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Expression of heat shock protein 70 in rat cornea during wound healing following alkali burn*

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Abstract

BACKGROUND: Many factors can influence the wound healing of cornea following alkali burn, heat shock protein can promote the rapidly recovery and clear of the degenerated and injured protein.

OBJECTIVE: To investigate whether the expression of heat shock protein 70 after corneal alkali burn in rats is related to corneal wound healing process.

METHODS: After the rat eye was checked without inflammation and other lesions, oxybuprocaine eye drops were dropped into the rat eye for twice. Conjunctival sac liquid was sucked by swab, and then the unified specification filter paper of 5 mm diameter was soaked in 1 mol/L NaOH solution for 10 seconds, then the filter paper was placed in the center of the rat cornea for 30 seconds to make the rat corneal alkali burn model. The corneal specimens were obtained at 6 hours, 1, 3, 7, 14 and 21 days after alkali burn.

RESULTS AND CONCLUSION: Reverse transcription polymerase chain reaction, immunohistochemistry staining and Western blot observation showed that the expression level of heat shock protein 70 increased at 1 day after alkali burn and reached its peak at 7 days. Then, it decreased at 14 days. Hematoxylin-eosin staining and transmission electron microscope observation showed that the corneal injury was most obvious at 6 hours after burning and the wound was gradually recovered at 7 days after burning. Alkali burned cornea can induce heat shock protein 70 synthesis in a time-dependent manner, also in accordance with the corneal repair procedure. Induced heat shock protein 70 plays an important role in wound healing.

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INTRODUCTION

Corneal alkali burn is a serious ocular trauma and may cause severe and permanent visual impairment[1]. Healing following alkali burn is characterized by the extended inflammatory cells infiltration in injured corneas, recurrent epithelial defects, stromal edema, and neovascularization. These characteristics interfere with proper healing processes and result in the formation of scar tissue and recurrent corneal erosion[2]. Present clinical therapies are limited. A number of growth factors and cytokines are believed to be involved in the tissue destruction and late scarring that occurs in the cornea after alkali burn[3-4]. However, the precise mechanisms of alkali injury and the wound healing response remain poorly understand.

Although heat shock proteins (HSPs) were usually synthesized in cells exposed to higher temperature, they are also upregulated by other forms of stress[5]. HSPs have been called a molecular chaperone and are thought to control cell death or protect damaged cells. The role of HSPs in interacting with other proteins and maintaining appropriate states of protein folding prior to the synthesis of other proteins, their intracellular transport or signal-specific activation, provides cells a mechanism for enhance the production of these proteins following thermal stress when the needs to inhibit inappropriate associations of partially denatured proteins will be increased. HSPs chaperone denatured proteins in the stressed cells, giving the proteins time to reestablish their normal structure and escaping the risk of cell death[6-7]. Furthermore, cytoplasmic HSPs are involved in the caspase-3 and caspase-9 apoptosis pathway. HSPs help to prevent immediate apoptosis of the cell after a shock, allowing repair mechanisms to function and giving the cell a chance to survive.

The HSP70 family is the most conserved and best-studied family. There are several HSP70 family members, including highly stress-inducible HSP70 (also known as HSP72 or HSP70i), constitutively expressed heat shock cognate protein HSC70 (HSP73), a mitochondrial HSP75 (mtHSP75) and Grp78 (BiP) localized in the endoplasmic reticulum[8-9]. Under various stress conditions, the synthesis of stress-inducible HSP70 enhances the ability of

stressed cells to cope with the increased concentrations of unfolded and/or denatured proteins. For this reason, we purposed to examine the pattern of inducible HSP70 expression induced by corneal alkali burn *in vivo* and to explore the relationship of the changes with corneal damage repair.

MATERIALS AND METHODS

Design

A self-controlled animal experiment.

Time and setting

All experiments were performed at the Experimental Animal Center of the Sichuan University from June 2010 to July 2011.

Materials

Animals

Sixty adult Wistar rats (either male or female) weighing 200−250 g were purchased from the Experimental Animal Center of the Sichuan University, China. All experiments were carried out according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. We performed slit lamp (SLM-3, KongHua Company, Chong Qing, China) biomicroscopic examinations before preconditioning to exclude any other defects such as corneal abnormality or cataract.

Reagents and equipments

Reagent and instrument	Source
0.4% oxybuprocaine	Alcon, Texas, USA
Trizol Reagent	Invitrongen, Carlsbad, CA, USA
One-Tube Expand RT-PCR Kit	TaKaRa, Dalian, China
A video-densitometer	Bio-Rad, California, USA
Anti-HSP70 monoclonal antibody, anti-rat IgG	Boster, Wuhan, China
Elivision^{TM} Kit	Maixin Bio, Fuzhou, China
Microscope	Olympus, Tokyo, Japan
A image collection system	Nikon ECLIPSE E600, Tokyo, Japan
Image-pro-plus Image Analyzer	Sichuan University, Chengdu, China
Transmission electron microscope	Hitachi, Tokyo, Japan

Methods

Establishment of corneal alkali burn models in rats

Preoperative examinations were performed under anesthesia with 10% chloral hydrate by intraperitoneal injection (3 mg/kg). The eyes were then treated with 0.04 mL oxybuprocaine in the concentration of 0.4%. After application, the eyes were washed with saline and blotted dry. A 4 mm filter paper was saturated in 1 mol/L NaOH, randomly applied to the center of corneas for 30 seconds, and excess rinsing the eyes with 0.9% NaCl solution for 5 minutes[10]. The other eye was treated with 0.9% NaCl solution filter paper alternatively, and served as controls. Rat cornea was examined using a slit-lamp daily. Atropine and erythromycin ointment were instilled into the operated eye twice a day. Any animal with clinically suspected ocular infection or corneal perforation was immediately excluded from the study.

Preparation of the specimens

There were six experimental groups according to the time that eyes were enucleated at 6 hours, 1, 3, 7, 14 and 21 days following alkali burn (n=10 in each group. five rats in each group used for reverse transcription-PCR and Western blot, the other five rats used for immunohistochemical observation under transmission electron microscope). Corneas without alkali burn were served as controls. Each cornea was cut into two halves.

Reverse transcription-PCR analysis of corneal HSP70 mRNA expression

Total ribonucleic acid from the cornea was isolated after 6 hours, 1, 3, 7, 14 and 21 days contusion, experimental rats were sacrificed and total RNA was isolated from half of each cornea using Trizol Reagent. Complementary deoxyribonucleic acid (cDNA) was synthesized and subjected to RT-PCR for detecting HSP70 mRNAs using a One-Tube Expand RT-PCR System Kit. The following primer pairs were used: HSP70 cDNA primer, up-stream: 5'-ATG AAG GAG ATC GCC GAG G-3'; down-stream: 5'-AGG TCG AAG ATG AGC ACG TTG-3' (amplifying a 238 bp fragment); β-actin cDNA primer, up-stream: 5'-GCT ACG TCG CCC TGG ACT TC-3'; down-stream: 5'-GTC ATA GTC CGC CTA GAA GC-3' (amplifying a 526 bp fragment which was used as the internal standard). Amplification was performed in a thermal cycler using the following conditions: 30 cycles of 94 °C for 30 seconds, 56 ℃ for 20 seconds, 72 ℃ for 30 seconds, and 72 ℃ for 30 seconds. PCR products were analyzed by electrophoresis in 0.8% agarose gels. The intensities of the amplified cDNA fragments were estimated using a video-densitometer.

Hematoxylin-eosin staining observation of corneal pathological changes and immunohistochemical observation of corneal HSP70 expression

HSP70 detection was performed at 6 hours, 1, 3, 7, 14 and 21 days after contusion. Corneas were dissected and fixed with 10% buffered formaldehyde at room temperature overnight, immersed in a series of graded isopropanol solutions, rinsed several times with PBS, and then embedded in paraffin. Serial 4−5 μm thick paraffin sections were cut and mounted onto microscope slides. For hematoxylin-eosin staining, slides were refixed, washed, and stained with hematoxtlin-eosin. For immunohistochemical staining, slides were deparaffinized, rehydrated, and pretreated

with 0.3% hydrogen peroxide in PBS to decrease endogenous peroxidase activity. Sections were incubated with an anti-HSP70 monoclonal antibody (1:200) at room temperature for 45 minutes, and the primary antibodies were localized by immunoperoxidase using a commercial kit (Elivision^{TM} Kit). Sections were rinsed with PBS then incubated with appropriated biotinylated secondary antibodies for 30 minutes at room temperature. Sections were rinsed again with PBS before being exposed to horseradish peroxidase labeled streptavidin for 30 minutes. After several washes with distilled water, bound antibody was visualized with a 3,3'-diaminobenzidine-nickel detection system. Sections were counterstained with hematoxylin-eosin and coverslipped with a mounting medium. Slides were examined and documented with a microscope equipped with bright-field illumination. Images were recorded by image collection system attached to the microscope. The values of average optical density were measured by Image-pro-plus Image Analyzer in positive results from immunohistochemical experiments in order to analyze the expressional difference of HSP70 in semiquantitative conditions.

Western blot observation of corneal HSP70 expression

Western blot analysis of HSP70 was performed on the control and alkali burn groups. Corneas in each Western blot groups were homogenized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis buffer and boiled for 4 minutes. The proteins from corneas extracts were separated on an SDS-PAG and were transferred to nitrocellulose membranes. The membrane was blocked by incubation in 0.1% Tween-20 of Tris-buffered saline containing 5% nonfat dried milk for 1 hour. The membrane was incubated with rat monoclonal antibodies of HSP70 (1:1 000) for 3 hours and then incubated with goat anti-rat IgG (1:5 000;) for 1 hour. 0.1% Tween-20 of Tris-buffered saline was used to shake and wash the membrane at room temperature for three times, 5 minutes each time. At last, added 3,3'-diaminobenzidine staining.

Observation of the corneal ultrastructure by transmission electron microscope

Corneas prepared for transmission electron microscope observation were fixed with 3% buffered glutaraldehyde and followed by postfixed with 1% buffered osmium tetroxide. After fixation, the corneas were dehydrated in graded alcohols and embedded in epoxy resin. Semithin sections were taken at various levels, stained with paraphenylenediamine, and examined by optical microscope. Representative areas were ultrathin-sectioned, double stained with uranyl acetate-lead citrate, and examined with H-600IV transmission electron microscope.

Main outcome measure

Expression of corneal HSP70, pathological and ultrastructural changes of cornea after alkali burn.

Statistical analysis

SPSS 12.0 software was used for analysis. All values were reported as mean±SD. Statistical evaluation was performed using analysis of one-way analysis of variance test. The 0.05 level of significance was accepted for statistical difference.

RESULTS

Quantitative analysis of experimental animals

All 60 rats were included in the final analysis.

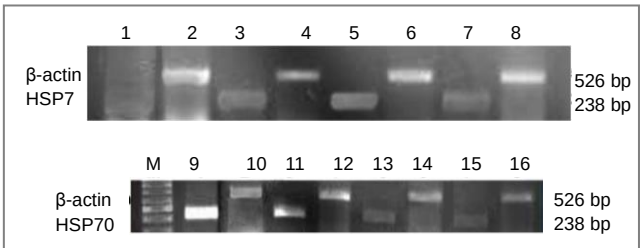
Pathological changes of rat cornea after alkali burn

Hematoxylin-eosin staining of alkali burned and control corneas showed that at 6 hours after the alkali burn, the corneal epithelium in the burned area completely disappeared and polymorphonuclear neutrophils (PMNs) began to infiltrate, which involved the superficial stromal layer. The opaque and distinct edematous margin appeared in the injured central corneal stroma. One day after the alkali burn, the corneal epithelium covered stroma was irregularly thickened in non-ulcerated regions and not accompanied by an identifiable basement membrane or Bowman's layer. The intercellular space was widened and PMNs infiltration was more. Three days after the alkali burn, the corneal epithelium covered the wound region, but the cell layers were fewer, accompanied with the superficial cells vacuolar degeneration, the inflammatory cell infiltration, and the peripheral neovascularization extended from the limbus toward the central cornea. Seven days after the alkali burn, the number of epithelial cell layers multiplied and the cells were arranged neatly, which had a certain restoration compared with the former. However, the surface of the epithelium of alkali burned cornea was rough and exhibited excessive sloughing. In addition, the inflammatory cell infiltration and neovascularization were obvious. The base membrane was thinner and discontinuous in many regions of the alkali burned cornea. At 14−21 days after the alkali burn, the corneal epithelium was recovered. Regeneration of the base membrane showed more clear linear patterns. The stroma showed fewer inflammatory cells but more fibroblasts and stroma scar formed.

Expression of HSP70 mRNA and protein in rat corneas

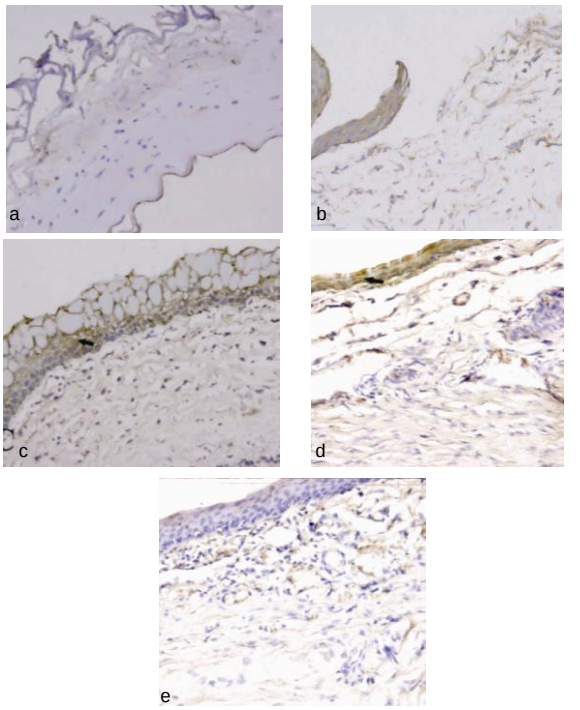
HSP70 expression pattern was elucidated with reverse transcription-PCR, Western blot and immunohistochemical staining. The expression of HSP70 mRNA in rat corneas was weakly positive or negative, but increased by 6 hours after alkali burn,

reached its peak at 7 days, and decreased gradually by 14 days (Figure 1). Immunostaining of alkali burned corneas for HSP70 expression showed a time-dependent pattern (Figure 2, Table 1). The expression of HSP70 could be weakly detected in both control corneas and traumatic corneas at 6 hours after alkali burn. But it increased at 1 day after the alkali burn and was strongest in the regenerated epithelium layer of corneas at 7 days. After that, it began to fall from 14 days to 21 days later. Moreover, HSP70 protein expression was mainly in the cytoplasm of the regenerative epithelial cells and the cell nucleus expressed weakly positive or negative. In addition, the expression of HSP70 was analyzed by Western blot (Figure 3). The results were consistent with the results of PCR and immunohistochemistry staining.



M: Marker; Lanes 2, 4, 6, 8, 10, 12, 14, 16: Expression of β-actin; Lane 1, 15: expression of HSP70 in the control group; Lanes 3, 5, 7, 9, 11, 13: Expression of HSP70 at 6 h,1, 3, 7, 14 and 21 d after alkali burn, respectively

Figure 1 Expression of heat shock protein 70 (HSP70) mRNA at different time points after corneal alkali burn in rats (Reverse transcription-PCR)



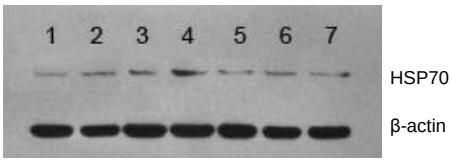
a–e: Expression of HSP70 at 6 h, 1, 3, 7 and 14 d after corneal alkali burn, respectively (staining)

Figure 2 Expression of heat shock protein 70 (HSP70) at different time points after corneal alkali burn in rats (Immunohistochemistry staining, ×400)

Table 1 Analysis of heat shock protein 70 expressions at different time points after corneal alkali burn in rats ($\bar{x}\pm s$, n=5)

Group	Absorbance value
Control	0.22±0.05
6 h	0.23±0.05
1 d	0.33±0.06[ab]
3 d	0.87±0.14[abc]
7 d	1.09±0.15[abcd]
14 d	0.33±0.07[abde]

[a]P < 0.05, *vs.* control group; [b]P < 0.05, *vs.* 6 h; [c]P < 0.05, *vs.* 1 d; [d]P < 0.05, vs. 3 d; [e]P < 0.05, *vs.* 7 d

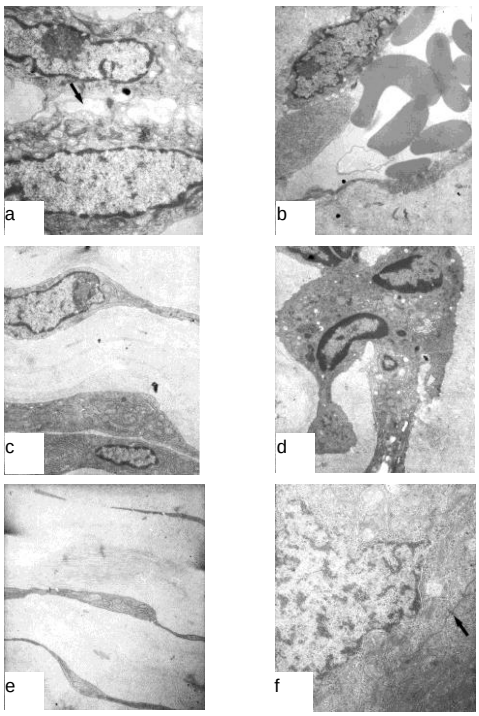


Lane 1, 2, 3, 4, 5, 6, 7: Expression of HSP70 at 6 h, 1, 3, 7, 14 and 21 days after alkali burn and control, respectively

Figure 3 Expression of heat shock protein 70 (HSP70) at different time points after corneal alkali burn in rats (Western blot). The level of β-actin expression serves as internal standard

Ultrastructural changes of rat cornea after alkali burn

At 6 hours and 1 day after the alkali burn, the corneal epithelia began to fall off and PMNs infiltration occurred. At 3 days after the alkali burn, the corneal epithelia started to regenerate. At 7 days after the alkali burn, the thickness of the regenerative epithelium was uneven, the polar arrangement of the cells in some regions was unclear, the cellular space was wide and formed the crevices with different widths, the bridge corpuscle structure between the cells was decreased obviously and some regions disappeared (Figure 4a). Neovascularization were found around the newborn collagen fibers in the stroma, the wall of the capillary vessel was thickened and the red cells could be seen in the cavity (Figure 4b). Fibroblasts were transformed from the static type to the secretion type. The mitochondria, rough surfaced endoplasmic reticulum and Golgi apparatus multiplied in the stroma, and different degrees of edema and degeneration could be seen (Figure 4c). Part of the mitochondria had vacuolar degeneration. The stroma collagen fibers were thicker, intersection and fracture, arranging in disorder. There was also a lot of neutrophils infiltration in the stroma of the cornea and the cytoplasm of the neutrophils contained a lot of phagolysosomes (Figure 4d). At 14 and 21 days after the alkali burn, the corneal epithelia were almost recovered, the regenerative epithelial intercellular space was narrowed and the bridge corpuscle structure was obvious. The fibrocytes in the corneal stroma became the main cell type (Figures 4e, f).



a: Regenerative epithelial cells with magnificant intracellular space (arrow); b: Proliferating vascular channels; c: Fibroblasts (secretion type); d: Polymorphonuclear neutrophils; e: Fibroblasts (static type); f: Regenerative epithelial cells with bridge corpuscle structure (arrow)

Figure 4 Observation of the rat corneas at 7 d (a–d) and 21 d (e–f) after alkali burn under transmission electron microscope (×15 000)

DISCUSSION

It is well known that HSP70 is the major form of HSP induced in eye injuries. For example, the research on HSP70 expression under high intraocular pressure showed that HSP70 played an important role in promoting the survival of ganglion cells in neurodegenerative disease, which might suggest a novel pathway for neuroprotection in patients with glaucoma[11-12]. In addition, other research showed that HSP70 could promote the regeneration and wound healing of the corneal epithelium after excimer laser ablation[13]. Few studies have been performed on HSP70 induction in alkali burned cornea.

The basic pathological change of the corneal injury mechanism after alkali burn is the fast infiltration of PMNs to the alkali burned region. PMNs generates a lot of superoxide radicals, proteolytic enzyme and active oxygen, which produce non-specific injury on the corneal tissues and cause the structural function abnormality. The generation of heat shock proteins can improve the protective action of cells on the damage factors, but the molecular mechanism is much more complicated. It may be explained by the chaperoning function of HSP70 on proteins that are important for the survival of cells. HSP70 participate in the folding and assembly of nascent and unfolded peptides, and they facilitate protein transport to specific subcellular compartments and disposal by degradation[14-15]. In addition, HSP70 are involved in multiple stages of the apoptosis pathway and function to inhibit apoptosis[16-17]. Moreover, some studies showed that heat shock protein could promote the injure recovery and elimination of the degenerative proteins, which can maintain the self-stability of the cells[11, 13]. Our study confirmed the hyperexpression of HSP70 in alkali burned cornea. Therefore, we thought that when corneal alkali burn occurred, the expression of HSP70 can promote the cells to maintain the self-stability, thus facilitate the recovery of the damaged cornea, while the exact mechanism needs further investigation.

Corneal neovascularization and subsequent opacification remain the most frequent causes of blindness following alkali burn injury. Most of this poor prognosis is due to the limbal stem cell damage and delayed epithelial healing. The main strategies in managing such injuries include prompt washing to reduce further damage, decreasing inflammation, and promoting healing and epithelialization to prevent ulceration and perforation[18]. HSP70 can be synthesized in a wide variety of cells such as rat islet cells, cardiomyocytes and cultured hepatocytes in response to heat or cold treatment, and it is known that the degree of HSP70 induction varies according to the recovery time after the thermal stress[19-21]. Our results demonstrated that alkali burned cornea could induce HSP70 synthesis in a time-dependent pattern, also in accordance with the corneal repair procedure. Moreover, the expression of HSP70 was confined to the corneal epithelia. Epithelial healing in the cornea depends on cell migration, proliferation and the integrity of epithelial basement membrane. Regenerated epithelium has an increased capacity of synthesizing HSP70, a known self-protective factor, and the maximal induction of HSP70 coincides with the wound healing in the alkali burned corneas. The capacity of the regenerating and healing corneal epithelia to form the protein has the pathologic significance. At 1−2 weeks after the alkali burn, the cornea goes into the recovery stage. It is useful to induce stress-inducible HSP70 to defend and treat corneal alkali burn.

In conclusion, our study demonstrated that the expression of HSP70 was in a time-dependent pattern, also in accordance with the corneal repair procedure. How HSP70 regulates corneal repair and the roles of other members of the HSP family is not yet known. Further experimental studies are required.

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大鼠角膜碱烧伤后热休克蛋白 70 的表达*

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文章亮点：实验发现大鼠角膜碱烧伤后热休克蛋白 70 的表达与角膜损伤修复过程相一致，提示热休克蛋白 70 参与了大鼠角膜碱烧伤后细胞的自我保护及修复过程。

关键词：角膜；碱烧伤；热休克蛋白；大鼠；修复

摘要

背景：角膜碱烧伤后损伤修复受许多因素的影响，热休克蛋白可促进变性、损伤蛋白质的迅速恢复或清除。

目的：观察大鼠角膜碱烧伤后热休克蛋白 70 的表达及其与角膜损伤修复的关系。

方法：检查大鼠眼无炎症及其他病变后，奥布卡因滴眼液点眼 2 次，棉签吸除结膜囊液体，将统一规格直径 5 mm 的滤纸片浸泡于 1 mol/L NaOH 溶液中 10 s，然后置于大鼠角膜中央 30 s 制作大鼠角膜碱烧伤模型。分别于碱烧伤后 6 h，1，3，7，14，21 d 取材。

结果与结论：RT-PCR、免疫组织化学染色、Western blot 结果均显示热休克蛋白 70 mRNA 和蛋白在角膜碱烧伤后 1 d 即开始升高，7 d 时达高峰，14 d 后开始下降。苏木精-伊红染色及电镜观察显示角膜损伤在烧伤后 6 h 即较明显，烧伤后 7 d 逐渐恢复。提示大鼠角膜碱烧伤后热休克蛋白 70 的表达与碱烧伤后角膜损伤修复过程一致，参与了大鼠角膜碱烧伤后细胞的自我保护及修复过程。

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