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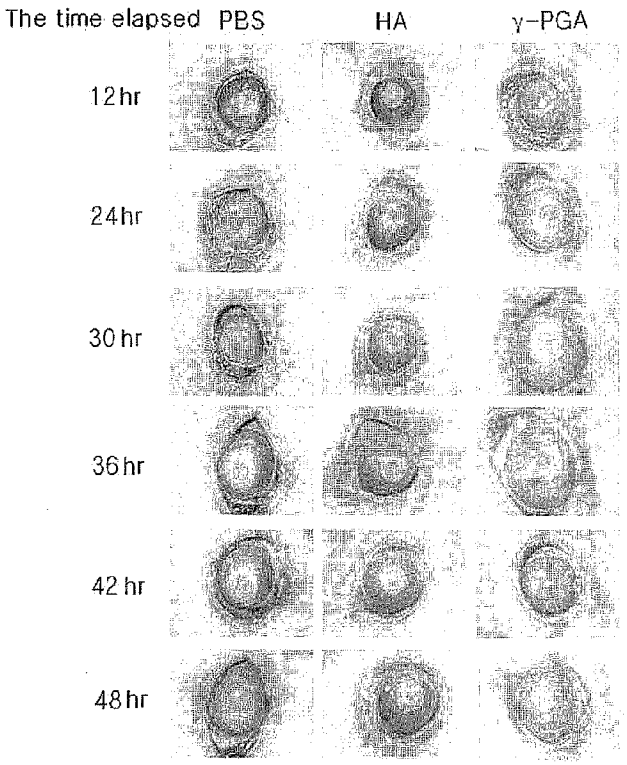
[Continued on next page]

(54) Title: PHARMACEUTICAL COMPOSITION FOR TREATING CORNEAL WOUND COMPRISING POLY-GAMMA-GLUTAMIC ACID

FIG. 1



(57) Abstract: The present invention relates to a pharmaceutical composition for treating corneal wounds, which comprises poly-γ-glutamic acid as an active ingredient. The pharmaceutical composition according to the present invention relieves inflammation by inhibiting a hyaluronidase enzyme which is activated upon the development of inflammation, and it maintains the ability of hyaluronic acid to stimulate corneal epithelial cell proliferation by inhibiting the degradation of hyaluronic acid. Thus, the composition is useful for the treatment of corneal wounds.

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Pharmaceutical Composition for Treating Corneal Wound Comprising Poly-Gamma-Glutamic Acid

TECHNICAL FIELD

The present invention relates to a pharmaceutical composition for corneal wounds, which comprises poly-γ-glutamic acid (γ-PGA).

BACKGROUND ART

The cornea is a transparent avascular tissue at the very front of the eye and forms the anterior 1/6 of the eyeball. The cornea consisting of epithelium, Bowman's membrane, stroma and endothelium functions as a protective barrier protecting the pupil and the inside of the eye and functions to refract and focus light.

The corneal epithelium which is the outermost layer of the cornea has an excellent regenerative ability like the epithelium of the skin and doe not bleed when damaged, because it is avascular. However, it contains numerous nerve fibers, and thus, people may feel a cold sore or severe pain in the eye even with a small damage, and frequent tears are induced. Such corneal wounds are caused by endogenous factors such as diseases including dry eye syndrome, and exogenous factors including contact lens wear and irritation caused by excessive eye exposure to UV rays, and also occur after surgery. The corneal wounds may proceed to keratitis such as viral corneal ulcers by viral infection associated with epithelial defects. Contamination of the corneal wounds with bacteria or fungi may lead to loss of eyesight, and when the cornea is severely damaged, recurrent corneal erosion may occur.

For corneal wound healing, the rapid regeneration and covering of corneal

epithelium must be achieved. The mechanism of corneal epithelial wound healing consists of adhesion, migration, proliferation and differentiation of epithelial cells, and factors promoting this mechanism can be used as agents for treating corneal wounds. Corneal cells and cells such as polymorphonuclear leukocytes (PMNs) produce matrix metalloproteinases such as stromelysin, gelatinase and collagenase, and other proteases to degrade collagen fibers, and inhibitors of these proteases were used to treat wound and their effectiveness of wound treatment was reported. Wound healing regulators involved in the adhesion and migration of epithelial cells include fibronectin, hyaluronic acid, IL-6, etc., and the proliferation of epithelial cells is stimulated by epidermal growth factor (EGF), fibroblast growth factor (FGF), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), etc. Also, vitamin A is known to facilitate the differentiation of epithelial cells.

Among them, hyaluronic acid is a therapeutic agent for treating typical ocular surface disease. Hyaluronic acid, which is a disaccharide polymer capable of binding a large amount of water, is an important component of the extracellular matrix (ECM) and is involved in tissue expansion during wound healing to allow cells to migrate. It is known that hyaluronic acid has a protective effect on the corneal epithelium and endothelium, stimulates the migration and proliferation of the corneal epithelium and promotes hemidesmosome formation. Endogenous hyaluronic acid is known to be synthesized by corneal epithelial cells, keratinocytes, corneal endothelial cells, iris pigment epithelial cells, lens epithelial cells, etc.

Substances which are currently being applied in clinical practice include only fibronectin and hyaluronic acid, the effect of which is mainly limited to the promotion of adhesion and migration of epithelial cells. Moreover, growth factors such as EGF and FGF have problems in that they have the risk of inducing angiogenesis and are difficult to prepare. Accordingly, the effect of these substances on the treatment of corneal wounds is not sufficient, and also has the risk of side effects. Thus, there is an urgent need to develop a more stable and

excellent therapeutic agent which ameliorates the fundamental causes of corneal wounds.

Meanwhile, poly-gamma-glutamic acid is a polymer consisting of D, L-glutamic acid which is produced from the genus *Bacillus* strain. The present inventors obtained a patent relating to poly-gamma-glutamate with high molecular weight and a method for using the same (Korean Patent Registration No. 10-0399091), and a patent relating to a method for producing γ-PGA using a halophilic *Bacillus subtilis* var. chungkookjang that produces γ-PGA with high molecular weight (Korean Patent Registration No. 10-0500796). Also, we obtained patents relating to an anticancer composition, an immune adjuvant, and an immune enhancing agent, which contain γ-PGA (Korean Patent Registration Nos. 10-0496606; 10-0517114; and 10-0475406). In addition, we have identified the effects of γ-PGA by continuously developing applications for γ-PGA, including studies on the use of γ-PGA in medical applications, such as development of a hyaluronidase inhibitor containing poly-gamma-glutamic acid (Korean Patent Registration No. 10-0582120).

Accordingly, the present inventors have made extensive efforts to develop a novel use of poly-γ-glutamic acid which is a natural amino acid polymer. As a result, the present inventors have found that poly-γ-glutamic acid is effective in the treatment of corneal wounds, because it functions to relieve inflammation by inhibiting a hyaluronidase enzyme which is activated upon the development of inflammation, and it inhibits the degradation of hyaluronic acid to maintain the ability of hyaluronic acid to stimulate corneal epithelial cell proliferation, thereby completing the present invention.

SUMMARY OF INVENTION

It is an object of the present invention to provide a pharmaceutical composition

containing poly-γ-glutamic acid having a therapeutic effect on corneal wounds.

To achieve the above object, the present invention provides a pharmaceutical composition for treating corneal wounds, which comprises poly-γ-glutamic acid as an active ingredient.

Other features and embodiments of the present invention will be more apparent from the following detailed description and the appended claims.

BRIEF DESCRIPTION OF DRAWINGS

FIG. 1 is photographs of fluorescence-stained ocular defects taken in order to examine corneal wound healing effect of poly-γ-glutamic acid after dropping poly-γ-glutamic acid into the eyes of a rabbit corneal wound model rabbits.

DETAILED DESCRIPTION OF THE INVENTION, AND PREFERRED EMBODIMENTS

In the present invention, it was confirmed that a natural amino acid polymer, poly-γ-glutamic acid is effective in the treatment of corneal wounds, because it functions to relieve inflammation by inhibiting a hyaluronidase enzyme which is activated upon the development of inflammation and it inhibits the degradation of hyaluronic acid to maintain the ability of hyaluronic acid to stimulate corneal epithelial cell proliferation.

Accordingly, in one aspect, the present invention relates to a pharmaceutical composition for treating corneal wounds, which comprises poly-γ-glutamic acid as an active ingredient.

Poly-γ-glutamic acid used in the present invention may be produced by chemical

synthesis or microbial fermentation. Preferably, poly-γ-glutamic acid used in the present invention can be produced by microbial fermentation, and more preferably, by the fermentation of *Bacillus Subtilis var. chungkookjang*. Also, the poly-γ-glutamic acid preferably has an average molecular weight of 1-15,000 kDa.

The poly-γ-glutamic acid according to the present invention can inhibit the activity of a hyaluronidase enzyme that degrades hyaluronic acid. Accordingly, it can stimulate angiogenesis and cell proliferation, which are the functions of hyaluronic acid, thus making it possible to achieve smooth tissue regeneration to regenerate the corneal epithelium.

Also, the poly-γ-glutamic acid according to the present invention functions to relieve inflammation by inhibiting a hyaluronidase enzyme which is activated upon the development of inflammation.

In order to secure the safety of the poly-γ-glutamic acid according to the present invention, an ocular irritation test of the poly-γ-glutamic acid was performed by Biotoxtech Co., Ltd., an institute approved as a Good Laboratory Practice (GLP) facility. As a result, it was proven safe for ocular use.

In the inventive pharmaceutical composition for treating corneal wounds, poly-γ-glutamic acid can be contained at a therapeutically effective concentration, which is suitably determined depending on the patient's age, disease severity or the degree of treatment. For example, the poly-γ-glutamic acid may be contained at a concentration of 0.001-5 wt%, and preferably 0.01-3 wt%. If the concentration of the poly-γ-glutamic acid is less than 0.001 wt% on a dry weight basis, the pharmacological action of the poly-γ-glutamic acid cannot be expected, and if it exceeds 5 wt% on a dry weight basis, a further increase in pharmacological action will not be expected, and the viscosity of the resulting composition can be excessively increased.

The pharmaceutical composition for treating corneal wounds can be prepared into ophthalmic formulations by a conventional method of adding, to the poly-γ-glutamic acid, additives, including an isotonic agent such as sodium chloride or potassium chloride, a buffer such as sodium hydrogen phosphate or sodium dihydrogen phosphate, a stabilizer such as sodium ether, a preservative such as ethyl paraben, butyl paraben or benzalkonium chloride, a pH adjusting agent such as sodium hydroxide and diluted hydrochloric acid, and an ointment base such as white Vaseline or liquid paraffin. As used herein, the term "ophthalmic formulation" is meant to include ophthalmic solutions, eye wash solutions, ophthalmic ointments or freeze-dried formulations, that is, all formulations associated to ophthalmic treatments.

In the present invention, examples of diseases induced by corneal wounds include, but are not limited to, conjunctival and corneal epithelial defects, corneal epithelial abrasions, corneal ulcers, infectious ocular diseases and the like.

Examples

Hereinafter, the present invention will be described in further detail with reference to examples. It is to be understood, however, that these examples are for illustrative purposes only and are not to be construed to limit the scope of the present invention.

Example 1: Production of poly-gamma-glutamic acid

3L of a basal medium for γ-PGA production (GS medium containing 5% L-glutamic acid: 5% glucose, 1% $(NH_4)_2SO_4$, 0.27% KH_2PO_4, 0.42% $Na_2HPO_4 \cdot 12H_2O$, 0.05% NaCl, 0.3% $MgSO_4 \cdot 7H_2O$, 1ml/l vitamin solution, pH 6.8) was inoculated with 1% culture broth of *Bacillus subtilis var. chungkookjang* (KCTC 0697BP) and then cultured at a stirring speed of 150 rpm, an air injection

rate of 1 vvm and a temperature of 37℃ for 72 hours. Then, the cells were removed from the culture broth after completion of the culture using a filter press, thus obtaining a γ-PGA-containing sample solution.

2N sulfuric acid solution was added to the γ-PGA-containing sample solution and left to stand at 10°C for 12 hours to collect a γ-PGA precipitate. The collected precipitate was washed with a sufficient amount of RO water to obtain γ-PGA using a Nutsche filter. Molecular weight of the obtained poly-gamma-glutamic acid was measured using GPC (gel permeation column), and as a result, it was confirmed that poly-gamma-glutamic acid having a molecular weight of 1-15,000kDa was produced, and then separated according to molecular weight to collect poly-gamma-glutamic acid having an average molecular weight of 5,000 kDa. The collected γ-PGA was used in the following examples.

Example 2: Investigation of effect of poly-γ-glutamic acid on treatment of corneal wounds

30 female New Zealand white rabbits weighing 2.5 kg were systemically anesthetized by intravenous injection of pentobarbital, and then the ocular surface of each rabbit was anesthetized by injection of 0.5% proparacaine. A 5.5 mm-diameter circular filter paper containing 1N NaOH absorbed therein was brought into contact with the central portion of the cornea for 60 seconds to create corneal alkali burn in only one eye of each rabbit, and then the eyes were washed with balanced salt solution (BSS®, Alcon, USA) for 120 seconds. Then, in the control group, phosphate buffered saline (PBS) was dropped onto the eyes of 10 rabbits four times a day, and in the treatment group, 0.1% HA (hyraulronic acid) having a molecular weight of 1,200,000 Da was dropped onto the eyes of 10 rabbits among 20 rabbits four times a day and 0.1% γ-PGA having a molecular weight of 5,000,000 Da was dropped onto the eyes of the remaining 10 rabbits four times a day.

Immediately after creating the wounds and at 12 hours, 24 hours, 30 hours, 36 hours, 42 hours and 48 hours after creating the wounds, the corneal epithelial defects of each rabbit were stained with 2% fluorescein solution, and then photographed using Nikon D80 with Micro-Nikkor 105 mm 1:4 objects. The wound healing procedure was compared between the treatment group and the control group by measuring changes in the epithelial defect areas using AutoCAD 2007 (Autodesk, Inc.).

The area of the initial epithelial defects created using 1N NaOH was 23.83±0.79㎟ (in the range from 22.68 to 25.59㎟) for the PBS-treated group, 23.73±1.03㎟ (in the range from 22.75 to 26.24㎟) for the HA group, and 23.77±0.67㎟ (in the range from 22.93 to 25.01㎟) for the γ-PGA group, and did not show any difference between the three groups (p=0.957). The wound healing rate was 0.466±0.059㎟/hr (in the range from 0.382 to 0.572 ㎟/hr) for the PBS-treated group, 0.490±0.055 ㎟/hr (in the range from 0.442 to 0.573 ㎟/hr) for the HA-treated group, and 0.531±0.076 ㎟/hr (in the range from 0.456 to 0.660 ㎟/hr) for the γ-PGA-treated group, and did not show any difference between the PBS group and the HA group (p=0.361). There was also no difference in the wound healing rate between the HA group and the γ-PGA group (p=0.189), but the wound healing rate of the γ-PGA-treated group was significantly higher than that of the PBS-treated group (p=0.048).

Example 3: Ocular irritation test of poly-γ-glutamic acid

An ocular irritation test of poly-γ-glutamic acid was carried out using three 16-week-old male NZW rabbits. As a test substance, 10% poly-γ-glutamic acid solution was applied to the rabbits. In a test group, 0.1 ml of the test substance was applied into the conjunctival sac of the right eye, and at 1 hr, 24 hr, 48 hr, 72 hr and 96 hr after applying the test substance, the ocular defects of the cornea, the iris and the conjunctiva were observed. Ocular irritancy was scored according to the Draize criteria and the ocular irritation scores were classified according to the method of

Kay and Calandra.

As a result, as shown in Table 1 below, ocular irritation in the cornea, the iris and the conjunctiva was not shown in all the animals when observed at 1 hr, 24 hr, 48 hr, 72 hr and 96 hr after the application of poly-γ-glutamic acid. The mean total score (MTS) was zero (no irritability). During the observation period, there was no death of animals, and abnormal changes in general conditions and body weight caused by the application of the test substance was not shown. From the above results, it was concluded that the test substance, poly-γ-glutamic acid did not irritate the ocular tissue of the rabbits under the test conditions.

Table 1

Test group	No. of animals	Mean total score (MTS)					MMTS
		1 h[(1)]	24 h	48 h	72 h	96 h	
G1 test substance	3	0	0	0	0	0	0

MMTS: maximum mean total score

[(1)] hour after application

Formulation Examples 1 to 4: Preparation of ophthalmic solutions containing poly-γ-glutamic acid

As shown in Table 2 below, ophthalmic solutions were prepared by mixing poly-γ-glutamic acid with isotonic agents (sodium chloride and potassium chloride), buffers (sodium hydrogen phosphate and sodium dihydrogen phosphate), a stabilizer (sodium ether), a preservative (benzalkonium chloride), a pH adjusting agent (sodium hydroxide) and sterile purified water.

Table 2

Component	Formulation Example 1	Formulation Example 2	Formulation Example 3	Formulation Example 4

Poly-γ-glutamic acid (g)	0.1	0.5	0.1	0.5
Sodium chloride (g)	0.2	0.2	0.2	0.2
Potassium chloride (g)	-	0.1	-	0.1
Sodium hydrogen phosphate (g)	0.1	0.1	0.1	0.1
Sodium dihydrogen phosphate (g)	0.1	-	0.1	-
Sodium ether (g)	0.1	0.1	0.1	0.1
Benzalkonium chloride (g)	0.01	0.01	0.01	0.01
Sodium hydroxide (g)	added to pH 7			
Sterile purified water (ml)	added to 100%[(2)]			

[(2)] based on 100 ml of ophthalmic solution

Formulation Examples 5 to 8: Preparation of ophthalmic ointments containing poly-γ-glutamic acid

As shown in Table 3 below, ophthalmic ointments were prepared by mixing poly-γ-glutamic acid with isotonic agents (sodium chloride and potassium chloride), buffers (sodium hydrogen phosphate and sodium dihydrogen phosphate), a stabilizer (sodium ether), a preservative (benzalkonium chloride), a pH adjusting agent (sodium hydroxide) and ophthalmic ointment bases (white Vaseline and liquid paraffin).

Table 3

Component	Formulation Example 5	Formulation Example 6	Formulation Example 7	Formulation Example 8
Poly-γ-glutamic acid (g)	0.1	0.5	0.1	0.5
Sodium chloride (g)	0.2	0.2	0.2	0.2
Potassium chloride (g)	-	0.1	-	0.1
Sodium hydrogen phosphate (g)	0.1	0.1	0.1	0.1
Sodium dihydrogen phosphate (g)	0.1	-	0.1	-
Sodium ether (g)	0.1	0.1	0.1	0.1
Benzalkonium chloride (g)	0.01	0.01	0.01	0.01
Sodium hydroxide (g)	added to pH 7			
White Vaseline (g)	90	90	90	90

Liquid paraffin (g)	added to 100%[3]

[3] based on 100 g of ophthalmic ointment

INDUSTRIAL APPLICABILITY

As described above in detail, the pharmaceutical composition for treating corneal wounds, which comprises poly-γ-glutamic acid according to the present invention relieves inflammation by inhibiting a hyaluronidase enzyme which is activated upon the development of inflammation, and it maintains the ability of hyaluronic acid to stimulate corneal epithelial cell proliferation by inhibiting the degradation of hyaluronic acid. Thus, the composition of the present invention is useful for the treatment of corneal wounds.

Although the present invention has been described in detail with reference to the specific features, it will be apparent to those skilled in the art that this description is only for a preferred embodiment and does not limit the scope of the present invention. Thus, the substantial scope of the present invention will be defined by the appended claims and equivalents thereof.

THE CLAIMS

What is Claimed is:

1. A pharmaceutical composition for treating corneal wounds, which comprises poly-γ-glutamic acid as an active ingredient.

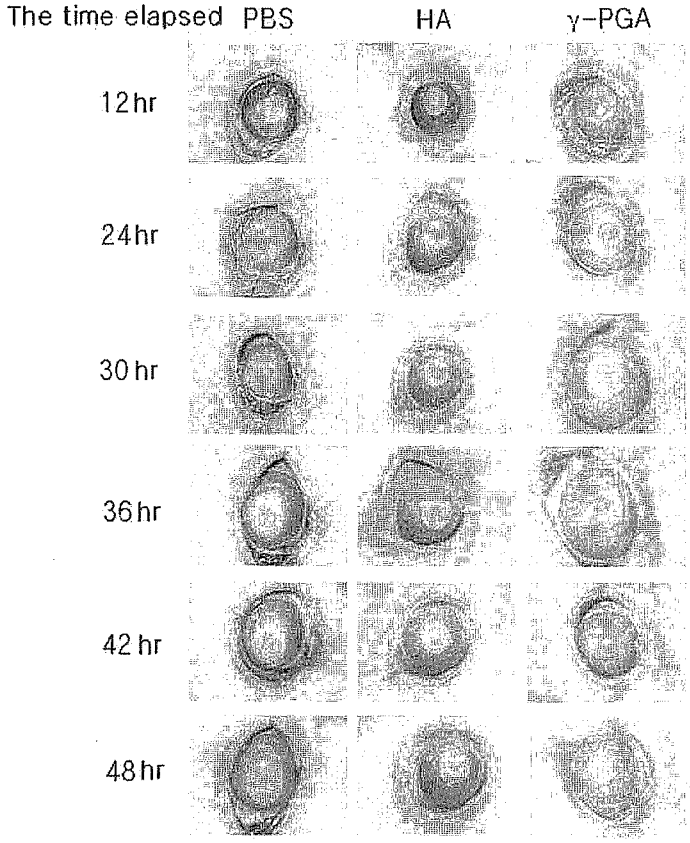
2. The pharmaceutical composition for treating corneal wounds according to claim 1, wherein an average molecular weight of the poly-γ-glutamic acid is 1-15,000kDa.

3. The pharmaceutical composition for treating corneal wounds according to claim 1, which is an ophthalmic solution.

DRAWINGS

1/1

FIG. 1



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2008/001051

A. CLASSIFICATION OF SUBJECT MATTER

A61K 31/765(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN(CA), eKIPASS(KIPO internal)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KR 10-582120 B1 (BIOLEADERS CORPORATION et al.) 15 May 2006 Cited in the application, see abstract, example 2, claim 4	1 - 3
Y	KR 10-2001-41337 A1 (WAKAMOTO PHARMACEUTICAL CO., LTD.) 15 May 2001 See abstract, page 2	1 - 3
A	WO 2006/90968 A1 (BIOLEADERS CORPORATION et al.) 31 August 2006 See the whole document	1 - 3
A	KR 10-475406 B1 (BIOLEADERS CORPORATION et al.) 25 February 2005 Cited in the application, see the whole document	1 - 3
A	KR 10-496606 B1 (BIOLEADERS CORPORATION et al.) 13 June 2005 Cited in the application, see the whole document	1 - 3
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☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
28 JUNE 2008 (28.06.2008)	**30 JUNE 2008 (30.06.2008)**

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Authorized officer
LEE, Dong Wook
Telephone No. 82-42-481-8163

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2008/001051

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