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Effect of Exopolymers from *Aureobasidium pullulans* on Formalin-Induced Chronic Paw Inflammation in Mice

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Abstract The effects of the exopolymers of *Aureobasidium pullulans* SM-2001 containing β-1,3/1,6-glucan on formalin-induced chronic inflammation were observed. Doses of 62.5, 125, and 250 mg/kg of the exopolymers were orally administered once a day for 10 days to formalin-induced chronic inflammatory mice (0.02 ml of 3.75% formalin was subaponeurotically injected into the left hind paw), and then the bilateral hind-paw thickness and volume were measured daily, while the paw wet-weight, histological profiles, and histomorphometrical analyses were conducted at termination. The results were compared with those for diclofenac, indomethacin, and dexamethasone (intraperitoneally injected) 15 mg/kg-dosed groups. All the animals were sacrificed 10 days after dosing. As a result of the formalin injection, a marked increase in the difference between the intact and formalin-induced paw thickness and volume was detected in the formalin-injected control compared with that in the intact control with time, plus at the time of sacrifice, the difference in the paw wet-weights was also dramatically increased. In a histological and histomorphometrical analysis, severe histological profiles of chronic inflammation were detected in the formalin-injected control with a marked increase in the thickness of the skin of the dorsum pedis. However, these formalin-induced chronic inflammatory changes were significantly and dose-dependently decreased by the exopolymer treatment. In conclusion, the exopolymer treatment inhibited the chronic inflammatory response induced by formalin injection in the mice. However, somewhat low efficacies were detected compared with those for the diclofenac-, indomethacin-, and dexamethasone-treated groups.

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Inflammation is an essential protective process preserving the integrity of organisms against physical, chemical, and infective insults. However, the inflammatory response to certain insults invariably and erroneously leads to the damage of normal tissues [10]. Chronic inflammation is an inflammatory response of prolonged duration: weeks, months, or even indefinitely, where the extended time course is provoked by the persistence of the causative stimulus for inflammation in the tissue. The formalin-injected hind paw of chronic inflammatory mice has generally been used as a classic method for detecting the efficacy of anti-inflammatory treatments. The effect of a drug is then based on observing changes in the paw's thickness, volume, weight, and histopathology [1, 11, 17]. The most widely used anti-inflammatory drugs are diclofenac and indomethacin, as cyclo-oxygenase inhibitors, and dexamethasone, as a well-known glucocorticoid, plus they are generally used as the control drugs in the development of new anti-inflammatory drugs [1, 4].

β-Glucan is a fiber-type complex sugar (polysaccharide) derived from the cell wall of Baker's yeast, oat, and barley fiber, and various medicinal mushrooms, such as maitake. The two primary uses of β-glucan are to enhance the immune system [5, 8] and lower blood cholesterol levels [3, 14], yet anti-osteoporotic effects have also been recently demonstrated [22]. Although the effect of enhancing the immune system can worsen or even induce inflammation, β-glucan activates macrophages or neutrophils and can

remove the cellular debris resulting from oxidative damage, thereby speeding up the recovery of damaged tissue [6, 19]. Meanwhile, an immunomodulatory agent can reduce the inflammation previously observed [18]. For example, nitric oxide (NO) plays an important role in inflammation, and NO synthase inhibitors can reverse several classic inflammatory symptoms [2]. The antioxidative effect of β-glucan has also already been demonstrated in various studies [13, 15, 20]. Therefore, it can be postulated that β-glucan will have a favorable effect on reducing or speeding up the recovery from local inflammation induced by irritants.

The exopolymers of *Aureobasidium pullulans* SM-2001, a UV-induced mutant, have previously been shown to be mixtures of various polymers, including β-glucan (half of the dry material is -1,3/1,6-glucans) [21]. Accordingly, in the present study, the effects of the exopolymers of *A. pullulans* SM-2001 on formalin-induced chronic inflammation were observed in comparison with those produced by diclofenac, indomethacin, and dexamethason, all well-documented anti-inflammatory drugs.

MATERIALS AND METHODS

Experimental Animals

Fifty-six male ICR mice (6-wks-old upon receipt; Jung-Ang Lab. Animal Co., Korea) were used after acclimatization for 7 days. The animals were allocated seven per polycarbonate cage in a temperature (20–25°C) and humidity (40–45%) controlled room. The light:dark cycle was 12 h:12 h, while food (Samyang, Korea) and water were supplied *ad libitum*. The animals were fasted overnight before being sacrificed (about 18hrs; water not restricted), and all procedures were undertaken in accordance with the National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals.

Test Articles, Grouping, and Dosing

The exopolymers of pullulans SM-2001 (Glucan Corp. Ltd., Korea), diclofenac (Suzhou Leader Chemical Co., China), indomethacin (Fluka, Switzerland), and dexamethasone (Sigma, U.S.A.) were used in this study. The animals were divided into eight groups of 7 mice: intact control; formalin-injected control; 62.5, 125, and 250 mg/kg exopolymer-dosed groups; and diclofenac, indomethacin, and dexamethasone 15 mg/kg-dosed groups. The exopolymers dissolved in distilled water were orally administered once a day for 10 days, while the diclofenac, indomethacin, and dexamethasone dissolved or suspended in saline were intrapeitoneally administered.

Induction of Chronic Inflammation

One hour before dosing the test articles, a subaponeurotic injection of 0.02 ml of 3.75% formalin (Sigma, U.S.A.) was administered to the left hind paw (planta pedis) on the first and third days of the experiment. The right hind paw was considered as the control. In the case of the intact control, the same volume of saline as that used in the other dosing groups, including the vehicle control, was administered in the same region using the same method.

Paw Thickness Measurement

One hour before the formalin injection or 2 h before dosing the test articles, the thicknesses of both hind paws were measured using an electronic digital caliper and recorded once a day for 10 days. The difference in thickness between the intact paw and induced paw was calculated as follows:

Difference in paw thickness (mm)
=thickness of induced paw−thickness of intact paw

Paw Volume Measurement

One h before the formalin injection or 2 h before dosing the test articles, the lengths of the long axis (longitudinal; excluding dactyl region) and short axis (cross) of both hind paws were measured using an electronic digital caliper and recorded once a day for 10 days. The difference in the paw volume between the intact paw and induced paw was calculated as follows:

Paw volume (mm^3)=1/2(length of long axis
×length of short axis×thickness of paw)
Difference in paw volume (mm^3)
=(volume of induced paw−volume of intact paw)

Paw Wet-Weight Measurement

At sacrifice, the wet weight of both paws was measured, and to reduce any deviation due to individual body weight differences, the relative weight (%) was calculated using the body weight at sacrifice and absolute weight, with the difference between the intact and induced paws calculated as follows:

Relative weight (%)
=(Absolute weight/Body weight at sacrifice)×100
Difference in weight (g)
=(weight of induced paw−weight of intact paw)

Histology and Histomorphometry

The dorsum pedis (including the subcutaneous regions) was separated from the hind paws, and longitudinally trimmed. Thereafter, they were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned (3–4 μm), and stained with Masson's trichrome. The histological profiles of the paws were observed compared with that for the intact control. The thickness of the skin (including the subcutaneous tissue) of the dorsum pedis was calculated using automated image analysis (analySIS Image Processing;

SIS, Germany) under a light microscope (Zeiss, Germany) (×50). The thickness of the regular regions, including the epithelium and subcutaneous tissue, was measured.

Statistical Analyses

All data were calculated as the mean±SD, and the statistical analyses conducted using the Mann-Whitney U-Wilcoxon Rank Sum W test (MW test) with SPSS for Windows (Release 6.1.3., SPSS Inc., U.S.A.).

RESULTS

Changes in Paw Thickness

A significant ($p<0.01$) increase in the difference of thickness between the induced and intact paws was detected in the formalin-injected control compared with that in the intact control from 1 day after dosing. However, the differences in all the dosed groups were significantly ($p<0.01$ or $p<0.05$) decreased compared with that in the formalin-injected control from 4 or 6 days after dosing. A clear dose-dependent decrease in the paw thickness difference was detected in the exopolymer-dosed groups (Table 1).

Changes in Paw Volume

A significant ($p<0.01$) increase in the volume difference between the induced and intact paws was detected in the formalin-injected control compared with that in the intact control from 1 day after dosing. However, the differences for all the dosed groups were significantly ($p<0.01$ or $p<0.05$) decreased compared with that for the formalin-injected control from 3 to 6 days after dosing. A clear dose-dependent decrease in the paw volume difference was detected in the exopolymer-dosed groups (Table 2).

Changes in Paw Wet Weight

A significant ($p<0.01$) increase in the absolute and relative weights of the induced hind paws was detected in the formalin-injected control compared with that in the intact control, also leading to a significant increase in the difference in the wet weight between the intact and induced paws. However, the increase in the paw wet weights was dramatically decreased when compared with that in the vehicle control, although significance ($p<0.01$ or $p<0.05$) was restricted to certain groups of absolute weights. A clear dose-dependent decrease in the paw wet-weight difference was detected in the exopolymer-dosed groups (Table 3).

Histopathology of Induced Paw

Histopathological changes related to chronic inflammation, such as severe fibrosis, the formation of necrotic debris, and infiltration of inflammatory cells, were observed in the formalin-injected control, leading to the hypertrophy of subcutaneous regions. In all the dosed groups, including the exopolymer groups, these histopathological changes were dramatically decreased compared with those in the formalin-injected control. In addition, a clear dose-

Table 1. Changes in the difference between intact and induced paw thicknesses in intact and formalin-injected mice.

Group	Days after dosing										
	0[a]	1	2[a]	3	4	5	6	7	8	9	10
Controls											
Intact	−0.03± 0.09	0.02± 0.16	0.08± 0.18	0.03± 0.13	0.01± 0.08	−0.01± 0.07	−0.04± 0.09	0.02± 0.07	−0.02± 0.03	0.00± 0.05	0.02± 0.02
Formalin-injected	0.02± 0.05	1.38± 0.50*	1.33± 0.59*	1.78± 0.60*	1.51± 0.47*	1.32± 0.27*	1.62± 0.63*	1.99± 0.61*	1.55± 0.51*	1.79± 0.57*	1.81± 0.66*
Diclofenac	0.05± 0.07	1.70± 0.26*	1.50± 0.47*	1.67± 0.41*	1.13± 0.41*	0.64± 0.28*,#	0.38± 0.26**,#	0.51± 0.29*,#	0.43± 0.39*,#	0.60± 0.35*,#	0.53± 0.35*,#
Indomethacin	−0.04± 0.12	1.52± 0.36*	1.41± 0.12*	1.43± 0.53*	0.93± 0.42*,##	0.59± 0.29*,#	0.73± 0.51*,##	0.29± 0.32**,#	0.47± 0.20*,#	0.50± 0.35*,#	0.58± 0.31*,#
Dexamethasone	0.02± 0.13	1.57± 0.85*	0.76± 0.77	1.51± 0.29*	0.96± 0.48*,#	0.62± 0.36*,#	0.40± 0.33**,#	0.26± 0.34#	0.42± 0.23*,#	0.44± 0.34*,#	0.28± 0.19**,#
Exopolymers of *A. pullulans* SM-2001											
250 mg/kg	−0.02± 0.05	1.39± 0.27*	1.17± 0.49**	1.44± 0.55*	0.77± 0.63*,##	0.62± 0.49*,##	0.68± 0.40*,#	0.64± 0.59*,#	0.60± 0.32*,#	0.44± 0.29*,#	0.53± 0.39*,#
125 mg/kg	0.00± 0.04	1.67± 0.61*	1.24± 0.30*	1.38± 0.44*	0.83± 0.57*,##	0.70± 0.45*,##	0.85± 0.77*	0.65± 0.66*,#	0.88± 0.63*,##	0.74± 0.58*,##	0.62± 0.43*,#
62.5 mg/kg	−0.01± 0.08	1.37± 0.55*	0.82± 0.58*	1.29± 0.42*	1.22± 0.52*	0.94± 0.51*	0.78± 0.29*,##	1.00± 0.34*,#	0.92± 0.32*,##	0.98± 0.51*,##	0.95± 0.43*,##

Mean±SD, mm (n=7); [a]Day of formalin injection; *$p<0.01$ and **$p<0.05$ compared with that for intact control; #$p<0.01$ and ##$p<0.05$ compared with that for formalin-injected control.

Table 2. Changes in the difference between intact and induced paw volumes in intact and formalin-injected mice.

Group	Days after dosing										
	0[a]	1	2[a]	3	4	5	6	7	8	9	10
Controls											
Intact	1.04± 8.82	6.34± 3.60	8.65± 6.78	3.85± 5.56	1.42± 2.61	−1.24± 7.75	−0.91± 2.02	0.25± 2.45	0.16± 1.42	0.57± 2.16	1.68± 2.03
Formalin-injected	1.01± 1.50	82.56± 18.98*	69.61± 26.83*	100.41± 36.53*	86.40± 24.35*	78.91± 18.50*	81.95± 24.34*	107.90± 35.93*	87.71± 28.01*	105.66± 40.65*	106.75± 43.64*
Diclofenac	3.26± 2.63	81.81± 19.88*	68.39± 24.81*	92.44± 25.30*	46.39± 16.76*,#	32.56± 18.29*,#	21.84± 10.77*,#	24.78± 13.89*,#	29.99± 12.83*,#	38.52± 19.11*,#	33.21± 16.12*,#
Indomethacin	−1.18± 2.76	85.90± 18.51*	73.72± 16.88*	70.66± 15.72*	45.62± 16.07*,#	29.50± 11.88*,#	32.33± 12.98*,#	23.64± 14.64*,#	27.00± 9.88*,#	26.58± 15.19*,#	31.05± 10.93*,#
Dexamethasone	1.19± 6.34	67.56± 38.44	40.04± 36.77	66.84± 14.43*	45.49± 19.93*,##	21.74± 7.72*,#	18.77± 9.44*,#	15.53± 11.76*,#	19.57± 7.12*,#	16.38± 8.80*,#	23.47± 6.66*,#
Exopolymers of *A. pullulans* SM-2001											
250 mg/kg	0.81± 10.18	59.25± 17.31*	52.96± 31.00*	60.12± 19.05*,##	37.33± 26.10*,#	30.42± 24.08*,#	42.60± 25.94*,##	38.31± 31.75*,#	42.42± 27.95*,##	36.98± 19.04*,#	46.29± 35.68*,#
125 mg/kg	0.21± 2.68	80.38± 30.78*	57.73± 22.36*	53.85± 28.18*,##	49.24± 27.86*,##	36.04± 27.76*,#	43.90± 27.20*,#	42.71± 29.44*,#	50.08± 32.56*	49.80± 30.92*,##	51.26± 31.09*,##
62.5 mg/kg	5.38± 11.45	75.76± 36.57*	54.63± 32.75*	75.74± 27.29*	66.92± 36.05*	57.52± 41.65*	46.87± 26.74*,##	58.07± 22.28*,##	57.68± 20.36*,##	57.73± 34.95*,##	57.61± 27.56*,##

Mean±SD, mm^3 (n=7); [a]Day of formalin injection; *$p<0.01$ compared with that for intact control; #$p<0.01$ and ##$p<0.05$ compared with that for formalin-injected control.

dependency was also demonstrated in the exopolymer-dosed groups (Figs. 1A–1H).

Histomorphometrical Analyses of Induced Paw

A significant ($p<0.01$) increase in the thickness of the dorsum pedis of the induced paw was detected in the vehicle control compared with that in the intact control. However, this increased thickness was dramatically and significantly ($p<0.01$ or $p<0.05$) decreased in all the dosed groups when compared with that in the formalin-injected control. In addition, a clear dose-dependency was also demonstrated in the exopolymer-dosed groups (Table 4).

DISCUSSION

Formalin-injected hind-paw chronic inflammatory mice are generally used to detect the efficacy of anti-inflammation [1, 11, 17]. In the present study, the effects of the exopolymers of *A. pullulans* SM-2001 (half of the dry material is -1,3/1,6-glucans), a UV-induced mutant of *A. pullulans* [21], on formalin-induced chronic inflammation were observed in comparison with diclofenac, indomethacin, and dexamethasone, all well-documented anti-inflammatory drugs. As a result of the exopolymer treatment, the changes due to chronic inflammation, such as a marked increase in the induced paw thickness, volume, and weight, histopathological

Table 3. Changes in the difference between intact and induced paw thicknesses in intact and formalin-injected mice.

Group	Absolute weight (g)			Relative weight (%)		
	Intact paw	Induced paw	Differences	Intact paw	Induced paw	Differences
Controls						
Intact	0.193±0.011	0.197±0.011	0.004±0.004	0.635±0.032	0.649±0.031	0.013±0.014
Formalin-injected	0.199±0.006	0.335±0.089*	0.136±0.088*	0.680±0.037	1.150±0.341*	0.470±0.318*
Diclofenac	0.194±0.009	0.307±0.090**	0.112±0.085**	0.644±0.021	1.014±0.291**	0.370±0.280**
Indomethacin	0.199±0.005	0.297±0.060*	0.098±0.061*	0.658±0.015	0.987±0.224*	0.330±0.211*
Dexamethasone	0.167±0.007*,#	0.241±0.039**,##	0.073±0.036*	0.702±0.027*	1.011±0.172*	0.309±0.158*
Exopolymers of *A. pullulans* SM-2001						
250 mg/kg	0.203±0.025	0.302±0.078*	0.099±0.079**	0.694±0.010	1.039±0.300*	0.346±0.290**
125 mg/kg	0.197±0.014	0.301±0.063*	0.103±0.063*	0.668±0.038	1.016±0.199*	0.349±0.208*
62.5 mg/kg	0.195±0.015	0.305±0.046*	0.110±0.043*	0.656±0.035	1.026±0.143*	0.370±0.145*

Mean±SD, g or % (n=7); Relative weight (%)=(absolute paw weight/body weight at sacrifice)×100; Differences=induced paw weight−intact paw weight; *$p<0.01$ and **$p<0.05$ compared with that for intact control; #$p<0.01$ and ##$p<0.05$ compared with that for formalin-injected control.

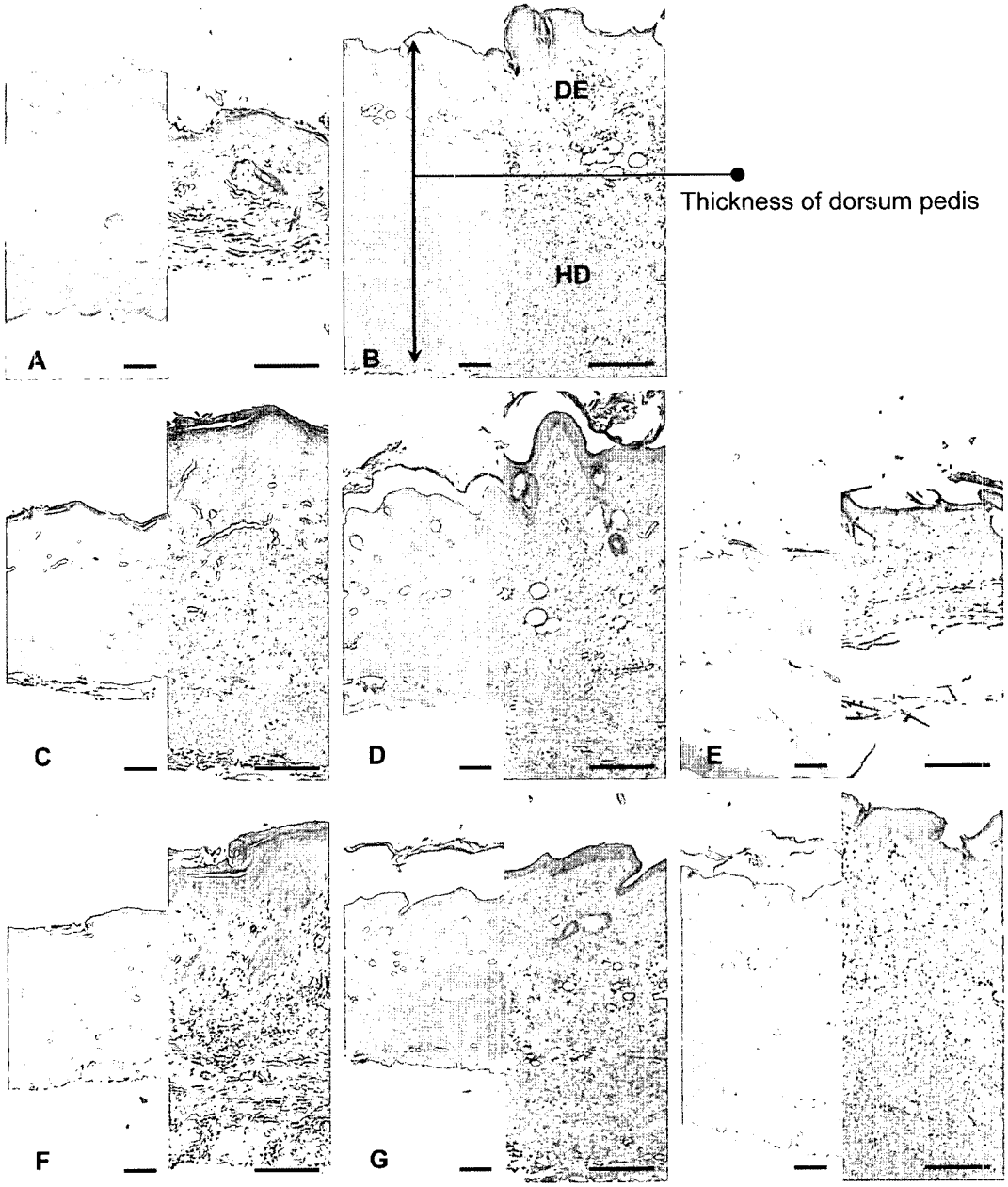

Fig. 1. Histological profiles of induced paws: detected in intact control (**A**), formalin-injected control (**B**), and diclofenac (**C**), indomethacin (**D**), dexamathasone (**E**), exopolymer 250 (**F**), 125 (**G**), and 62.5 (**H**) mg/kg-dosed groups.
Note that the classic histopathological profile of chronic inflammation, including the formation of necrotic debris in the epithelium (EP), plus severe fibrosis and infiltration of inflammatory cells, mainly lymphocytes in the hypodermis region (HD), were detected in the formalin-injected control. However, these signs were dramatically decreased in all the dosed groups tested. Masson's trichrome stain, scale bars=200 μm.

changes, such as severe fibrosis, the formation of necrotic debris, and infiltration of inflammatory cells, and an increase in the thickness of the dorsum pedis, were all significantly ($p<0.01$ or $p<0.05$) and dose-dependently inhibited. Therefore, these results are considered as direct evidence that exopolymers improve the chronic inflammatory response induced by formalin. The previously reported immunomodulatory [6, 19] and antioxidative effects [13, 15, 20] of β-glucans are thus considered as the major mechanisms of the anti-inflammatory effect exhibited by the exopolymers in the present study, as immunomodulatory agents can reduce inflammation [18], whereas NO synthase inhibitors can reverse several classic inflammatory symptoms [2].

After a local injection of formalin, marked increases in the paw thickness, volume, and weight were detected as the general chronic inflammation response, and these

Table 4. Changes in histomorphometry analysis of induced paw in intact and formalin-injected mice.

Group	Thickness of dorsum pedis
Controls	
Intact	351.14±122.95
Formalin-injected	1584.69±318.53*
Diclofenac	838.52±192.14*,#
Indomethacin	803.00±154.99*,#
Dexamethasone	427.77±115.65*,#
Exopolymers of *A. pullulans* SM-2001	
250 mg/kg	890.98±105.64*,#
125 mg/kg	1024.03±73.13*,#
62.5 mg/kg	1208.39±121.49*,##

Mean±SD, μm (n=7); *$p<0.01$ compared with that for intact control; #$p<0.01$ and ##$p<0.05$ compared with that for formalin-injected control.

increases have already been used as valuable markers for testing anti-inflammatory effects [9, 17, 23, 24]. In the present study, the increased paw thickness, volume, and weight were dose-dependently inhibited by the exopolymer treatment. Consequently, these inhibitions were considered as direct evidence that the exopolymers used in this study had a favorable effect on reducing the chronic inflammatory response.

Histopathologically, severe fibrosis, the formation of necrotic debris, infiltration of inflammatory cells, mainly lymphocytes, and hypertrophy of subcutaneous regions are used as signs of chronic inflammation after a local injection of formalin [7, 12, 16], also resulting in a marked increase in the thickness of the dorsum pedis. However, in the present study, these histopathological changes and the thickness of the dorsum pedis were dose-dependently decreased after treatment with the exopolymers. Consequently, these inhibitions were considered as direct evidence that the exopolymers used in this study had a relatively favorable effect on reducing the chronic inflammatory response.

Accordingly, based on the current results, it was concluded that the exopolymers of *A. pullulans* had a rather favorable effect on reducing the chronic inflammatory response induced by formalin injection in mice. However, somewhat low efficacies were detected compared with those for the diclofenac-, indomethacin-, and dexamethasone-treated groups.

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