·Basic Research·

Comparison of the therapeutic effects of extracts from *Spirulina platensis* and amnion membrane on inflammation-associated corneal neovascularization

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Abstract

• AIM: To compare the therapeutic effects of polysaccharide extract from *Spirulina platensis* (PSP) and extract from amnion membrane (AME) on alkali burn-induced corneal neovascularization (CorNV).

• METHODS: PSP and AME were extracted from dry powder of *Spirulina platensis* and human aminion membrane respectively. Murine CorNV was induced by applying 1N sodiumhydroxide (NaOH) solution directly on the mice corneas. PSP and AME extracts were administered topically on the corneas 4 times daily for 7 days. The therapy effects of PSP and AME extracts were evaluated daily using slit-lamp. At the end of the therapy, corneas were harvested for H&E staining, masson trichrome staining, immunohistochemical study, and semi-quantification reverse transcriptive PCR (RT-PCR) was utilized for measurement of inflammation-related molecules.

• RESULTS: Topical application of PSP extract had significant therapeutic effects on CorNV that could be shown in various assays of the corneas. Compared with AME extract, PSP extract treatment was more effective in suppressing CorNV in terms of vessel length and levels of cluster of differentiation 31 (CD31) proteins or the angiogenesis related genes like vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP2) and matrix metalloproteinase-9 (MMP9). PSP also inhibited inflammation more markedly by more effectively inhibiting mononuclear and polymorphonuclear cells infiltration into the corneal stroma and reducing levels of stromal cell-derived factor-1 (SDF1), tumor necrosis factor-alpha (TNFα) and macrophage inflammatory protein-3 (MIP3a). In additon, corneas of PSP group had a more regular and compact architecture of collagen with thinner corneal thickness than in the AME group.

• CONCLUSION: Polysaccharide extract from *Spirulina platensis* inhibited alkali burn-induced inflammation and CorNV more effectively than AME extract at the studied doses, thus may be used for the therapy of corneal diseases involving neovascularization and inflammation.

• KEYWORDS: *Spirulina platensis;* aminion membrane; corneal neovascularization; inflammation

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INTRODUCTION

Corneal neovascularization (CorNV), namely the abnormal formation of new blood vessels in previously avascular cornea, is a significant sight-threatening event that usually leads to loss of central vision or even results in blindness. CorNV is almost always accompanied by inflammation and occurs in many corneal disorders, such as corneal chemical burns, thermal burns, infections and autoimmune diseases. Alkali burn of the corneal surface is one of the most devastating injuries to the eye [1,2]. The difficulty in the treatment of such situation results partially from lack of knowledge about host reactions to the injury factors.

Placental amniotic membrane (AM) has been used as a temporary or permanent graft in clinical practice when handling burn or persistent infections to the skin, open trauma and dermal infections. In recent years, AMs are used extensively for ocular surface reconstruction [3,4], such as in patients with chemical or thermal burns [5,6] as well as in conditions of corneal infection [7-9]. Clinical experience has

revealed that inflammation and angiogenesis can be markedly suppressed by AM [10-12] in the anterior segment environment. Beside the original membrane materials, amniotic membrane extract (AME) is also being used to treat inflammation and angiogenesis-related diseases and proven to be more convenient for application [13-16].

In searching for substitutes to AME, we came up to spirulina, an aquatic plant that has long been used in several countries as a supplement in human and animal food either as a health drink or in tablet form because of its alimentary value [17]. In addition to its special alimentary benefits, researchers are paying attention to its potential usefulness in health care and clinical applications due to it many biological activities [18-20]. In our previous study, we demonstrated that polysaccharide extract from *Spirulina platensis*(PSP) exhibits anti-angiogenic and anti-inflammatory properties in vivo and in vitro and may be a novel natural agent for prevention and cure of inflammatory CorNV-related diseases [21]. The current study further assessed the anti-angiogenic and anti-inflammation effects PSP on alkali-burn CorNV by comparison with AME, and provided more evidence to support the potential usefulness and advantages of PSP in controlling CorNV.

MATERIALS AND METHODS

Materials

Preparation of polysaccharide extract from *Spirulina platensis* PSP was prepared as described previously [21]. In brief, dry powder of *Spirulina platensis* was incubated with 95% (v/v) ethanol overnight, torrefied, and reincubated in NaOH (pH 10.0) solution at 80℃ for 4-6 hours, then centrifuged and collected the supernatant, adjusted pH to 7.0, precipitated with 5% trichloracetic acid (TCA) at 4℃ overnight, centrifuged and collected the supernatant, then precipitated with 5% TCA for another 3 hours, centrifuged and the supernatant was precipitated with ethanol (1/5:V/V) at 4℃ overnight. The precipitate was washed twice with acetone and then was lyophilized in a freeze dryer, and stored at -20℃ . This precipitate, mainly containing *Spirulina* polysaccharides (PSP), was dissolved in normal saline and filtered with 0.22μm filtration membrane and the stock polysaccharides concentration was measured using anthrone-sulfuric acid method. For topical application, the stock PSP was adjusted to 100μg/mL in sodium chloride eye drops compound (Nanjing Liye Pharmceutical Co., LTD, Nanjing, China).

Preparation of extract of amnion membrane The whole procedure of preparing human AME was carried out aseptically. The entire frozen human AM was sliced into small pieces and homogenated thoroughly in saline buffer. The homogenate was centrifuged at 6 000g for 15 minutes and the supernatant was collected. The protein concentration of AME was measured using a BCA protein assay kit (Pierce, Rockford, IL, USA) and adjusted to 100μg protein/mL in above eye drop before use.

Methods

Evaluation of alkali-induced corneal burn All animal experiments were carried out following the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The female C57BL/6 mice of 6-8 weeks old were used as experimental animals. Under general anaesthesia with intraperitoneal ketamine and chloropromazine, the alkali burn CorNV model was established by direct application of 3μL of 1 N NaOH to both corneas for 30 seconds. Then, the eyes were rinsed with 20 mL of normal saline immediately after burn. The mice were divided into PSP, AME and control groups randomly, eight mice in each group. PSP, AME or carrier eye drops were applied topically (5μL) to the burned eyes 4 times daily for seven consecutive days, respectively. At day 7, eyes were photographed under a slit lamp and CorNV was quantified using a method for determining corneal angiogenesis [17]. All mice were sacrificed and their eyes were harvested for further examination.

Histological studies Eyeballs were fixed with formalin and embedded with paraffin. Serial sections (4μm) were made and processed for hematoxylin and eosin (H&E) staining and Masson trichrome staining following conventional protocols. For immunohistochemical assay (IHC), eyeballs were snap-frozen in optimal cutting temperature (OCT) compound (Sakura Finetechnical, Tokyo, Japan). OCT-embed sections (6μm) were prepared and fixed in ice-cold acetone for 10 minutes followed by conventional IHC protocols, using PE-conjugated anti-CD31 mAb (1:100, BD Pharmingen, CA, USA) or mouse anti-SDF1 mAb (1:50, Santa Cruz, CA, USA) as primary antibodies. For SDF1 staining, rhodamine-conjugated goat anti-mouse IgG (1:100, Santa Cruz, CA, USA) was used as secondary antibody. The sections were counterstained with DAPI and viewed under an Eclipse TE2000-U microscope (Nikon, Tokyo, Japan). Negative controls were performed by omitting primary antibodies.

Semi quantitative reversce transription PCR Total RNA from mouse corneas was extracted using a NucleoSpin RNA Ⅱ kit (Macherey-Nagel, Germany) and 1μg of RNA was reverse transcribed using a PrimeScript RT Reagent Kit (Takara, Japan) according to the manufacturers' instructions. Semi-quantitative PCR was performed using the resultant cDNA and specific primers (Table 1) to each gene. The house-keeping gene GAPDH was used as an internal control. PCR amplification were performed as following: denaturation for 2 minutes at 95℃, followed by 35 cycles or 30 cycles (GAPDH) of 1 minute at 95℃, 30 seconds at 60℃ and 1 minute at 72℃ with an additional extension for

Table 1 Primers used for semi quantitative reversce transription PCR

Gene	Forward primer	Reverse primer	Product size (bp)
VEGF	GAGCAGAAGTCCCATGAAGTG	CATGGTGATGTTGCTCTCTGA	213
MMP-2	CCCGATCTACACCTACACCAA	AAACCGGTCCTTGAAGAAGAA	217
MMP-9	CGTCGTGATCCCCACTTACTA	AAGATGAACGGGAACACACAG	237
SDF1 TNFα	CAGTCAGCCTGAGCTACCGA	TCTTCAGCCGTGCAACAATC	126
MIP3a	AAGGGATGAGAAGTTCCCAAACCA	CCTTGTCCCTTGAAGAGAACC	264
GAPDH	GAGCTATTGTGGGTTTCA	GCTGTGATCATTTCCTCCTTG	210
	GGTGAAGGTCGGTGTGAACGGA	TGTTAGTGGGGTCTCGCTCCTG	246

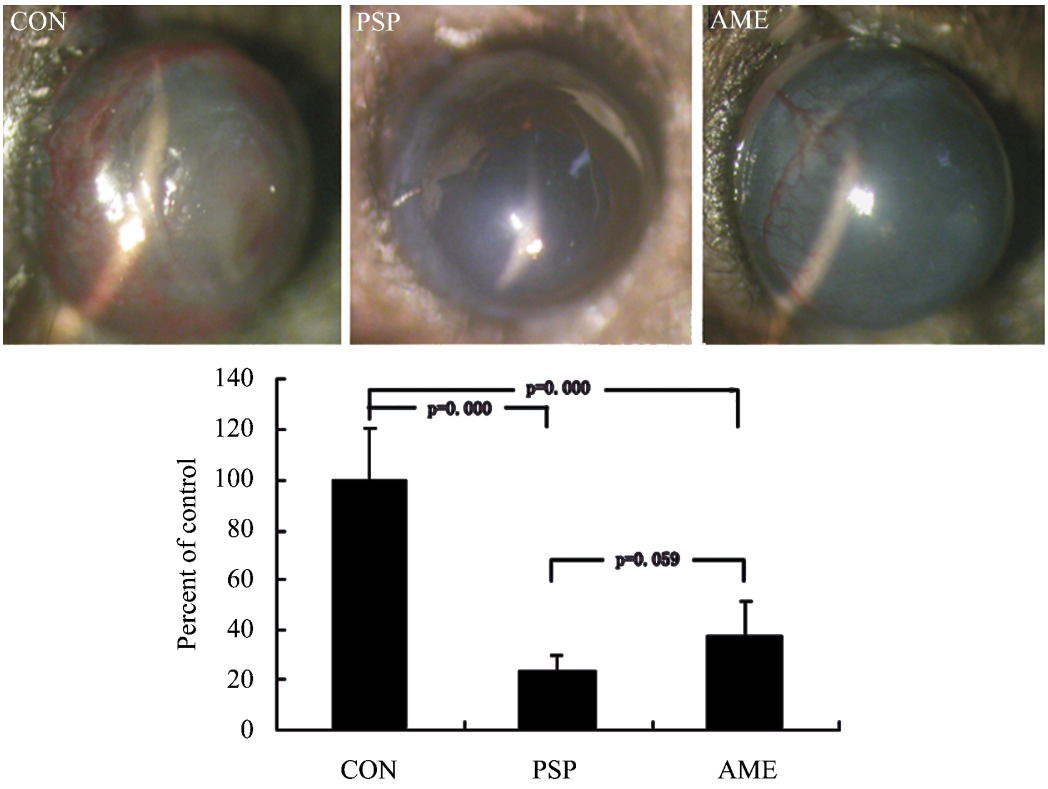

Figure 1 Macroscopic observation of corneal neovascularization Upper: the macroscopic photos of PSP (100μg/mL) treated, AME treated (100μg protein /mL) or control mice cornea after alkali burn. Below: The relative vessel length grows in cornea of three groups.

10 minutes at 72℃ at the end. PCR products were resolved in 1.5% agarose gel, stained with ethidium bromide, and photographed with a high-resolution digital camera under UV illumination.

RESULTS

Inhibition Effects of PSP and AME on CorNV *in vivo* In the animals of control group, vessels sprouted out from the limbus and reached central cornea by day 7 after alkali burn; when PSP and AME were administered topically during the 7 days time, CorNV development was markedly inhibited (Figure 1). Quantification statistics showed that the average length of vessels in PSP and AME treated group were 24% and 37% of that in the control group respectively, indicating that PSP was more effective than AME in inhibiting the growth of new vessels into cornea under our experimental situation.

Histological observation When detected with HE staining, corneas of control mice possessed many new vessels as well as lot of mononuclear and polymorphonuclear cell infiltration in the stromal layer. Corneas treated with AME contained much less new vessels and mononuclear cells and polymorphonuclear cell infiltration in stroma, while there were almost no new vessels or inflammatory cells infiltration in corneas treated with PSP (Figure 2A). Masson trichrome staining showed that the corneas of mice in control group had a much dispersed stroma layers and fluffier collagen fibers, while AME or PSP treatment greatly ameliorated the destruction of corneas, with PSP being more efficient in doing so (Figure 2B).

Immunohistochemitry for CD31 and SDF1 Staining for CD31, which is a specific marker of vascular endothelial cells, could reveal characteristics of blood vessels more accurately. As shown in Figure 3A, the corneas of control mice contained large number of vessels, diameter of which could be up to about 50μm, whereas PSP or AME treated corneas exhibited negligible immunoreactivity for CD31. SDF-1 is an important lymphocyte chemoattractant cytokine expressed in fibroblast in the cornea. Figure 3B showed

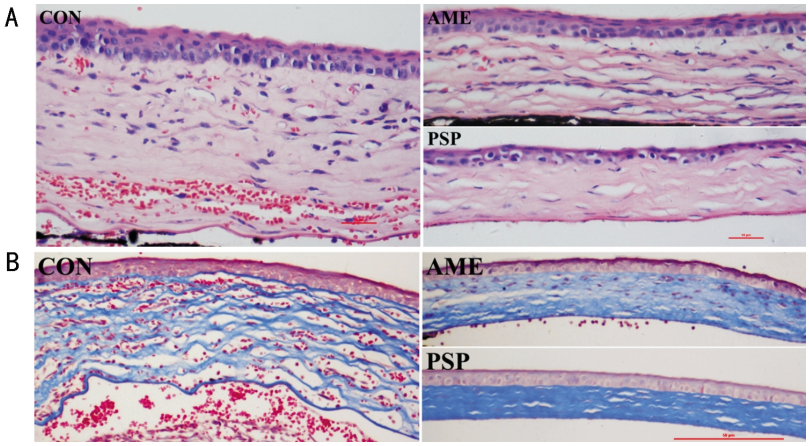

Figure 2 Histology of chemically burned corneas The sections from 3 different groups were stained with H&E (A) or masson trichrome (B). Representative sections from each group are shown. Bar, 50μm.

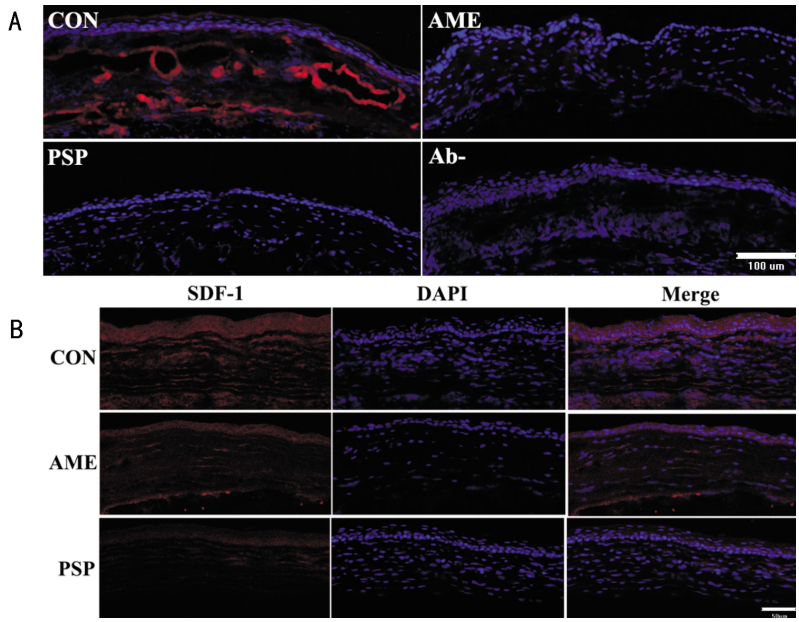

Figure 3 CD31and SDF1 expression profile in alkali burned mouse cornea The sections from 3 different groups were stained with CD31 (A) or SDF1 (B). Three specimens from different mice were studied in each group; representative micrographs are shown in this figure. A, Bar 100μm; B, Bar 50μm.

decreased expressions of SDF1 in PSP or AME-treated corneas than in control corneas, and the decrease of SDF1 in PSP group was more significant than in AME group.

Inhibition effects of PSP and AME on the expression of genes associated with angiogenesis or inflammation in burned cornea Semi quantitative RT-PCR analysis of the mRNA level of some angiogenesis and inflammation related genes demonstrated that both angiogenesis-related factors (VEGF, MMP2 and MMP9) and inflammation-related factors (SDF1, TNFα and MIP3a) were repressed significantly by PSP treatment. But AME treatment only significantly decreased expression of SDF1 and MIP3a without affecting other genes (Figure 4).

DISCUSSION

Under normal physiological conditions, corneal tissue maintains its avascular transparent characteristics to fully perform its role in the refraction and transmmission of light [2223].

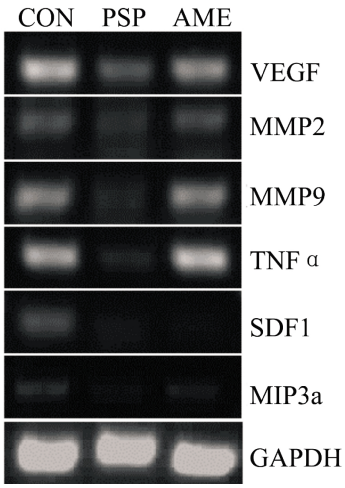

Figure 4 Changes in angiogenesis- and inflammation-related genes in PSP -treated or AME -treated CorNV corneas The expression mRNA level of VEGF, MMP2, MMP9, TNFα , SDF1 and MIP3a in corneas from 3 different groups was detected using semi quantitative RT-PCR analysis.

Neovascularization is the formation of new vascular structures in areas that were previously avascular, thus CorNV alters corneal function and is often clinically significant. CorNV is usually associated with inflammatory disorders of the ocular surface. Therefore, approaches to control or prevention of inflammation may be utilized for CorNV management.

Previous studies [24,25] have shown that chemical burn induced CorNV is accompanied with corneal inflammation in ways that are also seen in inflammatory corneal diseases. Thus, the alkali-induced CorNV serves as a suitable animal model for the study of inflammatory CorNV. In our previous study [21], we were able to demonstrate the anti-angiogenic ability of PSP using this CorNV model. In fact several other natural products have also been shown to posses anti-angiogenic properties and affect CorNV in experimental animal models. These products include genistein [26], shark cartilage [27], curcumin [28, 29], propolis extract [30] and AME [13-16], among which AME was most popular in ocular context. By comparing efficacy of PSP and AME at similar dose (both 100μg/mL, though for polysaccharide and protein respectively) in the model of chemical burn-induced inflammatory CorNV, our present experiment showed that PSP was more effective in inhibiting CorNV at all levels, namely gross appearance (Figure 1), histological or cellular levels (Figure 2,3), and molecular level (Figure 3,4).

Among the inflammatory factors that might be involved in CorNV, we studied SDF1, TNFα and MIP3a as representatives to determine the mechanisms of anti-inflammatory and anti-angiogenic effects of PSP and AME. SDF1 is the principal ligand for CXCR4, and the SDF-1/CXCR4 ligand/receptor pair is an important contributor to several types of ocular neovascularization [31]. TNFα is known to be one of the key regulators of inflammation, and can mediate angiogenesis [32]. MIP3a (CCL20) can produce by corneal epithelial cells and stromal keratocytes, and recruit CCR6-expressing cells such as dendritic cells into inflamed cornea [33]. Surprisingly, we found that PSP blocked expression of all three factors, but AME only significantly, though less effectively than PSP, inhibited expression of SDF1 and MIP3a without affecting TNFα (Figure 4), implying that TNFα might be less possibly involved in pathogenesis of chemical burn induced CorNV. Beside confirming our previous finding that PSP greatly decreased MMP2 and MMP9 expression in CorNV [21], we also found that AME had minimal effect on expression of these two enzymes in the CorNV model used in this study (Figure 4). As with the possible effect of AME or AM, controversial data exist in references. For example, both using chemical burn induced CorNV model in rabbits, Takahashi *et al*[34] reported inhibition of MMP2 and MMP9 expression by AM, while Rigal-Sastourné *et al* [35] observed strong upregulation of MMP2 and MMP9 induced by AM application. We have no explanation but we assume that the difference between our MMP observations with references might because of that we used mouse instead of rabbits and AM extract instead of intact AM. Thus the actual effect of AME or AM on MMPs might be more complicated and deserves more investigation.

CONCLUSION

In summary, we compared the anti-angiogenic and anti-inflammation effects of PSP in inflammatory CorNV with that of AME, a biological components being used to manage similar situations in clinical practice. The results showed that PSP was even better than AME in inhibiting inflammation and angiogenesis under our experiment situation, proposing a promising usefulness of PSP in clinical application. Further studies should focus on more exhaustive molecular mechanism and safety issues associated with potential ocular applications of PSP.

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