·Basic Research·

Ultrastructural pathology of corneal neovascu – larization after photodynamic therapy in rabbits

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Abstract

• AIM: To investigate the ultrastructural pathogenesis of photodynamic therapy (PDT) for the experimental corneal neovascularization (CNV) by Hematoporphyrin Derivate (HPD) as photosensitizer and Argon laser as light source.

• METHODS: Experimental CNV models were induced in 7 white rabbits using alkali burn. Six weeks after models establishment, animals with CNV were injected with HPD intravenously, and 48 hours after the injection, 7 eyes were irradiated with argon laser (power 800mw, wavelength 514.5nm, spot diameter 200μ m, exposure time 2ms). The irradiated CNV was observed by light microscopy and scanning electron microscopy.

• RESULTS: Histopathological study indicated that there was a striking decrease in the number of the CNV, vascular endothelium became degeneration and necrosis, some vessels were atrophy and attenuated, and vessels cavity were blocked by some thrombosis. No obvious abnormal histopathological findings were noted in surrounding tissues.

• CONCLUSION: The high precise action on CNV and minimal damage to surrounding tissues with PDT by HPD as photosensitizer suggested that PDT might be an effective and safe modality in the treatment of CNV.

• KEYWORDS: corneal neovascularization; photodynamic therapy; histopathology

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INTRODUCTION

Corneal neovascularization (CNV) is caused by many ocular conditions, including inflammatory, infectious, degenerative, traumatic, ischemic, immunologic diseases of the cornea [1]. CNV is associated with a high incidence of corneal allograft rejection. CNV can lead to corneal scarring, edema, lipid deposition and result in significant visual impairment. In addition, current treatments for CNV including corticosteroids, Argon laser photocoagulation and corneal transplantation, can result in many considerable complications and normal ocular tissues damage. Photodynamic therapy (PDT) has been safely and effectively used to treat cancer and choriodal neovascularization, we study the efficacy and mechanism of PDT for experimental CNV with light microscope and electronic microscope on the level of ultramicrostructure.

MATERIALS AND METHODS

Materials Seven healthy white rabbits (Laboratory Animal Center, Tongji Medical College, Huazhong University of Science and Technology) weighing 2-3kg, were used in this study. Basic slitlamp examination did not show any abnormity of the anterior segment of the eye. The left eye was as the experimental eye, and the right eye as control. Animals were anaesthetized with 0.25% dicane topically. The CNV was induced by using an alkali burn technique. The filter paper was round and diameter was 7mm, infused with alkali (2mol/L) and applied to the central part of the cornea for 30 seconds and rinsed the conjunctival sac for 2 minutes with saline. The animals were observed and antibiotic ointment with tetracycline were administrated daily.

Methods Six weeks after alkali burn of cornea, CNV was observed significantly, the vessels invaded into the entire cornea. Hematoporphyrin derivate (HPD) (Beijing Pharmacy Research Institue, Beijing, China) with the dosage of 10mg/kg was administrated intravenously. Forty-eight hours later, PDT was applied to the branches of the corneal limbus neovascularization using an Argon green (wave length 514nm) laser (Orion 3001, Rodenstock, Germany) with a spot size of 200μm and a duration of 2 seconds and at power setting of 800 milliwatts. Animals need to be fed in dark room for about one week. Slitlamp examination was performed after alkali burn of the cornea to observe the CNV formation and take a photograph of the ocular anterior segment, evaluate the areas of the CNV, the changes of the diameter of the neovessel cavity and degeneration of the vessels. At the same time to stain the corneal epithelium with fluorescence to evaluate the repair of the corneal

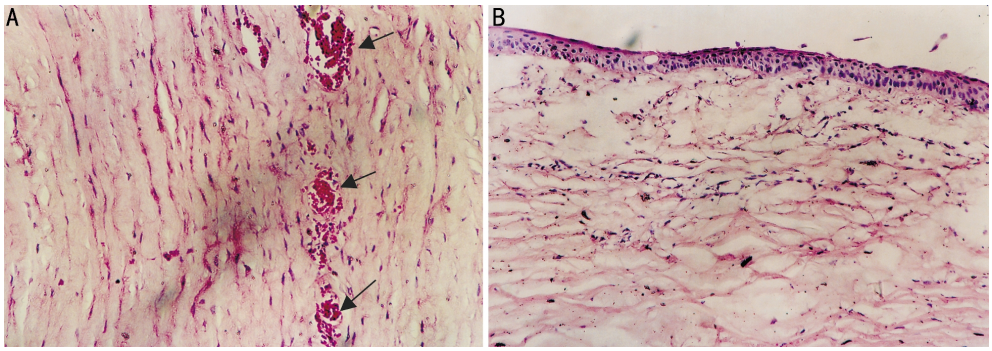

Figure 1 Corneal neovascularization (HE×100) A: Before therapy (arrow); B: 56 days after therapy

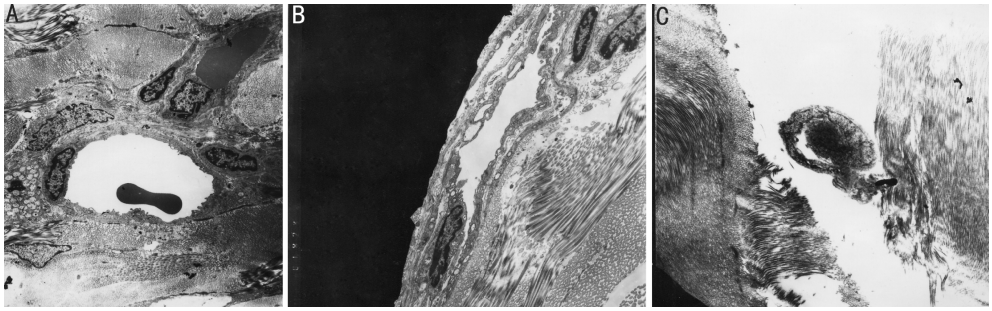

Figure 2 Corneal neovascularization (TEM×6 300) A: Before therapy; B:14 days after therapy; C: 28 days after therapy

epithelial cells, the other ocular structures including iris, anterior chamber and lens also be observed daily. After the animals were killed at 2, 14, 28, 42, and 56 days following PDT and 1 day before PDT by CO_2 overdose, the eyes were enucleated and the cornea and limbus were abladed into two equal parts to make histological specimen. The specimen was fixed in 40g/L buffered formaldehyde. The 4μm paraffin embedded sections of the cornea and corneal limbus stained with haematoxylin and eosin and periodic acid Schiff stain was used for light microscopy; The non-paraffin embedded corneal tissue stained with uranylacetate-lead citrate was available for electron microscopy (Opton EM10C, Germany).

RESULTS

CNV was evident from 3 days after alkali burn and developed maximally 14 days after animal model induction, 6 weeks after the animal model induction, the modality and areas of the CNV were basically stabilization, PDT was carried out at this time. One to two days after PDT, the majority of the eyes showed no fluorescein staining in the area of PDT, the neovessels shrinkled and attenuated at 14 days after PDT; 28 days after PDT, corneal neovessels were atrophy, the cavity of the neovessel became shutdown, there was marked clearing of the cornea; 42 to 56 days following PDT, persistent vascular shutdown was observed, there was no evidence of toxic damage to the corneal epithelium, endothelium, iris and lens. Some animals had skin erythema. There were numbers of new vessels at the cornea and corneal limbus prior to the PDT, the blood capillary filled with erythrocytes and had a continuous basement membrane and was covered by single layer pericyte, there were plasma cells and lymphocytes and hemorrhage around the neovessels (Figure 1A). Forteen days after the PDT, the number of the vessels decreased significantly, the lumen of the vessels became narrower than pretreatment, the cavity collapsed and erythrocytes degenerated. 28 days following the PDT, the corneal neovessels decreased markedly and the wall of the neovessels shutdown and atrophy, some vessels became "ghost" vessels; 56 days after PDT, there were plenty of fibroblast cells infiltrated around the atrophic vessels (Figure 1B). Corneal epithelium and endothelium appeared normal, iris and lens also appeared unchanged.

Ultrastructure

Prior to the PDT, the cavity of the corneal neovessels was filled with erythrocytes, the structure of the endothelial cell of the vessel was observed clearly, there were plenty of mitochondrion, granular endoplasmic recticulum, ribosome and golgi apparatus. Fourteen days after the PDT, the endothelial of the neovessel degenerated and the continuous basement membrane of the cavity was damaged, the collapsed vessels with fibrin and metamorphic erythrocytes can be observed in the treated vessels (Figure 2A); 28 to 56 days after PDT, the nuclei of the endothelial of the neovessels were pyknotic, cavity of the vessels were closed and attenuated, and the neovessels were occluded by the extensive thrombosis (Figures 2B and 2C).

DISCUSSION

Corneal neovascularization (CNV) is a potential complication of corneal inflammation, corneal trauma, penetrating keratoplasty. CNV destroys the normal microenvironment of

the cornea, breaks in the physiologic state of immune privilege and increases the risk of corneal graft rejection. CNV is one of the most important risk factors of immunologic graft rejection after penetrating keratoplasty and results in the graft failure [2]. Currently available treatment for CNV including topical corticosteroids, angiogenesis inhibitors[3] and argon laser photocoagulation[4]. These treatments have many clinical limitations for their side effects and complications, and the clinical application of these treatments were restricted. Long-term use of the corticosteroids may lead to serious side effects such as corticosteroid-induced glaucoma, cataract and corneal ulceration. The angiogenesis inhibitors have been used in animal model, the safety and efficacy for the patients have not been proved. Argon laser Photocoagulation based on the thermal effect of laser, and had been used for CNV and achieved some effects ,but this treatment requires high amount of thermal energy to damage the neovessels, higher laser energy will result in the degeneration, vaporization and even carbonization of the tissue and lead to inflammation of the tissue. The increased vascular permeability due to inflammation will lead to inflammatory cells infiltration and cytokine exudation. These cytokines will stimulate the occlusion neovessels recanalization and new neovessels formation. In our previous study, we quantitatively anlayzed the areas of the CNV only irradiated by Argon laser (Argon laser photocoagulation) and treated by PDT using computerized analysis technique. The results revealed that the mean areas of CNV after Argon laser photocoagulation had no significant decrease. On the contrary, there was a significant decrease of the mean areas of CNV after PDT, the results indicated that the effects of the Argon laser photocoagulation was not the ideal method to treat the CNV. PDT was a kind of traditional treatment for tumor, now PDT provides a utilization for the choroidal neovascularization such as age-related macular degeneration (AMD)[5], choroidal melanoma, closure of the iris neovascularization and eyelid basal cell carcinoma. PDT did not put into use in the clinical therapy for the CNV. PDT involves the administration the photosensitizer and irradiated the target tissue by laser. We use Hematoporphyrin derivate (HPD) as photosensitizer, HPD was a kind of compounds and Dihaematoporphyrin ethers (DHE) was the photosensitive portion of the compounds. DHE has high affinity with the endothelial cells of the vessel and come to the highest dosage of the tissues 48 to 72 hours later after HPD injected intravenously[6]. The photosensitizer can be selectively absorbed and retained by the neovacular endothelial cells, HPD has an absorption at a long wavelength and enables light to penetrate to the target tissue and form complex with low density lipoprotein (LDL) and be easily combined by the surface receptor on the endothelial cell membrane. Application of the low energy laser to the target neovessels (CNV) generates the photosensitizer from the single state to an excited triple state to release cytotoxic free radicals and cause neovasular endothelial cell lipid peroxidation and damage the structure and function of the neovessel endothelial cell. Red blood cells and leukocytes, aggregated platelets and fibrin will form the photo-thrombosis to block the lumen of the neovessels. In our previous study, the mean areas of the neovascularization decreased significantly after the PDT. In this study, the histopathyological results revealed that the structure of the endothelial cell of the neovessel has plenty of mitochondrion, granular endoplasmic recticulum, ribosome and golgi apparatus. But after the PDT these abundant cell apparatuses became pkynosis and disappeared ultimately, and resulted in the degeneration, and necrosis of the endothelial cell of the neovessel, the continuity of the basement membrane of the vessels broken and thrombosis occluded the cavity, all of these histopathological changes led to the neovessels attenuate and vanish. There was no obvious damage to the adjacent ocular tissue.

PDT with intravenous HPD can result in the selectively CNV endothelial cell damage and photothrombosis formation, and result the neovessels occlusion without causing damage to the surrounding normal ocular tissue, appears to be more safe and effective than other treatment such as photocoagulation etc.. PDT has the advantage of higher selectivity to the target CNV and lower damage to normal ocular tissue. The experimental results indicate that PDT will be an effective and safe method to decrease the CNV. HPD is a kind of photosensitizer and has such complications as red and swollen, itch and pigmentation of the exposure area. To decrease the side effects of drug and increase the utilization ratio, the next research will pay more attention to the new dosage forms, the best method and most suitable time of administration and delivery system of the photosensitizer, at the same time, the research about the optimal treatment parameters of the laser should not be neglect. Along with the research and development of the new photosensitizers with minimal side effects and good clinical outcomes, PDT will be an effective and safe clinical therapy for CNV.

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