Inhibition of Laser-Induced Choroidal Neovascularization by Hematoporphyrin Dimethylether-Mediated Photodynamic Therapy in Rats

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This study aimed to investigate the effect of hematoporphyrin dimethylether (HDME)-mediated photodynamic therapy for laser-induced choroidal neovascularization (CNV) in adult Brown Norway rats. HDME was administered via tail vein at 14 d after the laser photocoagulation, and the rats received irradiance with a laser light at 570 nm at 15 min after injection. CNV was evaluated by fundus photography, fundus fluorescein angiography, optical coherence tomography, and hematoxylin and eosin staining. We found that CNV was occurred at 7 d after photocoagulation and reaching peak activity at 14 d after photocoagulation. There is a significant reduction in the total area of the fluorescein leakage and the number of strong fluorescein leakage spots on 7 d after HDME-mediated photodynamic therapy (PDT). The results suggest that HDME-mediated PDT inhibits laser-induced CNV in rats, representing a promising therapy for wet age-related macular degeneration.

Key words photodynamic therapy; hematoporphyrin dimethylether; choroidal neovascularization; age-related macular degeneration

Age-related macular degeneration (AMD) is a progressive, degenerative disease of the central retina causing irreversible blindness or loss of vision. AMD can be divided into two subtypes as wet (neovascular) and dry (atrophic), characterized by the presence of choroidal neovascularization (CNV). In wet AMD which is responsible for 80–90% of severe vision loss associated with AMD, CNV extends through Bruch's membrane into the sub-retinal space or sub-retinal pigment epithelium. The CNV can leak blood and fluid, and are accompanied by fibrous tissue, which often leads to damage of the retinal tissues and vision loss. At present, the removal of CNV is the key to the treatment of AMD. Conventional management of CNV includes laser photocoagulation, transpupillary thermotherapy (TTT), sub-macular surgical removal of CNV and vascular endothelial growth factor (VEGF) inhibition. The efficacy of laser photocoagulation is not satisfactory. The Macular Photocoagulation Study Groups reported that the laser treatment of subfoveal CNV induced an immediate decrease in visual acuity, and they also showed that the neovascularization recurrence rate was rather high. The sub-macular surgical to remove CNV has great risk and surgical complications. VEGF trap and anti-VEGF antibodies for CNV are effective for maintaining and improving vision, however, a portion of patients do not respond to the treatment well. Moreover, current anti-VEGF drugs may increase the risk of complications such as the retinal pigment epithelium (RPE) tears, cardiovascular disease, nervous system disease (transient ischemic attack) and other systemic adverse events.

Photodynamic therapy (PDT) has been introduced into ophthalmology in the late 1990s as a treatment for CNV due to AMD. PDT is based on the use of photosensitizers that generate oxygen radicals when activated by light of the appropriate wavelength. Since Verteporfin (Visudyne; Novartis AG, Basel, Switzerland) has been approved of U.S.A. Food and Drug Administration (FDA) for the treatment of CNV due to subfoveal lesions, PDT with Verteporfin has been shown to be effective and saved up to now more than a million eyes from legal blindness. Verteporfin is semisynthetic product derived from natural products hemin. It mainly absorbs light at 689 nm, which allows deeper tissue penetration (>1 mm) but may damage normal tissues such as the RPE.
and photoreceptors. Therefore, the discovery and development of safe and effective photosensitizers for the treatment of AMD has attracted increasing attentions. It has been suggested that the laser light having a wavelength ranging from 430 to 600 nm may be safer for PDT of CNV.

Hematoporphyrin dimethylether (HDME) is a porphyrin-based photosensitizer, which exhibits five absorption bands from 300 to 650 nm (shown in Fig. 1). Previous studies show that HDME is more effective for tumor in vivo than Photofrin II. However, the efficacy and safety of HDME in treatment of CNV has not been reported. Herein, the experimental CNV in rats was induced by laser photocoagulation, and the effect of new photosensitizer HDME-mediated photodynamic therapy for laser-induced CNV in rats was evaluated by fundus color photography, fundus fluorescence angiography (FFA), optical coherence tomography (OCT) and histopathological analysis.

MATERIALS AND METHODS

Chemicals and Reagents Hematoporphyrin dimethylether (HDME) and verteporfin were supplied by Shanghai Xianhui Pharmaceutical Co., Ltd. All the chemicals and reagents were of analytical grade and used without any purification, and all the reagents were obtained from Sinopharm Chemical Reagent Co., Ltd.

Animals Seven week-old male Brown Norway (BN) pigmented rats were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Their weights ranged from 170 to 210 g. All animal protocols were approved by the Animal Care and Use Committee of Donghua University.

Laser-Induced CNV Eighteen BN rats were used to establish the experimental CNV by laser photocoagulation. The rats were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg). Laser photocoagulation-induced rupture of Bruch’s membrane was performed on the right eyes using the following laser parameters: 659 nm, 100 µm spot size, 120 mW intensity, and 0.1 s duration (VISULAS Trion, ZEISS, Germany). Six laser spots were applied between the major retinal vessels adjacent to the optic disc. Only laser lesion with a subretinal bubble indicating perforated Bruch’s membrane was considered effective and thus included in the study.

Evaluation of Experimental CNV Sixteen BN rats were used to establish the experimental CNV by laser photocoagulation. On day 0, laser photocoagulation was performed as described above. The coagulated lesions of twelve rats (n=72) were assessed on days 1, 7, 14, 21 by means of ophthalmoscopy, fundus color photography, fluorescein angiography, OCT and another four rats were used for histopathological analysis on days 14.

Evaluation of the Effects of HDME-Mediated PDT on Experimental CNV Twelve male rats were randomly assigned into control group, HDME group and verteporfin group, each group includes four rats and underwent laser photocoagulation as described above. At 14 d after the laser photocoagulation, the rats in PDT group were injected with HDME or verteporfin at a dose of 10 mg/kg via the tail vein. PDT was performed at 15 min after administration by a laser instrument for ophthalmology (Anywave Technology Co., Ltd., Beijing, China) on the eyes using the following laser parameters: 1800 µm spot size, 600 mW/cm², and 83 s duration.

The wavelength of light used in HDME group is 570 nm when that in verteporfin group is 689 nm. The rats in control group were without any treatment.

Fundus Color Photography and FFA Fundus color photography and FFA were performed under systemic anesthesia and pupil dilation using a hand-held retinal camera (Genesis-Df, KOWA, Japan) and a 5.4 mm contact fundus lens. For FFA, fluorescein sodium (100 mg/mL, 0.5 mL/kg) was injected.
into the tail vein of anesthetized rats. Pictures were taken from 3 to 360 s after injection. The laser lesions were studied using fluorescein angiography to evaluate CNV development and its activity.

**OCT** OCT scanning was performed using an OCT system, (Spectralis OCT, Heidelberg, Germany). Rats were positioned on a custom cassette that allowed aligning the mouse eye for imaging. Hydration with normal saline was used to preserve corneal clarity. Volume analysis centered on the optic nerve head was performed, using 100 horizontal, raster, and consecutive B-scan lines, each one composed by 2000 A-scans.

**Histopathological Analysis** Rats were given a lethal dose of sodium pentobarbital (50 mg/kg intraperitoneally), and eyes were enucleated. The anterior segments were removed, and the posterior eyecups were post-fixed in 4% paraformaldehyde (PFA) for 2 h and transferred to 30% sucrose/phosphate buffered saline (PBS) overnight. Then tissue sections at 6 µm thickness were obtained from paraffin embedded tissue blocks. After washed in xylene to remove the paraffin, rehydrated with serial dilutions of ethanol, and washed in distilled water, sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope (Olympus, Japan).

**Evaluation and Statistical Analysis** CNV formation

![Fig. 4. The Intensity of FFA Leakage Grade (a.) and the Fluorescence Leakage Size (b.) at 1 Day, 7, 14 and 21 Days after Photocoagulation (**p<0.01, significantly different from 1 d after photocoagulation)**

![Fig. 5. OCT Images (a) and Light Microscopy (b) of Retinal Lesions with H&E (×200) from Control and 14 Days after Photocoagulation (GCL, Ganglion Cell Layer; INL, Inner Nuclear Layer; ONL, Outer Nuclear Layer)]
and the effect of PDT treatment were evaluated by fundus color photography, FFA, OCT and histopathological analysis. The intensity of staining in late-phase (100–140 s after dye injection) fluorescein angiography was graded as follows: no staining, Score 0; slightly stained, score 1; moderately stained, score 2; strongly stained, score 3. The area of the CNV lesion was measured three times and averaged using Image pro-plus 6.0. Student’s t-test was used to measure differences using the SPSS16.0 software package, and only p-values less than 0.05 were considered significant.

RESULTS

Fundus Color Photography Evaluation of Experimental CNV Figure 2 is the fundus color photography taken before photocoagulation, 7, 14, and 21 d after photocoagulation. It was showed that the photocoagulation spots formed gray and white scars and the scars became deeper with the increase of time after photocoagulation from 7 to 14 d. There was no significant change between 14 and 21 d after photocoagulation.

FFA Evaluation of Experimental CNV FFA is showed in Fig. 3, weak fluorescein staining was observed on day 7, the intensity increased on day 14, and the strong fluorescein staining was still present on day 21. The intensity of FFA leakage grade and the fluorescence leakage size at 1, 7, 14 and 21 d after photocoagulation are displayed in Fig. 4. At 1 d after photocoagulation, there was no spot showing strong staining (score 2 or more). At 7 d after photocoagulation, most of the spots showed strong staining at score 2, the number of spot show strong staining at score 3 were increased with the increase of time until 14 d after photocoagulation. A significant increase in fluorescence leakage size from 1 to 14 d after photocoagulation was observed, and there was no significant change between 14 and 21 d after photocoagulation.

Fig. 6. Fundus Fluorescence Angiography
a. The intensity of FFA leakage grade b. The fluorescence leakage size of model group, HDME-PDT group and verteporfin-PDT group at 7 d after PDT (**p<0.01, significantly different from model group) c. Fundus fluorescence angioigraphy taken before and 7 d after HDME-PDT and verteporfin-PDT. At 7 d after PDT, some photocoagulation spots (gray arrow indication) showed weaker staining intensity in fundus fluorescence angiography than that before PDT.
OCT and Histopathological Analysis Evaluation of Experimental CNV  The OCT image and light microscopy of retinal lesions with H&E (Fig. 5) showed that the layers of retina and choroid in control rats were distinct and the structures were integrated. At 14d after photocoagulation, we observed hyperreflectivity in the RPE and the outer photoreceptor region. The outer plexiform layer (OPL) folded toward the outer nuclear layer (ONL) forming an arch pattern and the hyper-reflective subretinal lesion were differentiated easily from the surrounding retina (Fig. 5a). These findings correlated with the histological findings which revealed disruption and necrosis of the outer retina and RPE (Fig. 5b). Furthermore, a prominent subretinal fibrovascular complex was observed by H&E staining.

The Effects of HDME-Mediated PDT on Experimental CNV  At 7d after HDME-PDT and verteporfin-PDT, the

![Fig. 7. OCT Images of Retinal Lesions before and 7 Days after HDME-PDT and Verteporfin-PDT](image)

At 7d after PDT, the integrity of retina and choroid was recovered to a certain extent. The arch pattern forming by outer plexiform layer (OPL) and outer nuclear layer (ONL) became smaller.

![Fig. 8. CNV Size (a) and Light Microscopy (b) of Retinal Lesions with H&E (×200) from Model Group and Treatment Group at 7 Days after PDT](image)

At 7d after PDT, the CNV size was significantly decreased compared with that in the model group.
score given to the staining in FFA were lower than model group and the fluorescence leakage size was smaller than that in model group (Fig. 6). The number of spots that showed strong dye staining (score 3) were decreased and the number showing weak staining (score 1 and score 0) increased at 7 d after HDME-PDT and verteporfin-PDT (Fig. 6a). The fluorescence leakage size of decreased by 51.14 and 68.37% compared with model group, respectively (Fig. 6b). As shown in Fig. 6c, at 7 d after HDME-PDT and verteporfin-PDT, some photocoagulation spots (gray arrow indication) showed weaker staining intensity in fundus fluorescence angiography than that before PDT. In verteporfin-PDT group, the retinal blood vessels was fuzzy and the whole fundus appeared weak fluorescence leakage compared with HDME-PDT group.

Figure 7 is OCT images of retinal lesions before and 7 d after HDME-PDT. At 7 d after PDT, the integrity of retina and choroid was recovered in a certain extent. The results showed that the arch pattern forming by outer plexiform layer (OPL) and outer nuclear layer (ONL) all became smaller in visudyne-PDT and HDME-PDT groups. After 7 d, the arch pattern size was decreased by 41.67% in HDME-PDT group, especially. This corresponded to a significantly decrease of CNV size seen on H&E staining compared with model group (Fig. 8). The CNV size of HDME-PDT group and verteporfin-PDT group were decreased by 48.79 and 67.56% compared with model group, respectively.

DISCUSSION

CNV is the hallmark of wet AMD and is responsible for most vision loss attributed to the AMD. Hence, a new therapy for CNV in AMD has been long-awaited. The development of the laser-induced CNV model, which has had a significant impact on our understanding of the mechanism and treatment of CNV, has become the most widely used model in wet AMD research. Since many animal models, including monkey, rabbit, cats and rats, have been developed and used to evaluate the effect of various antiangiogenic agents on CNV. Among the numerous animal models, the pigmented rats have become the most widely used model in wet AMD research. Since many animal models, including monkey, rabbit, cats and rats, have been developed and used to evaluate the effect of various antiangiogenic agents on CNV. Among the numerous animal models, the pigmented rats have been used by several groups because its retina is similar to the human retina and is homogeneously pigmented and exhibited a high incidence of CNV after photocoagulation. Herein, we establish the experimental CNV in the subretinal space of BN pigmented rats by laser photocoagulation.

The experimental CNV were confirmed by fundus color photography, FFA, OCT and histopathological analysis. Similar to previous reports, the results of fundus color photography, FFA, OCT and histopathological analysis with H&E confirmed the presence of CNV at 14 d after photocoagulation. And the CNV remained at a high level at 21 d after photocoagulation. On the basis of the time course of laser-induced CNV, we opted to perform HDME-PDT at 14 d after photocoagulation.

PDT has been shown to be effective in closing both experimental CNV in animal models as well as subfoveal CNV in humans. PDT is an approved therapeutic procedure to treat cancers using combinations of chemical photosensitizers and light. Currently, photosensitizers most commonly used clinically or in clinical trials are porphyrin derivatives. Porphyrins are a class of naturally occurring compounds which have become intensively studied in last decades due to their unique photochemical properties. HDME is a porphyrin-based photosensitizer.

In the present study, we found that HDME-mediated PDT and verteporfin-mediated PDT results in a significant reduction of the total area of the fluorescein leakage and the number of strong fluorescein leakage spots. Although the effect of HDME was lower than verteporfin, the drug safety was higher. So the HDME had an ability which could be used for retreatment of CNV. In addition, the retinal blood vessels of HDME-PDT group was more clear than verteporfin-PDT group. The results suggest that HDME-mediated PDT is a promising therapy for CNV due to wet AMD.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES

11) Lewis H, Medendorp SVB. Tissue plasminogen activator-assisted surgical excision of subfoveal choroidal neovascularization in age-related macular degeneration: a randomized, double-masked trial.


Siemiatycki JS, 24.5. 2015.}


