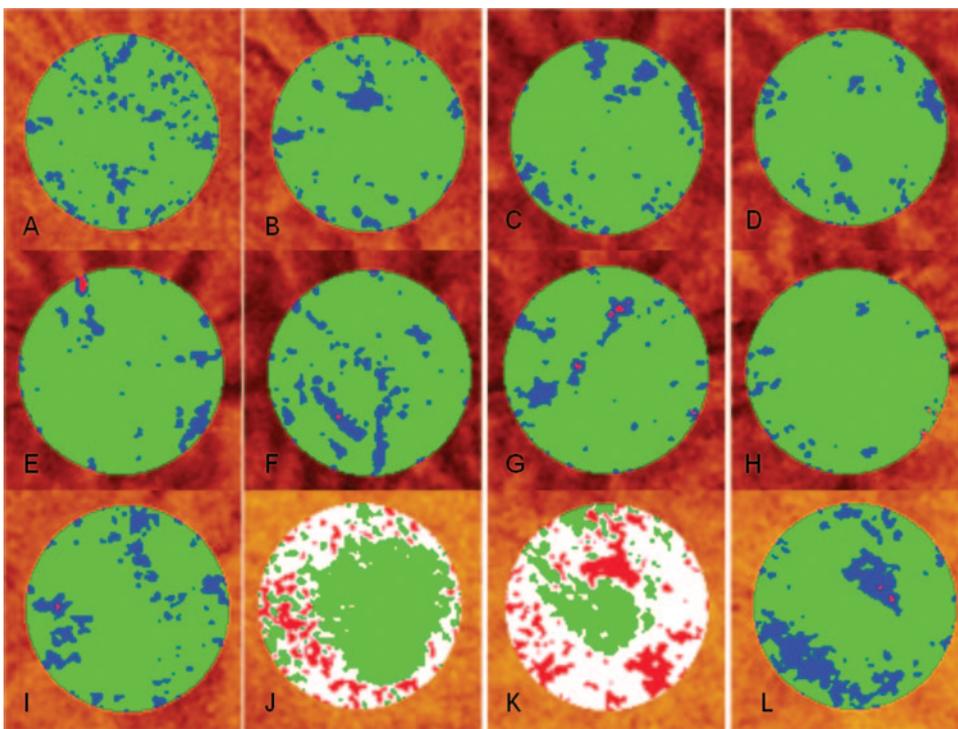


FIGURE 5. Follow-up confocal scanning laser ophthalmoscopic images of optic nerve heads after laser irradiation at 100 (A-D), 120 (E-H), or 140 (I-L) mW with an 810-nm laser for 60 seconds. No definite morphologic changes were observed after laser irradiation at both 100 and 120 mW. Definite morphologic changes were found at 1 day (J) and 3 days (K) after laser irradiation at 140 mW for 60 seconds, respectively. Images obtained 1 week after irradiation appeared to show recovery (L).

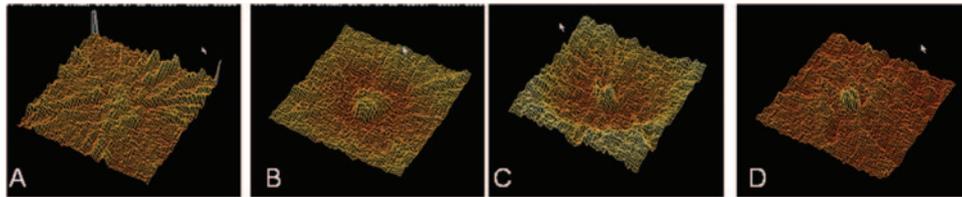


FIGURE 6. Confocal scanning laser ophthalmoscopic pseudo-3-D images of the optic nerve head of a rat irradiated with 140 mW for 60 seconds using an 810-nm laser: before laser treatment (A) and 1 day (B), 3 days (C), and 1 week (D) after laser treatment. Pseudo-3-D images showed optic nerve head depression after 1 day (B) and 3 days (C). At 1 week, the entire retina, including the optic nerve head surface, was depressed.

Hsp70 expression in the optic nerve heads was quantified by Western blot (Fig. 10). With each power change, Hsp70 was induced more in TTT eyes (at a power of ≥ 60 mW) than in untreated control eyes, in which the differences of Hsp70 expression at these power levels were not distinct. However, when the exposure duration was increased, Hsp70 expression increased progressively. Quantitative values were obtained by densitometry.

DISCUSSION

Our study demonstrated Hsp70 hyperexpression in the optic nerve head tissue by transpupillary thermal elevation. At the power level of 140 mW or higher, Hsp70 hyperexpression was

induced, but the optic nerve head structure was not maintained, and blood vessel as well as peripapillary tissue photo-coagulation was observed (Fig 1). In the eyes irradiated with 120 mW for 1 minute, a slight whitening of peripapillary tissues was observed after laser application in only one case (Fig. 9D). At powers >140 mW for 1 minute, peripapillary whitening was observable during laser irradiation in all cases. However, at 100 mW for 1 minute, no structural changes were detected in irradiated areas by biomicroscopy or by gross examination. At this level, the thermal effect of a TTT laser may be balanced by the cooling effect of blood, which creates an optimal stable thermal environment for Hsp induction.¹⁸ Mammalian cell death increases beyond 43°C,¹⁹ but at temperatures between 37°C and 43°C, Hsp70 protects cells from stress. In neural cells, it is suggested that Hsp70 induction may protect cells from heat shock.²⁰ This thermotolerance leads to cell survival on the one hand or to apoptosis (as opposed to necrosis) on the other.²¹ According to Polla et al.,²² Hsp70 is necessary for apoptosis rather than for necrosis or survival instead of apoptosis.²¹ According to our results, at 140 to 200 mW for 1 minute no inflammatory signs were detected grossly or by light microscopy. However, electron microscopy showed some lymphocyte-like cells on the optic nerve head (Fig. 8), which may have been caused by mild vitritis induced by photo-coagulation.

The optic nerve head is known as a primary site of glaucomatous optic nerve damage, which supports our rationale for targeting the optic nerve head with TTT. After TTT, Hsp was induced in the optic nerve head and in the RGCs. Hsp70 is known to be taken up by retinal cells after intravitreal injection.²³ Some of the Hsp70s taken up by retinal cells can undergo axonal transport from the RGCs into the optic nerve and subsequently down the contralateral optic tract to synaptic termini in the superior colliculus, and the Hsp70s not taken up by retinal cells remain in the retina itself.²⁴ Another possibility cannot be excluded that Hsp70 never gets to the RGCs and is

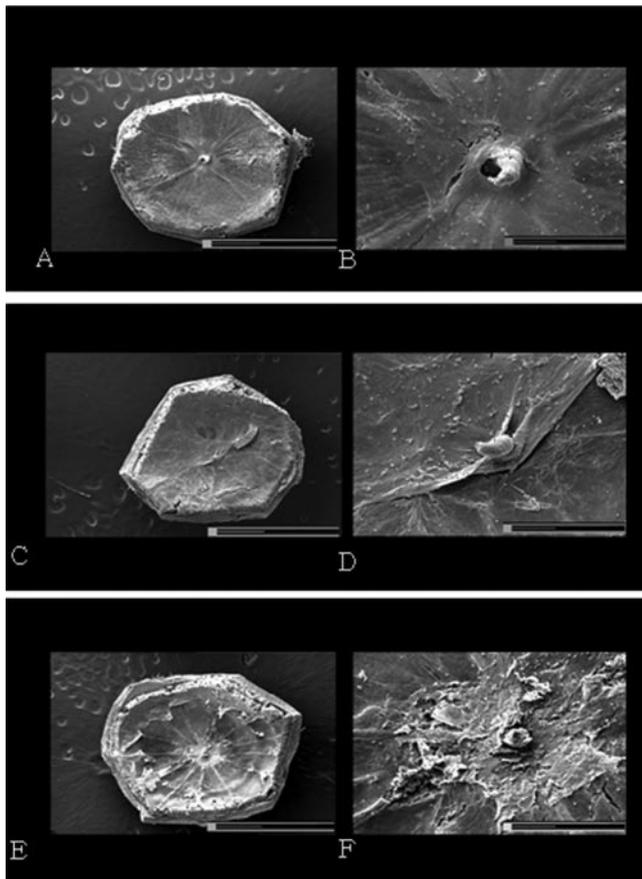


FIGURE 7. SEM images of optic nerve heads 1 week after laser irradiation with 100 (A, B), 120 (C, D), or 140 (E, F) mW for 60 seconds. The surfaces of the optic nerve heads showed smooth, normal contours after 100 mW (B), but membrane-like attachment of vitreous was observed after 120 mW (D) and irregular coarse attachment of vitreous after 140 mW (F). Magnification: (A, C, E) $\times 35$; (B, D, F) $\times 200$.

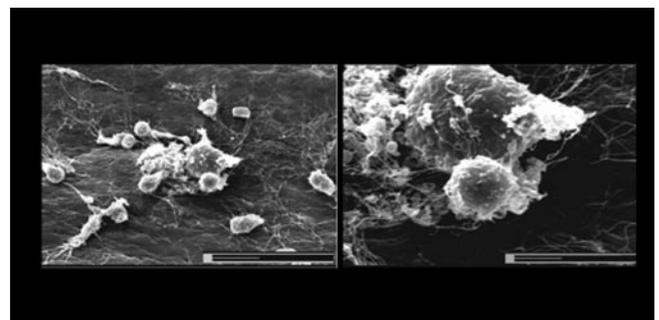


FIGURE 8. Magnification of cells on the optic nerve head. *Left:* An accumulation of inflammatory cells was observed on the retinal surface near the optic nerve head at 1 week after irradiation with 140 mW for 60 seconds. *Right:* photomicrograph showing an inflammatory cell. Magnification, *left*, $\times 1500$; *right*, $\times 5000$.

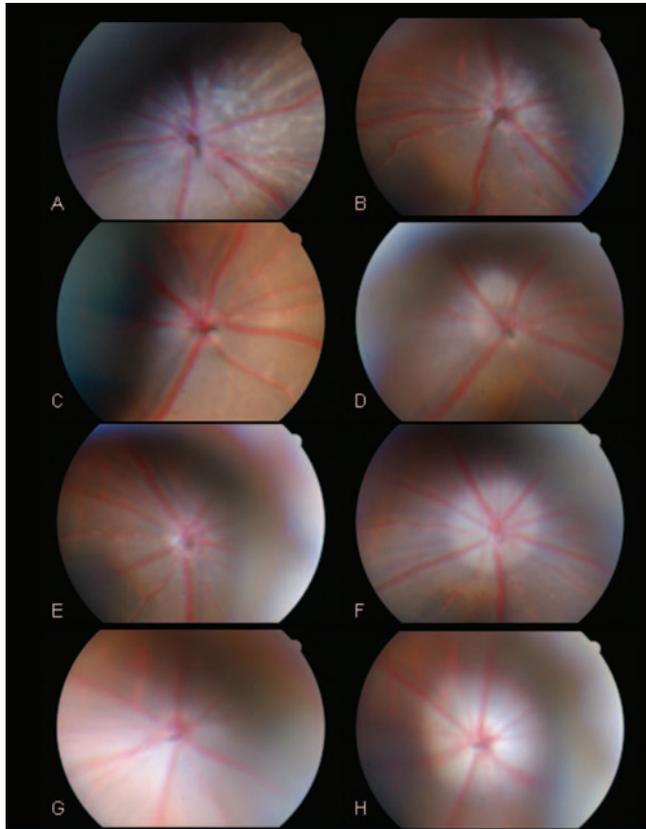


FIGURE 9. Fundus photographs before (A, C, E, G) and after (B, D, F, H) laser irradiation for different durations. A power of 100 mW was applied for 1 (B), 2 (D), 3 (F), or 5 (H) minutes. After 100 mW for 1 minute (B), optic nerve heads showed no changes in colors or shapes, whereas after 100 mW for 2 minutes (D), slight peripapillary whitening was observed. Longer durations also increased peripapillary whitening (F, H).

only induced in other cells of the optic nerve tissue. These findings support a concept that cell-to-cell transport of Hsp70 is possible, and RGCs themselves are not a unique target of

Hsp70 induction for neuroprotection. There is a possibility that a glial cell-to-RGC transport of Hsp70 may also contribute to neuroprotection.

In experimental studies on the optic nerve, temporal analysis is important. The confocal scanning laser ophthalmoscope can provide serial quantitative data on morphologic changes in the optic nerve head without killing experimental animals. Pseudo-3-D images obtained after 100 mW showed progressive optic nerve head cupping after 1 day and 3 days. At 1 week, the entire retinal area including the optic nerve head surface was depressed. These findings reflect generalized optic nerve head damage. Because of a backward movement of the reference plane, the recovered appearance of topographic images at 1 week after TTT could have been corrected by using pseudo-3-D imaging. Our confocal scanning laser ophthalmoscope results showed that after 100- or 120-mW power applied for 1 minute there was no damage, but there was an inflammatory change in the optic nerve head after laser irradiation at 120 mW. Thus, we conclude that 100 mW for 1 minute is optimal.

To observe the optic nerve head by SEM, the vitreous had to be removed. In control eyes and after 100 mW for 1 minute, vitreous was removed easily. However, this was difficult after 120 or 140 mW power for 1 minute. In SEM images, optic nerve heads showed membranlike attachment of vitreous after 120 mW and irregular coarse attachment of vitreous after 140 mW (Fig. 7). These findings may be due to inflammatory adhesion between the vitreous and retina. Moreover, the lymphocyte-like cells observed on the optic nerve head by SEM may be associated with a breakdown of the blood-retinal barrier by photocoagulation or autoimmune reaction.²⁵

The duration of irradiation is also an important factor in the laser's effect on the optic nerve head.²⁶ Thus, in the present study, we observed optic discs after its exposure to 100 mW for 1, 2, 3, and 5 minutes. Peripapillary whitening was observed for exposures exceeding 1 minute, and whitening areas enlarged as the exposure duration increased. Peripapillary arteriolar constriction was evident after 4 minutes of exposure.

Our Western blot results show that small amounts of Hsp are expressed constitutively in the optic nerve head. Such constitutive expression in the optic nerve head may be due to basal stress levels. By Western blot analysis, we observed that Hsp70 expression increased progressively with increasing

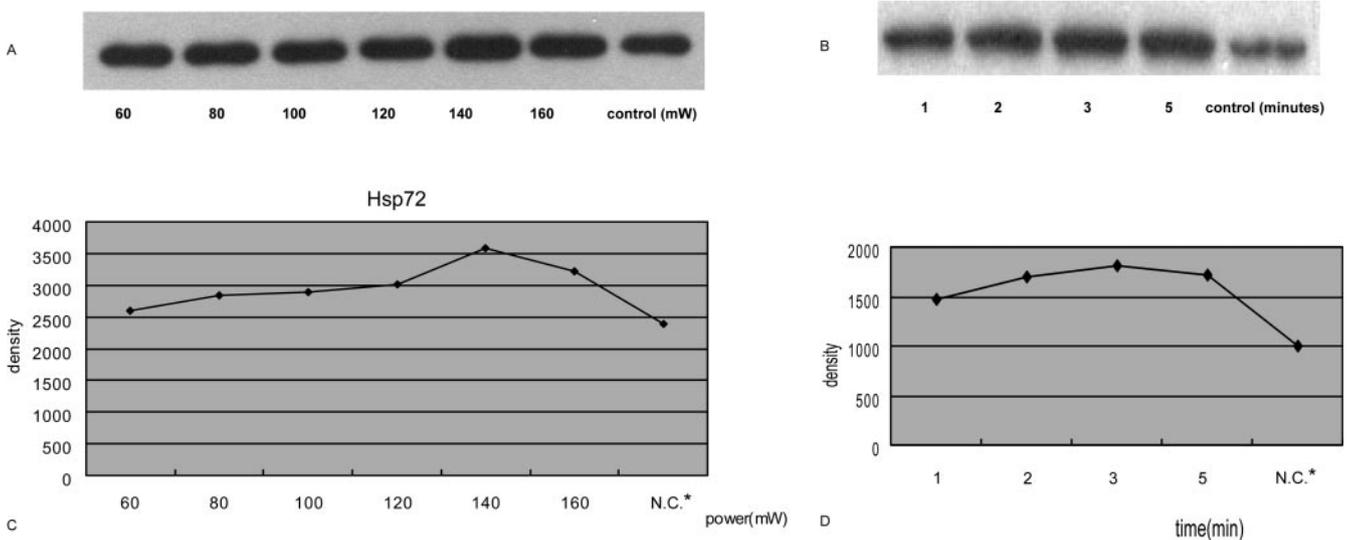


FIGURE 10. When power levels were modulated (A), Hsp70 expression was found to be higher after TTT treatment (all at 60 seconds) than in control eyes. With changes in exposure duration (B), Hsp70 expression was found to increase up to 3 minutes. (C, D) Densitometry of the Western blot shown in (A) and (B). *Normal control.

power, but decreased slightly at >140 mW. When exposure durations were extended, Hsp70 expression was also observed to decrease after 100 mW for 5 minutes.

The advantage of TTT in inducing Hsp is that it can be applied to selected areas such as on the optic nerve head where glaucomatous damage is known to develop. In addition, Hsp70 can be induced without a risk of systemic adverse effects.

In conclusion, our study confirms that TTT application to the optic nerve head induces Hsp70 expression, and demonstrates that the optimal laser setting for Hsp70 induction without tissue damage is 100 mW for 60 seconds. This result provides baseline data for future neuroprotective experiments in glaucoma that are designed to enhance natural cytoprotective mechanisms.

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