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Original Article

Testing of the Safety and the Effectiveness of Using *Samjeong* Pharmacopuncture Solution as Eye drops

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Key Words

Antibacterial effect; Eye drops; Eye irritation; *Samjeong* pharmacopuncture solution (SPS)

Abstract

Objective: This experimental study was designed to investigate the safety and the effectiveness of *Samjeong* pharmacopuncture solution (SPS) manufactured by using a the lowtemperature extract on process.

Methods: To identify the safety and the effectiveness of using SPS as eye drops, we performed applied eye irritation tests on rabbits and antibacterial tests for *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Aspergillus niger, Fusarium oxysporum, and Candida albicans.* The eye irritation test was performed according to the toxicity testing regulation of the Korea Food & Drug Administration (2009. 8. 24, KFDA 2009-116). After SPS had been applied on the left eye of the rabbits, eye irritation in the cornea, iris and conjunctiva was observed on the 1st, 2nd, 3rd, 4th & 7th day. After SPS had been dropped on bacterial species that cause keratitis, the minimum inhibition concentration and the size of the inhibition zone were measured. The anti-bacterial potency was also measured by taking the size of inhibition zone.

Results: After SPS had been administered on the left eye of the rabbits, none of nine rabbits were found to show abnormal signs or weight changes. After SPS had been administered on the left eye of the rabbits, no eye irritation in the cornea, iris and conjunctiva was observed on the 1st, 2nd, 3rd, 4th & 7th day. No specific response was detected in MIC for bacterial species *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Aspergillus niger, Fusarium oxysporum, and Candida albicans* after SPS had been applied.

Conclusions: This study suggests that SPS is a non-toxic and non-irritant medicine that does not cause any of eye irritation in rabbits, but it has no antibacterial effects on bacterial species that are well known to cause keratitis. These results suggest that more research is required on extracts from herbal medicines for treating keratitis.

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1. Introduction

Eye drops are a medical treatment applied to various ophthalmological diseases, so they must be safe and non-irritating as they directly affect the eyes. Especially, the fact that no blood vessels exist in the cornea makes localized eye-dropping more available and effective than oral medication; besides, effective cleansing of bacteria and toxins can be mostly achieved through the proper use of eye drops [1]. However, recently there have been no standardized pharmaceutical companies manufacturing eye drops for clinical use in korean medicine. For that reason, many pharmacopunctures manufactured by the Korea Pharmacopuncture Institute, which has aseptic facilities almost equivalent to the GMP level, are considered to be the most appropriate materials for making eye drops.

In order to utilize Pharmacopuncture solutions (PSS) from the Korean Pharmacopuncture Institute as eye drops, there have been constant experimental trials on ascertaining the safety and the effectiveness of saline solution, *Hwangryeonhaedoktang, Bovis Calculus* (B), *Fel Ursi* (U), *Bovis Calculus*

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& Fel Ursi (BU) *and Bovis Calculus* & *Fel Ursi* &+ *Moschus* (BUM) PSS [2-8]. However, PSS manufactured through distillation and alcohol immersion haven't shown any significant antibiotic efficacy in many testings so far. Thus, PSS with antibiotic effects need to be produced by using new extraction processes. Thereupon, this experiment on eye irritation and antibiotic effects was conducted with *Samjeong* pharmacopuncture solution (SPS), which was manufactured using a low-temperature extracting process.

2. Materials and methods

2.1. Materials

2.1.1. *Samjeong* pharmacopuncture solution

SPS used in this study was manufactured using a low-temperature extracting process and was provided by the Korea Pharmacopuncture Institute. The prescription is given in Table 1.

Table 1 Prescription of *Samjeong*

The Botanical Name of Herb	Relative Amount (g)
Taraxaci Herba	77.4
Lonicerae Flos	77.4
Rehmanniae radix	77.4
Forsythiae Fructus	77.4
Coptidis Rhizoma	47.6
Scutellariae Radix	47.6
Phellodendri Cortex	47.6
Gardeniae Fructus	47.6
Total Amount	500.0

2.1.2. Animals

In this study, animal experiments were conducted under the approval of the Pusan National University Hospital Institutional Animal Care and Use Commitee *(PNUH 2011-031)*. Nine New Zealand white rabbits (male: 5, female: 4) aged about 3~4 months (weight: 2.0~3.0 kg) were used in this study and snuffles, ear mites, coccidium infection states and conditions of fur and excrement were checked on arrival. They had been observed during one week of domestication at the breeding farm. Ophthalmological tests were performed on the conjunctiva, eyeball and cornea 24 hours before the experiment.

Rabbits were bred in the rabbit cage (420W×500D×310H ㎜) made of stainless and had free access to feed (Sinchon Co.) and water. The environment was maintained at a constant temperature (21±2℃) and humidity (60%).

2.1.3. Cell line and culturing strain

The cell line used in this experiment was provided by Korean Collection for Type Culture (KCTC). *Staphylococcus aureus* (KCTC 1916), *Staphylococcus epidermidis* (KCTC 1917) and *Pseudomonas aeruginosa* (KCTC 2004) were cultured in Trypic Soy Agar. *Aspergillus niger* (KCTC 6906) was cultured in Malt Extract Agar. *Fusarium oxysporum* (KCTC 16322) was cultured in Potato Dextrose Agar, and *Candida albicans* (KCTC 7965) was cultured in Yeast Mold Agar.

2.2. Methods

2.2.1. Eye irritation test

Eye irritation tests were conducted following the toxicity testing regulation of the Korea Food & Drug Administration (2009. 8. 24, KFDA 2009-116). Both eyes of all the laboratory rabbits had been tested 24 hours before the experiment began, among which rabbits with normal corneas were selected. SPS, 0.1 ㎖ was dropped on an eye of each of the nine rabbits, and after 20 ~30 seconds, three out of these were washed with 20 ㎖ of warm saline solution for a minute while the others were left untouched to be treated as a control group. After the application of SPS, changes in the weight and clinical symptoms on the 1st, 2nd, 3rd, 4th and 7th day were observed. The eye irritation tests were evaluated, with the maximum points set at 80 points on cornea response, 10 points on iris response and 20 points on the conjunctiva response, adding up to a total score of 110. If injury still remained on the affected eyes afterwards, the eye-dropping was applied every three days over 13 days (Tables 2, 3, and 4).

Table 2 Scale of weighted scores used for grading the severity of ocular lesions (cornea)

A. Opacity-degree of density (area which is most dense is taken for reading)	
No opacity and pyosis	0
Scattered or dense areas; every inch of iris is clearly visible	1
Easily discernible translucent areas; every inch of iris is slightly obscure	2
Opalescent areas; every inch of iris is invisible; the size of pupil is barely discernible	3
Opaque; iris is not observed	4
B. Area of corneal opacity	
One quarter (or less), but not zero	1
More than one-quarter ~ less than one-half	2
More than one-half ~ less than three-quarters	3
More than three-quarters up to whole area	4

Score equals A×B×5. Total maximum = 80.

Table 3 Scale of weighted scores used for grading the severity of ocular lesions (iris)

Values	
Normal	0
Remarkable folds, congestion, swelling, circumcorneal injection (any one of these or combination); iris still reacting to light	1
No reaction to light, hemorrhage, gross destruction (some of these or all)	2

Score equals A×5. Total maximum = 10.

"The mean index of ocular irritation (MIOI)" which had been obtained from the division of the total score of "the individual index of ocular irritation (IIOI)" by the number of rabbits, "the index of acute ocular irritation (IAOI)", which is the maximum value of "the mean index of ocular irritation (MIOI)" during observation and the day-7 IOII (indivisual ocular irritation index) were used in order to evaluate the degree of eye irritability (Table 5).

Table 4 Scale of weighted scores used for grading the severity of ocular lesions (conjunctiva)

A. Redness (refers to ocular and palpebral conjunctiva only)	
Normal	0
Vessels definitely injected above normal	1
More diffuse bright red. Each individual vessels is not observed easily.	2
Diffused beefy red	3
B. Chemosis	
No swelling	0
Any swelling above normal (included nictitating membrane)	1
Obvious swelling with partial eversion of eyelids	2
Swelling with eyelids about half closed	3
Swelling with eyelids about half closed to completely closed	4
C. Discharge	
No discharge	0
A little discharge (expect small amount of discharge observed in inner canthus of normal animal)	1
Discharge with moistening of the lids and hairs	2
Discharge with moistening of the lids, hairs and considerable area around the eye	3

Score equals (A+B+C)×2. Total maximum = 20.

Table 5 Irritation index of eye irritation

Rating	Evaluation value		
	IIOI	MIOI	Day-7 IAOI
Nonirritant	0~5	0 (after 48 hrs)	
Minimally irritant	5~15	≤5 (after 48 hrs)	
Mildly irritant	15~30	≤5 (after 4 days)	
Moderately irritant	30~60	≤20 (after 7 days)	≤30 (all of non-washing group) ≤10 (more than four of non-washing group)
Severely irritant	60~80	≤40 (after 7 days)	≤60 (all of non-washing group) ≤30 (more than four of non-washing group)
Extremely irritant	80~110		

IIOI: The individiual index of ocular irritation (total score of each animal).

MIOI: Mean index of ocular irritation (the amount of total score/tested animal No.).

Day-7 IAOI: The index acute ocular irritation (Max among MIOI on Day-7).

2.2.2. Antibacterial test (filter disc method)

Sterile water was added to the lyophilized ampule, and the medium was coated with 1~2 droplets of the cell line for 16~24 hours under 35±1℃. This single colony was then moved to the new medium and subcultured for another 16~24 hours under 35±1℃. A yeast fungus was cultured for 3 days and a mold was cultured for a week. Several colonies separated from the 10 ㎖ sterile saline solution were suspended, and the number of germs in the colony was counted up to 2.5~10×10^9 cells/㎖ through a microscope in order to be used as a source of inoculum. Mold was made to 2.5~10×10^9 cells/㎖ in the saline solution with 0.03% detergent and was used as the inoculum source. The prepared inoculum sources were coated with 0.4 ㎖ each on the medium and left to dry for 2~3 minutes with a plate lid slightly open on a clean bench. *Staphylococcus aureus, Staphylococcus epidermidis and Pseudomonas aeruginosa* were cultured in Tryptic soy agar, *Candida albicans* was cultured in Yeast malt agar, *Aspergillus niger* was cultured in Malt extract agar, and *Fusarium oxysporum* was cultured in Potato Dextrose Agar. SPS, 50 ㎕ of sample was dropped on the germ-seeded medium with a sterilized filter disc on top of it. Equal amounts of sterile water and Vioflox (Ofloxacin) were used for negative and positive control, respectively. The antibiotic potency was examined by measuring the clear zone after culturing (2~7days) each cell line at an appropriate temperature. To measure the minimal inhibitory concentration (MIC), we difuted an appropriate amount of the sample, and we performed the experiment in exactly the same way as above.

3. Results

3.1. Weight and general conditions

No abnormalities related to general conditions like weight, appearance, feed, water consumption, tremor, spasm, diarrhea, coma, drowsiness, contraction and dilatation of pupils, feces and urine, and disposal per day were found during this experiment (Tables 6 and 7).

Table 6 Weight change of New Zealand white rabbits for 7 Days

Day		Start	day 1	day 2	day 3	day 4	day 7	p-value
Weight (kg)	I	2.70 ±0.4	2.63 ±0.4	2.64 ±0.5	2.55 ±0.5	2.49 ±0.5	2.53 ±0.6	ns
	II	2.88 ±0.3	2.83 ±0.3	2.82 ±0.3	2.88 ±0.4	2.87 ±0.4	2.93 ±0.5	

Statistical significances were tested by using the one way analysis of variances among groups (SPSS 17.0). Values are represented as means ± S.Ds.

I: washed group after treatment with *Samjeong* Pharmacopuncture solution (n=3).

II: non-washed group after treatment with *Samjeong* Pharmacopuncture solution (n=6).

ns: non-significant.

Table 7 General condition of New Zealand white rabbits for 7 Days

Rabbit No. \ Day		Start	day 1	day 2	day 3	day 4	day 7
I	1 (F)	N	N	N	N	N	N
	2 (M)	N	N	N	N	N	N
	3 (M)	N	N	N	N	N	N
II	4 (F)	N	N	N	N	N	N
	5 (F)	N	N	N	N	N	N
	6 (F)	N	N	N	N	N	N
	7 (M)	N	N	N	N	N	N
	8 (M)	N	N	N	N	N	N
	9 (M)	N	N	N	N	N	N

I: washed group after treatment with *Samjeong* pharmacopuncture solution.

II: non-washed group after treatment with *Samjeong* pharmacopuncture solution.

F: Female, M: Male, N: Normal.

3.2. Eye irritation

After SPS had been administered only on the left eyes, no eye irritation on the affected cornea, iris and conjunctiva of the nine rabbits from both the washed and the non-washed groups was observed compared with the right eyes (control group) (Table 8, Fig. 1).

3.3. Antibacterial effect

SPS didn't show any antibacterial effects on *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger* and *Fusarium oxysporum.* On the other hand, Vioflox (Ofloxacin), the control group, shows strong antibacterial effects on *Staphylococcus aureus, Staphylococcus epidermidis* and *Pseudomonas aeruginosa,* forming an inhibition zone of over 1 ㎝. However, no antibacterial effects were seen on *Candida albicans, Aspergillus niger* and *Fusarium oxysporum* (Fig. 2).

Table 8 Eye irritation score of New Zealand white rabbits treated with *Samjeong* pharmacopuncture solution

Group	Rabbit No.	Check area	Tissue score	Day					Total score	I.I.O.I	M.I.O.I	I.A.O.I
				1	2	3	4	7				
I	1 (F)	Cornea	A×B×5	0	0	0	0	0	0/80	0	0	
		Iris	A×5	0	0	0	0	0	0/10	0	0	
		Conjunctiva	(A+B+C)×2	0	0	0	0	0	0/20	0	0	
	2 (M)	Cornea	A×B×5	0	0	0	0	0	0/80	0	0	
		Iris	A×5	0	0	0	0	0	0/10	0	0	
		Conjunctiva	(A+B+C)×2	0	0	0	0	0	0/20	0	0	
	3 (M)	Cornea	A×B×5	0	0	0	0	0	0/80	0	0	
		Iris	A×5	0	0	0	0	0	0/10	0	0	
		Conjunctiva	(A+B+C)×2	0	0	0	0	0	0/20	0	0	
II	4 (F)	Cornea	A×B×5	0	0	0	0	0	0/80	0	0	
		Iris	A×5	0	0	0	0	0	0/10	0	0	
		Conjunctiva	(A+B+C)×2	0	0	0	0	0	0/20	0	0	
	5 (F)	Cornea	A×B×5	0	0	0	0	0	0/80	0	0	0
		Iris	A×5	0	0	0	0	0	0/10	0	0	
		Conjunctiva	(A+B+C)×2	0	0	0	0	0	0/20	0	0	
	6 (F)	Cornea	A×B×5	0	0	0	0	0	0/80	0	0	
		Iris	A×5	0	0	0	0	0	0/10	0	0	
		Conjunctiva	(A+B+C)×2	0	0	0	0	0	0/20	0	0	
	7 (M)	Cornea	A×B×5	0	0	0	0	0	0/80	0	0	
		Iris	A×5	0	0	0	0	0	0/10	0	0	
		Conjunctiva	(A+B+C)×2	0	0	0	0	0	0/20	0	0	
	8 (M)	Cornea	A×B×5	0	0	0	0	0	0/80	0	0	
		Iris	A×5	0	0	0	0	0	0/10	0	0	
		Conjunctiva	(A+B+C)×2	0	0	0	0	0	0/20	0	0	
	9 (M)	Cornea	A×B×5	0	0	0	0	0	0/80	0	0	
		Iris	A×5	0	0	0	0	0	0/10	0	0	
		Conjunctiva	(A+B+C)×2	0	0	0	0	0	0/20	0	0	

I: washed group after treatment with *Samjeong* pharmacopuncture solution.

II: non-washed group after treatment with *Samjeong* pharmacopuncture solution.

Cornea A: Opacity, B: Area of cornea involved.

Iris A: Values.

Conjunctiva A: Redness, B: Chemosis, and C: Discharge.

IIOI: Individiual index of ocular irritation (total score of each animal).

MIOI: Mean index of ocular irritation (the amount of total score/tested animal No.).

Day-7 IAOI: The index acute ocular irritation (max among MIOI on Day-7).

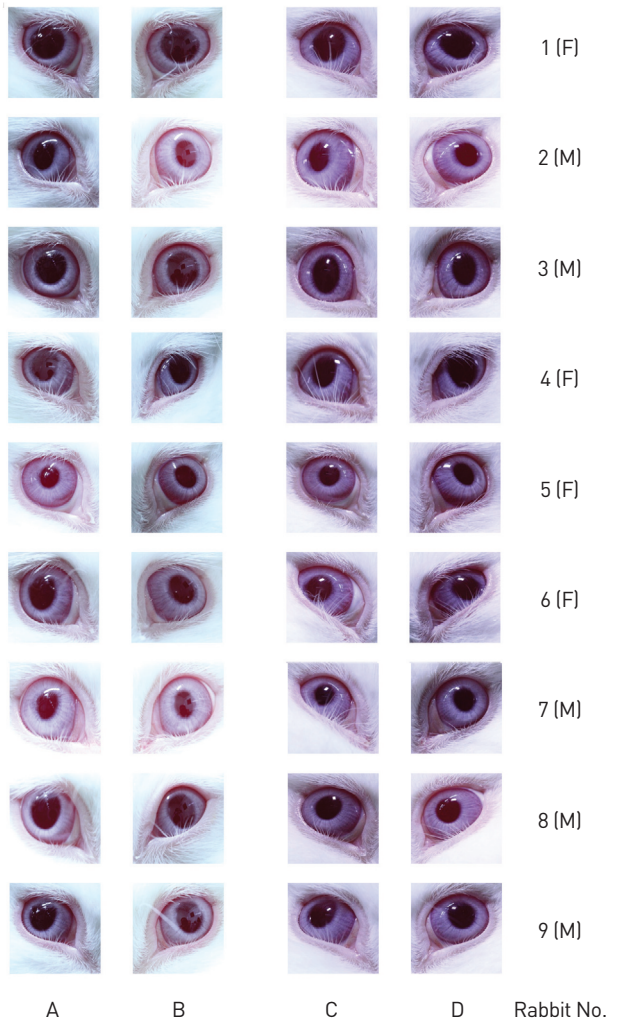

Figure 1 New Zealand white rabbit eye treated with *Samjeong* pharmacopuncture solution. Rabbit Nos. 1, 2, and 3 are the washed groups after treatment with *Samjeong* pharmacopuncture solution. Rabbit Nos. 4, 5, 6, 7, 8, and 9 are the non-washed groups after treatment with *Samjeong* pharmacopuncture solution. A is the first day of the experiment (Rt. eye), B is the first day of the experiment (Lt. eye), C is after 7 days (Rt. eye), and D is after 7 days (Lt. eye).

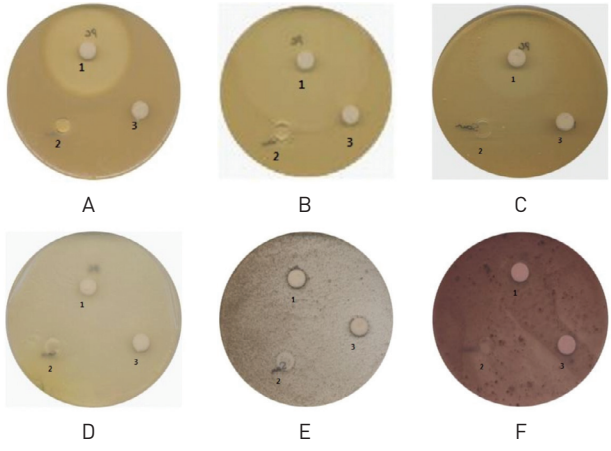

Figure 2 Inhibition zone of *Samjeong* pharmacopuncture solution. A: *Staphylococcus aureus*, B: *Staphylococcus epidermidis*, C: *Pseudomonas aeruginosa*, D: *Candida albicans*, E: *Aspergillus niger*, and F: *Fusarium oxysporum;* 1: Vioflox (Ofloxacin), 2: *Samjeong* pharmacopuncture solution, and 3: Normal saline.

4. Discussion

Currently, korean medical doctors are having difficulty treating ophthalmological patients because no standardized pharmaceutical company manufacture eye drops, which can possibly lead to a scale-down in our medical treatment range. Therefore, readily available forms of eye drops desperately need to be immediately developed in korean medical ophthalmology as various kinds of eye drops are already prevalent in western medicine. Especially, aseptic forms of eye drops are needed.

PSS is an aseptic treating material that is obtained from single or compound herbs through various extract on methods, and is applied in many clinical fields, chiefly by injection, for musculoskeletal or internal problems. However, the range of korean medical ophthalmological treatments could be expanded through this new method to aseptically manufacture PSS for eye drops.

To utilize PSS as eye drops, several experiments have chiefly focused on proving the safety and the effectiveness of anti-inflammatory herbs [2-8], and "A clinical case report of *Hwangryeonhaedok* pharmacopuncture soluton" was reported by the Korean Medical Society [9]. PSS manufactured through distillation and alcohol immersion have been found not to trigger any eye irritation in the safety and the effectiveness experiments so far, but different and improved extraction methods are still required because no significant antibacterial effects have been detected.

In low-temperature extraction, compound herbs are decocted and then separated to undergo decompression and low-temperature distillation. The outside temperature is maintained at 120℃, and the inside temperature is maintained at 60℃ during decompression and distillation. This method is recommended to maximize the efficacy of herbs and will replace the distillation method sooner or later [10].

Thus, by using SPS obtained from low-temperature extraction, we performed an antibacterial activity experiment on eye irritation by using six kinds of infectious keratitis-causing cell lines: *Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger* and *Fusarium oxysporum* [11-14]. No abnormalities were found in weight and general conditions during the experiment, and no eye irritation of the cornea, iris and conjunctiva in both the washed and the non-washed groups treated with SPS was observed, so the safety of SPS is assured.

In the antibacterial activity experiment, 50 ㎕ of SPS showed no antibacterial effects on the six kinds of cell lines with infectious keratitis-causing germs, yeast fungus and mold. Even an increased amount up to 200 ㎕ showed no such effects.

SPS is a non-toxic and non-irritant medicine which does not cause any of eye irritation in rabbits, but it has no antibacterial effects on bacterial species that are well known to cause keratitis. Consequently, the fact that SPS causes no eye irritation secures its safety, but the lack of effectiveness in antibacterial activity indicates that constant research on a new extract on method is still needed.

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