Hemin Promotes Corneal Allograft Survival Through the Suppression of Macrophage Recruitment and Activation

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Purpose. To explore the roles of hemin in preventing corneal allograft rejection (CGR) and the underlying mechanisms.

Methods. Hemin (30 mg/kg) was intraperitoneally injected into rats with a corneal allograft on alternate days, from the day of transplantation until euthanasia. The clinical signs of the corneal allografts were evaluated and recorded according to a previously published system. Corneal edema, macrophage infiltration, and phenotype, and the expression of chemokines, cytokines, and heme oxygenase (HO)-1 were detected by histology, real-time PCR, and Western blot. The rat macrophage cell line NR8383 was used to explore the mechanisms of action of hemin in vitro.

Results. Treatment with hemin significantly prolonged corneal allograft survival, with decreased corneal edema and fewer macrophages. Moreover, hemin treatment alleviated inflammation in the corneal grafts, as characterized by downregulated mRNA levels of proinflammatory mediators. In addition, hemin administration reduced the proportion of proinflammatory M1 macrophages and increased the proportion of anti-inflammatory M2 macrophages in the corneal grafts. Hemin treatment induced HO-1 expression in vivo and in vitro, whereas co-administration of zinc protoporphyrin IX (ZnPP), an HO-1 inhibitor, blocked the beneficial effects of hemin in preventing CGR.

Conclusions. Our results are the first to demonstrate that hemin, a Food and Drug Administration–approved drug, promotes corneal allograft survival. These findings indicate that hemin might be a potential alternative treatment for CGR.

Keywords: hemin, HO-1, corneal allograft rejection, macrophages

Materials and Methods

Rats

Female Wistar rats and Sprague–Dawley rats (6- to 8-weeks old) were acquired from the Guangzhou Animal Testing Center. The Institutional Animal Care and Use Committee of the Zhongshan Ophthalmic Center at Sun Yat-sen University approved all experimental protocols. All procedures involving animals adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were cared for and treated in strict pathogen-free conditions under a 12-hour light-dark cycle with regulated temperature (23 ± 2°C) and humidity (55 ± 10%).
Corneal Transplantation and Definition of Graft Rejection

A rat model of penetrating keratoplasty was established as previously described. Briefly, a donor corneal graft was prepared using a 3.5-mm diameter corneal trephine. Excising a circular 3.0-mm central area of the cornea made the recipient graft bed. The excised donor graft was set onto the recipient graft bed, and then sutured with eight interrupted 10-0 nylon sutures (Alcon, Sinking Spring, PA, USA) followed by the application of antibiotic ointment. The sutures were not removed till the end of observation period (day 30). Monitoring of clinical manifestations with a slit-lamp examination was carried out every second day. Graft transparency, edema, and neovascularization parameters were used to evaluate the corneal grafts according to the previously established scoring system. Three parameters have five grades (0–4 scores) respectively (Table). When the total scores of these three parameters are equal or greater than six scores, the graft is identified as rejection.

### Rat Treatment

Hemin (30 mg/kg, intraperitoneal [ip]) and ZnPP (50 μmol/kg, ip) obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) or physiologic saline (3 mL/kg, ip) was administered to rats with application of antibiotic ointment. The sutures were not removed till the end of observation period (day 30). Monitoring of clinical manifestations with a slit-lamp examination was carried out every second day. Graft transparency, edema, and neovascularization parameters were used to evaluate the corneal grafts according to the previously established scoring system. Three parameters have five grades (0–4 scores) respectively (Table). When the total scores of these three parameters are equal or greater than six scores, the graft is identified as rejection.

### Cell Culture and Treatment

The NR8383 rat macrophage cell line (ATCC, Rockville, MD, USA) was cultured in medium, which contained Ham’s F12K medium (Gibco BRL, Grand Island, NY, USA), 15% heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin, at 37°C and 5% CO₂. The macrophages were seeded in 24-well plates and cultured for 24 hours. Then, hemin (10 μM) and/or ZnPP (10 μM) was added to the cells for 1 hour, and cells were subsequently activated with IFN-γ (25 ng/mL; R&D Systems, Minneapolis, MN, USA) and lipopolysaccharide (LPS; 100 ng/mL; Sigma-Aldrich Corp.) for 24 hours.

### Histology

For histologic analysis, rat eyes from all groups were excised and fixed in 10% paraformaldehyde. Then, eyes were embedded in paraffin wax, sliced into 6-μm thick sections, and stained with hematoxylin and eosin (HE). The number of inflammatory cells was counted from three random fields. Corneal thickness was measured in three separate areas of the transplanted cornea. The mean of these three readings was calculated using a Leica microscope and Leica Application Suite 4.1.0 software (Buffalo Grove, IL, USA).

### Immunofluorescence

In brief, rat eyes were embedded in optimal cutting temperature compound, serially sectioned (8 μm), and stored at −80°C. The sections were permeabilized with 0.3% Triton X-100 for 10 minutes at 37°C. Subsequently, cryosections were blocked with 3% BSA for 1 hour at 37°C. The cryosections were then incubated with primary antibodies against HO-1 (Abcam, Cambridge, MA, USA), CD68 (Abcam), inducible nitric oxide synthase (iNOS; Abcam), and CD163 (Abcam) at 4°C overnight. After washing with PBS, the sections were incubated with Alexa Fluor 488 (Abcam) or Alexa Fluor 594 (Invitrogen, Rockford, IL, USA) secondary antibodies at room temperature for 1 hour. 4′,6-diamidino-2-phenylindole (DAPI) was applied to counterstain cell nuclei. All sections were evaluated with a fluorescence microscope. The counts of cell numbers were quantified by Adobe Photoshop CC (Adobe Systems, Inc., San Jose, CA, USA) from three random fields.

### Western Blot Analysis

Rat corneal lysates were separated by SDS-PAGE and electrotransferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Then, the membrane was blocked with 5% nonfat dry milk in tris-buffered saline and probed with antibodies against HO-1 (Abcam) and β-actin (Abcam). Subsequently, the membrane was incubated with an horse radish peroxidase-conjugated secondary antibody. The improved chemiluminescence reagent kit (Pierce, Rockford, IL, USA) was used to visualize the signals.

### Quantitative Real-Time PCR

The total RNA was obtained using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Then, RNA was quantified with a NanoDrop spectrophotometer (ND-1000; NanoDrop Technologies, Wilmington, DE, USA). Next, complementary (c)DNA was synthesized with an Omniscript RT Kit (Qiagen). Then, we used Absolute SYBR Green ROX Mix (Thermo, Waltham, MA, USA) to quantify the level of mRNA. Last, the results were analyzed by the 2^ΔΔCt method, and the expression level of each gene was normalized to the level of β-actin.

### Statistical Analysis

Student’s t-test or one-way ANOVA was used to analyze for significant differences (SPSS 18.0; IBM, Armonk, NY, USA). P < 0.05 was considered significant.

<table>
<thead>
<tr>
<th>Clinical Parameter</th>
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<tr>
<td>Clarity</td>
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<td>Mild opacity with clear anterior chamber</td>
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<td>None</td>
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<tr>
<td>Slight</td>
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<tr>
<td>New vessels invading the peripheral cornea</td>
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</tr>
<tr>
<td>New vessels growing up to peripheral graft</td>
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<td>New vessels invading the entire graft</td>
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RESULTS

Hemin Treatment Prolonged Corneal Allograft Survival

To explore the role of hemin in CGR, we performed corneal transplantations using Wistar rats as donors and Sprague-Dawley rats as recipients. The effect of hemin treatment on the corneal allografts was assessed for 30 days after the surgery. Physiologic saline (3 mL/kg, ip) was administered to the recipient as a vehicle control (hemin \(\Delta\) group) Corneal isograft (Sprague-Dawley to Sprague-Dawley) was performed as a negative control. Evidences of graft opacity, edema, and neovascularization were evaluated by slit-lamp microscopy (Fig. 1A). All corneal grafts in the hemin \((-)\) group were rejected within 15 days, with a mean survival time of 12 days, whereas all of corneal grafts in isograft group survived till the end of observation period (day 30; Fig. 1B). Compared with hemin \((-)\) group, hemin treatment delayed rejection onset and reduced the rate of rejection. Three of 10 allografts in hemin \((-)\) group were nonrejection whereas one of 10 allografts in hemin \((-)\) rats treated with physiological saline (Fig. 1B). However, the co-administration of HO-1 inhibitor ZnPP (50 \(\mu\)mol/kg) significantly abolished the hemin-mediated promotion of corneal allograft survival (Fig. 1B). The grafts of postoperative day (POD) 15 (the average day of rejection from neovascularization were evaluated by slit-lamp microscopy (Fig. 1A). All corneal grafts in the hemin \((-)\) group were rejected within 15 days, with a mean survival time of 12 days, whereas all of corneal grafts in isograft group survived till the end of observation period (day 30; Fig. 1B). Compared with hemin \((-)\) group, hemin treatment delayed rejection onset and reduced the rate of rejection. Three of 10 allografts remained nonrejection in hemin \((+\) group, whereas one of 10 allografts in hemin \((-)\) rats treated with physiological saline (Fig. 1B). However, the co-administration of HO-1 inhibitor ZnPP (50 \(\mu\)mol/kg) significantly abolished the hemin-mediated promotion of corneal allograft survival (Fig. 1B). The grafts of postoperative day (POD) 15 (the average day of rejection from
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The results demonstrated that TNF-α and iNOS mRNA expression in corneal allografts at POD4. Our findings suggested that hemin treatment significantly decreased the number of infiltrating macrophages prior to the visible rejection compared with the hemin (-) group (Fig.2B).

Because macrophage inflammatory protein (MIP)-1α and monocyte chemotactic protein (MCP)-1 are the two main chemokines implicated in macrophage recruitment, we explored the effects of hemin on these chemokines in the grafts at POD4. As shown in Figure 2C, the expression of MIP-1α and MCP-1 was reduced in the corneal allografts, which were treated with hemin compared with the grafts in untreated group. Moreover, we explored the effect of hemin on TNF-α and iNOS mRNA expression in corneal allografts at POD4. Our results demonstrated that TNF-α and iNOS mRNA expression in the hemin (+) group was markedly downregulated compared with the hemin (-) group (Fig. 2D).

Hemin Primarily Suppressed the Afferent Phase in Corneal Allograft Rejection

We next want to determine whether the hemin treatment effect to the afferent phase, efferent phase, or both in corneal allograft rejection. The surgery- and suture-induced inflammation is crucial for allograft antigen transport in afferent phase of corneal allograft rejection. Thus, corneal isografts were used to explore the role of hemin in the surgery- and suture-induced inflammation. We found that the surgery and suture also induced macrophage infiltration, whereas hemin treatment decreased the number of infiltrating macrophages (Figs. 3A, 3B). Similar with corneal isografts, the mRNA expression of MIP-1α, MCP-1, TNF-α, and iNOS increased by the surgery and suture was also decreased by hemin treatment in corneal isografts (Figs. 3C, 3D).

Next, we administrated hemin from the POD7 in corneal allografts (Fig. 3E). Our results showed that injection of hemin from the POD7 only slightly prolonged corneal allografts survival (Fig. 3F). Together, these results suggested hemin primarily suppressed the afferent phase in corneal allograft rejection.

Hemin Decreased the Proportion of Proinflammatory M1 Macrophages and Increased the Proportion of Anti-Inflammatory M2 Macrophages in Corneal Allografts

Macrophages are generally divided into proinflammatory M1 and anti-inflammatory M2 phenotypes. Thus, we explored the effects of hemin on macrophage subtype. Immunofluorescence was applied to examine the number of proinflammatory macrophages (CD68+/iNOS+ cells) and anti-inflammatory macrophages (CD68+/CD163+ cells) in corneal allografts at POD4 (Fig. 4A). The hemin (+) group exhibited a dramatically reduced proportion of proinflammatory macrophages but an increased proportion of anti-inflammatory macrophages compared with the hemin (-) group (Figs. 4B, 4C).

Hemin Suppressed Macrophage Activation by Inducing HO-1

The effects of hemin on the activation of macrophages were assessed using the rat macrophage cell line NR8383. The macrophages were activated with IFN-γ (25 ng/mL) and LPS (100 ng/mL) for 24 hours. Compared with the LPS group, IL-1β and TNF-α expression decreased notably in the hemin (+) group (Figs. 5A, 5B). On the contrary, the expression of the anti-inflammatory cytokine IL-10 was upregulated in the hemin (+) group (Fig. 5C). Meanwhile, hemin treatment significantly increased HO-1 gene expression, compared with vehicle groups (Fig. 5D). Furthermore, the suppression of TNF-α and iNOS in the hemin (+) group was significantly blocked by co-administration of the HO-1 inhibitor ZnPP (Figs. 5E, 5F). These results revealed that hemin-induced HO-1 contributed to the inhibition of macrophage activation.

Hemin Treatment Prolonged Corneal Allografts Survival by HO-1

Next, we assessed HO-1 level after hemin treatment in the corneal grafts at the average day of rejection (POD15). Hemin treatment dramatically upregulated HO-1 expression in the corneal allografts (Figs. 6A, 6B). In line with immunofluorescence results, real-time PCR results showed an obvious increase in HO-1 mRNA expression of the hemin-treated CGR rats relative to the level in non-hemin-treated CGR rats (Fig. 6C). Additionally, examination by Western blot showed increased HO-1 in hemin-treated rats compared with the hemin (-) rats (Figs. 6D, 6E).

HO-1 inhibitor (ZnPP) co-administrated with hemin significantly abolished the hemin-mediated promotion of corneal allograft survival (Fig. 1B). Co-administrated ZnPP also significantly decreased the inhibitory effects of hemin on macrophages infiltration (Fig. 6F), and increased the expression of MIP-1α, MCP-1, TNF-α, and iNOS in corneal allografts (Figs. 6G, 6H).

DISCUSSION

Macrophage aggregation in corneal grafts is a main feature of early-phase acute rejection. Reliable evidence has demonstrated that CGR can be completely prevented within a follow-up period of 100 days by macrophage depletion in rats. In this study, we found that hemin administration significantly prolonged corneal allograft survival, with decreased corneal edema, suppressed inflammatory cell infiltration, and reduced the number of macrophages. Moreover, hemin treatment reduced the expression of TNF-α, iNOS, MIP-1α, and MCP-1. Notably, hemin administration reduced the proportion of M1 macrophages and enhanced the proportion of M2 macrophages in corneal allografts of CGR rats. Together, these findings suggest that hemin treatment can reduce macrophage infiltration and activation, and suppress CGR.

Hemin induces an increase in HO-1 expression, and represses immune inflammation in various cells and organs. Previous studies have demonstrated that hemin is a potent anti-inflammatory agent for treating graft rejection in liver and kidney transplantation. Kato et al. found that the induction of HO-1 by hemin decreased oxidative stress levels in hepatic ischemia/reperfusion injury.
Hemin and dysfunction of liver grafts. Ferenbach and colleagues reported that hemin induced HO-1 in interstitial macrophages and provided protection against kidney ischemia and reperfusion injury in kidney transplantation. Nonetheless, the role of hemin in corneal transplantations has not yet been reported. Our study demonstrated that hemin therapy increased HO-1 expression to inhibit macrophage recruitment and protect against acute CGR. It has been reported that excessive macrophage infiltration and increased TNF-α and iNOS levels are associated with the development of CGR. Our study found that hemin-mediated upregulation of HO-1 caused a reduction in TNF-α and IL-1β expression, whereas it enhanced anti-inflammatory IL-10 expression for the suppression of macrophages. Collectively, our study shows, for the first time, the therapeutic effects of hemin in CGR and further supports the notion that hemin is a protective agent in organ transplantation.

**Figure 2.** Hemin inhibited macrophage recruitment and proinflammatory cytokine and chemokine expression in corneal allografts. (A) Fluorescence images showing the infiltration of macrophages in the allografts of CGR rats in the hemin (−) and hemin (+) groups at POD4. Corneal sections were stained by immunofluorescence for CD68 of macrophages as indicated (200x magnification; scale bar: 50 μm). (B) Counts of CD68+ cells in the corneal allografts at POD4 (n = 3 per group). (C) Hemin application significantly decreased the mRNA expression of chemokines (MIP-1α and MCP-1) in the corneal allografts at POD4 (n = 3 per group). (D) mRNA expression of TNF-α and iNOS decreased significantly after hemin treatment in the corneal allografts at POD4 (n = 3 per group). The data are presented as the mean ± SD. *P < 0.05, **P < 0.01.
FIGURE 3. Hemin primarily suppressed the afferent phase in corneal allograft rejection. (A) Fluorescence images showing the infiltration of macrophages in the isografts of CGR rats in the hemin (−) and hemin (+) groups at POD4. Corneal sections were stained by immunofluorescence for CD68 of macrophages as indicated (200x magnification; scale bar: 50 μm). (B) Counts of CD68+ cells in the corneal allografts at POD4 (n = 3 per group). (C) Hemin application significantly decreased the mRNA expression of chemokines (MIP-1α and MCP-1) in the corneal isografts at POD4 (n = 3 per group). (D) mRNA expression of TNF-α and iNOS decreased significantly after hemin treatment in the corneal isografts at POD4 (n = 3 per group). (E) Representative photographs of the corneal allografts which were treated with hemin from POD7 and the untreated group at POD15. (F) Kaplan–Meier survival curve of corneal allografts, which were treated with hemin from POD7 and the untreated group. Comparison of allograft survival between allograft hemin (−) (n = 8) and allograft hemin (+) group (n = 8) (allograft hemin (−) versus allograft hemin (+)), P = 0.0934; Log-rank [Mantel-Cox] test). The data are presented as the mean ± SD. *P < 0.05, **P < 0.01.
Different with vascularized organs, such as liver and kidney, the cornea is an avascular tissue. The nutrition of cornea come from corneal limbus vessels and aqueous humor, which derived from blood vessels. Therefore, the diffusion of hemin into cornea still relied on blood vessels, which is similar with its diffusion in liver and kidney. However, the diffusion of hemin into cornea is not directly through blood vessels. Therefore, the efficacy of hemin diffusion into cornea may be lower than the diffusion of hemin into liver and kidney. Thus, the dosage of hemin in humans with corneal transplantation may be higher than liver and kidney transplantation. The future studies are guaranteed to address this question.

Macrophages are divided into the following two main subtypes: (1) classically activated, or proinflammatory, M1 macrophages, and (2) alternatively activated, or anti-inflammatory, M2 macrophages.25 M1 macrophages produce proinflammatory cytokines that damage surrounding tissues, while M2 macrophages produce anti-inflammatory cytokines that inhibit inflammatory responses and promote tissue repair.25,30 Moreover, a recent study demonstrated that proinflammatory macrophages were abundantly present in rejected corneal grafts, but scarce in nonrejected grafts.31 Our results showed that hemin treatment reduced the recruitment and activation of macrophage in vivo and in vitro. Importantly, our study shows for the first time that hemin reduces the proportion of M1 macrophages but increases the proportion of M2 macrophages in graft rejection. These results further elucidated the anti-inflammatory mechanisms of hemin. Previous studies have demonstrated that hemin regulates the function of various immune cells, such as nature kill cells, neutrophils, dendritic cells, and different subtypes of T cells.32–35 Therefore, except for macrophage, multiple targets may contribute to therapeutic effects of hemin in CGR.

In the clinical setting, oral administration of hemin is a better route than injection. However, it is difficult to exactly quantify the dosage for oral administration of hemin in rats. Thus, we chose injections as administrated route in rats. But, oral administration of hemin is able to be exactly quantified in human. Ideally, preparations for treating corneal diseases are administered topically. However, it is difficult to prepare stable topical hemin drops for two reasons. First, hemin is an iron-containing metalloporphyrin, which is a macromolecular compound structure. Therefore, it is difficult for hemin to dissolve in water and penetrate the cornea. Second, hemin is not stable and is susceptible to light decomposition. These characteristics of hemin limit its local application. Thus, systematic administration of hemin was used in our study. In the future, our research will focus on overcoming the above difficulties in the topical use of hemin, in order to render it more suitable for the treatment of ocular diseases.

Whether the sutures are removed remains an issue in the rat penetrating keratoplasty model. Sutures alone have been shown to induce corneal neovascularization and are even used as a model of corneal neovascularization.36,37 In the rat penetrating keratoplasty model, retaining the interrupted sutures and exposing suture nodes to induce corneal neovascularization may lead to a high probability of graft

**Figure 4.** Hemin decreased the proportion of proinflammatory M1 macrophages and increased the proportion of anti-inflammatory M2 macrophages in corneal allografts. (A) Fluorescence photomicrographs showing M1 and M2 macrophages in the allografts of CGR rats from hemin (−) and hemin (+) group at POD 4, respectively. Tissue sections were immunofluorescence stained for markers of M1 and M2 macrophages as indicated (200× magnification; scale bar: 50 μm). (B, C) Quantitation of immunohistochemistry. Counts per square millimeter of CD68+/iNOS+ cells (M1 phenotype) and CD68+/CD163+ cells (M2 phenotype) in the corneal allografts at POD 4 (n = 5 per group). The data are presented as the mean ± SD. *P < 0.05, **P < 0.01.
rejection. Accordingly, this animal model possesses, to some extent, the characteristics of a high-risk transplant. Therefore, the sutures were not removed in some studies; in contrast, graft sutures were removed in other studies to mimic penetrating keratoplasty in patients. However, the suture removal time varied from 4 days to 2 weeks after operation and was not consistent among these studies. The optimal suture removal time should be determined in future studies.

**Figure 5.** Hemin suppressed macrophage activation by inducing HO-1. (A, B) Hemin application significantly attenuated the pro-inflammatory cytokines IL-1β and TNF-α in mRNA expression levels. (C, D) The mRNA expression of the anti-inflammatory cytokines IL-10 and HO-1 was increased significantly after hemin treatment. (E, F) TNF-α and iNOS suppression in the hemin+LPS group was significantly blocked by co-administration of the HO-1 inhibitor ZnPP. The data are presented as the mean ± SD. *P < 0.05, **P < 0.01.
FIGURE 6. Hemin treatment prolonged corneal allografts survival by HO-1. (A) Fluorescence images of anti-HO-1 (green) and DAPI (blue) staining at POD15 (400× magnification; scale bar: 20 μm). (B) The counts of HO-1 staining in the corneal allografts at POD15 (n = 5 per group). (C) HO-1 mRNA expression of the hemin (-) group and the hemin (+) group at POD15. (D) Western blot for HO-1 protein, compared with the β-actin standard at POD15. (E) Quantitation of Western blot for HO-1 in vivo at POD15 (n = 3 per group). (F) Coadministration of HO-1 inhibitor (ZnPP) significantly increased macrophages (CD68+ cells) infiltration in corneal allografts at POD4. (G) Hemin+ZnPP significantly increased the mRNA expression of chemokines (MIP-1α and MCP-1) in the corneal allografts at POD4 (n = 3 per group). (H) mRNA expression of TNF-α and iNOS increased significantly after hemin+ZnPP treatment in the corneal allografts at POD4 (n = 3 per group). The data are presented as the mean ± SD. *P < 0.05, **P < 0.01.
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