J Pharmacol Sci **99**, 353 – 363 (2005)

Journal of Pharmacological Sciences

Full Paper

Sclerosing Effect of OC-108, a Novel Agent for Hemorrhoids, Is Associated With Granulomatous Inflammation Induced by Aluminum

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Received July 25, 2005; Accepted October 7, 2005

Abstract. OC-108 is a novel sclerosing agent for hemorrhoids, containing aluminum potassium sulfate (alum) and tannic acid as its main ingredients. In clinical studies, OC-108 injection therapy for severe internal hemorrhoids proved to be highly effective, not only on bleeding but also for prolapse, and the effects were comparable to hemorrhoidectomy. The aim of this study was to elucidate the mode of action by administrating the agent s.c. to mice and rats. In response to OC-108 injection, inflammation with necrosis developed at an early stage followed by granuloma formation with fibrosis at the injection site. Necrotic debris with aluminum was observed in the granuloma for a long period. Alum, as well as OC-108, induced vascular permeability, leukocyte infiltration, and granuloma formation; however, tannic acid did not. On the other hand, tannic acid inhibited leukocyte infiltration induced by alum but did not inhibit granuloma formation. These results indicate that OC-108 causes sclerosis and retraction of hemorrhoids through fibrosis associated with granulomatous chronic inflammation induced by the main active ingredient alum and that the adjunct ingredient tannic acid reduces excessive acute inflammation induced by alum.

Keywords: OC-108, aluminum, tannic acid, granuloma, fibrosis

Introduction

Internal hemorrhoids are one of the commonest ailments that afflict mankind, exhibiting the cardinal symptoms of bleeding and prolapse. The main forms of treatment for the disease are conservative measures using topical medicine, injection therapy using sclerosing agents (sclerotherapy), rubber band ligation, and hemorrhoidectomy. Among them, sclerotherapy is a simple and safe palliative treatment in the management of symptomatic hemorrhoids and has few complications. In many countries, 5% phenol in almond oil (PAO) has been used as a confirmed sclerosing agent for hemorrhoids. This irritant solution is injected into hemorrhoids to induce inflammation, aiming to reduce blood flow inside the hemorrhoids and to fix hemorrhoids to the underlying muscular coat by secondary fibrosis induced by inflammation. The treatment is directed at the control of bleeding, but it provides only short-term benefits in the majority of patients. Moreover, it has been reported that the treatment is less effective for prolapse, the major symptom in patients with severe internal hemorrhoids (1, 2).

OC-108 is a novel sclerosing agent containing aluminum potassium sulfate (alum) and tannic acid as active components and some excipients in the formulation. It is pharmaceutically modified from *Xiaozhiling*, a Chinese agent for the treatment of internal hemorrhoids (3, 4). In clinical studies, OC-108 injection therapy for severe internal hemorrhoids proved to be highly effective, not only on bleeding but also for prolapse (5, 6), and the effects were comparable to hemorrhoidectomy (6). OC-108 is considered to induce persistent fibrosis (7) leading to sclerosis and retraction of hemorrhoids, although mechanisms of action of the agent have not been fully clarified.

In this study, OC-108 was injected s.c. into an air pouch in rats and examined for histological changes at the injection site chronologically to elucidate the pharmacological profile of the agent. The application

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of the air pouch model is thought to be advantageous to see retraction of granulomas in the pouch wall since the pouch technique has been devised as a procedure for the evaluation of inflammation and wound healing (8). Furthermore, to clarify the significance of active components in formulating OC-108, inflammation inducing actions of alum and tannic acid on mice and rats were evaluated by quantitative assays.

Materials and Methods

Animals

Male Wistar rats, Donryu rats, and CD1 (ICR) mice were obtained from Charles River Japan (Yokohama). Animals were housed under conditions of controlled temperature (23 ± 3°C) and humidity (55 ± 10%) with 12-h illumination cycles. They were allowed to acclimate for 1 week before the experiments, and at that time, rats were 6 weeks of age and mice were 5 weeks of age. All experiments were approved by the Animal Ethical Committee of Mitsubishi Pharma Corporation and performed in accordance with guidelines of The Japanese Pharmacological Society.

Compounds

OC-108 injection 4%, an undiluted solution of OC-108, was prepared at the Pharmaceutical Development Laboratories of Mitsubishi Pharma Corporation. PAOSCLE®, a preparation of PAO, was purchased from Torii Pharmaceutical Co., Ltd. (Tokyo). Alum, tannic acid, and saline, all of which are approved drugs in Japan, were purchased from Miyazawa Pharmaceutical Co., Ltd. (Tokyo), Fuji Chemical Industry Co., Ltd. (Wakayama), and Otsuka Pharmaceutical Factory, Inc. (Tokushima), respectively.

Just prior to use, OC-108 injection was diluted with an equal volume of saline. PAO was used as an intact solution. Alum and tannic acid were dissolved in saline and sterilized by filtration through a 0.22-μm filter. In this study, the concentrations of OC-108 and PAO were defined in order to conform with those used clinically.

Histopathological evaluation

Injection of test solutions into air pouches was performed by modifying the method of Selye (8). The dorsal region of Wistar rats was shaved, following which the animals were placed under light ether anesthesia. Then 4 ml of air was injected s.c. on the dorsum and a regular spherical or ellipsoid air pouch was formed. Two days later, the air was released through a needle, and then 2 ml of OC-108 was injected though the same needle under light ether anesthesia. Also, 2 ml of saline was used as a vehicle control, and 1 ml of PAO was used as a positive control. The rats were exsanguinated under ether anesthesia and the skin tissues at the injection sites were dissected out 1, 3, 7, 14, 28, 56, 84, 112, and 140 days after the injection. The skin tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, microtomed, and subjected to conventional staining with hematoxylin-eosin and elastica-van Gieson. To detect proliferating cells, immunohistochemical staining for proliferating cell nuclear antigen (PCNA) was performed using mouse monoclonal antibody to PCNA (Dako Cytomation, Copenhagen, Denmark) and ENVISION + kit/HRP-DAB for mouse antibody (Dako Cytomation), followed by nuclear counterstaining with methyl green. To examine aluminum localization, sections from saline- and OC-108-treatment groups were stained with 0.15% naphthochrome green (Aldrich Chem. Co., Milwaukee, WI, USA). Each section was examined under a light microscope. Histopathological assessment was performed by an observer unaware of the treatments using the grading criteria as outlined in Table 1.

The basis for selecting the injection volume was as follows: PAO produced mortal convulsions at 2 ml, whereas it produced no death at 1 ml in a preliminary study in rats. Therefore, 1 ml was selected as the maximal injectable volume of PAO. A 1-ml injection of PAO in rats was sufficient as a positive control, because the clinical injection volume of PAO is 15 ml at most (1, 2), while that of OC-108 in the clinical studies averaged 30 ml.

Evaluation of vascular permeability

The test solutions (0.025 ml/paw) were injected s.c. in the left planta of mice immediately after i.v. injection of Evans blue dye (50 mg/kg) into the tail vein. After 5 min, the mice were exsanguinated under ether anesthesia, and right and left back paws were cut into small pieces. The extravasated dye in the tissue was extracted by acetone/0.5% sodium sulfate solution (7:3, v/v) according to the method of Harada et al. (9). Absorbance of the extract at 620 nm was measured and the concentration of dye was quantified by a spectrophotometer (model V-560; JASCO, Tokyo). The amount of dye extravasation was calculated by subtraction of dye concentration in the right paw (untreated) from that in the left paw (test solution treated).

Evaluation of leukocyte infiltration

Leukocyte infiltration in the inflammatory exudates was assayed using a modification of Simmons' method (10). The dorsal region of Donryu rats was shaved, following which the animals were placed under light ether anesthesia. Sterilized pellets of polyurethane

Table 1. Grading criteria for histopathological findings

<Necrosis>
+: localized in a small area
++: multifocal or spreading to a moderate area
+++: spreading to a wide area

<Hemorrhage>
+: localized in a small area
++: multifocal or spreading to a moderate area
+++: spreading to a wide area

<Edema>
+: slightly sparse interstitium
++: clearly sparse interstitium
+++: markedly sparse interstitium

<Inflammatory cell infiltration>
+: localized around the injection site
++: spreading to a wide area
+++: densely spreading to a wider area

<PCNA stain>
+: a few cells with faintly stained
++: numerous cells with faintly stained or a few cells with densely stained
+++: numerous cells with densely stained

<Neovascularization>
+: scattered capillary growth
++: dense capillary growth
+++: hemangioma-like capillary growth

<Epithelioid cell granuloma>
+: nodule thickness <1 mm
++: nodule thickness ≥1 mm
+++: nodule thickness ≥2 mm

<Fibrosis>
+: sparse fibrous tissue
++: dense fibrous tissue
+++: highly dense fibrous tissue

<Cavitation>
+: cavity length <1 mm
++: cavity length ≥1 mm
+++: cavity length ≥2 mm

<Vacuoles>
+: a few vacuoles
++: numerous vacuoles with diameter <1 mm
+++: numerous vacuoles with diameter ≥1 mm

<Naphthochrome green stain (aluminum stain)>
+: a few cells with faintly stained
++: numerous cells with faintly stained or a few cells with densely stained
+++: numerous cells with densely stained

–: no change

sponge (50 ± 2 mg) soaked with 0.5 ml of the test solutions were implanted into a subcutaneous space at a scapula site through a slit incised in the lumber skin, and then the slit was sutured. The rats were exsanguinated under ether anesthesia at the indicated days after the implantation and the sponges were dissected out. Exudate was carefully squeezed from the sponges into tubes and the volume was measured. The number of leukocytes per unit volume of exudate was counted by hematology analyzer (Celltac®, model MEK-4200 and MEK-5258; Nihon Kohden, Tokyo). The cell number was calculated by multiplying the count and the volume of each exudate.

Evaluation of granuloma formation

A 2-ml aliquot of a test solution was injected into the air pouch as described above using Donryu rats. The rats were exsanguinated under ether anesthesia at the indicated days after the injection and the granuloma that had developed at the subcutaneous area was dissected out. Any fluid found in the pouch was drained from the tissue. The granuloma's weight was determined by an electronic analytical balance (model AC120S; Sartorius, Tokyo).

Statistical analyses

In the histopathological study, the grades of findings, –, +, ++, and +++, were replaced by the scores of 0, 1, 2, and 3, respectively. The mean scores were calculated in each group, and differences between OC-108 and PAO treated groups were evaluated using the Wilcoxon rank-sum test. In the other experiments, data are expressed as means ± S.E.M. Statistical significance of the inflammation inducing effects of each test solution was determined using Dunnett's multiple comparison test. Dose-response of alum was examined by linear regression analysis. Comparison between OC-108 and alum treated groups, or between alum treated group and alum with tannic acid treated group, was performed using Student's *t*-test. Differences were assessed with two-sided test, with an alpha level of 0.05. Statistical analyses were done with the SAS system, ver. 6.12 (SAS Institute Inc., Cary, NC, USA) and a pre-validated FORTRAN program coded by the Biometrics Section of Mitsubishi Pharma Corporation.

Results

Histological changes at the injection site of OC-108 (tissue sclerosing action)

General condition of the rats in saline and OC-108 groups was normal throughout the experiment. In PAO group, 69 rats were normal, while 3 rats died 1 day after the injection for unknown reasons.

Histopathological findings after injection of OC-108 are shown in Table 2. By OC-108 treatment, necrosis of the subcutaneous tissues around the pouch was observed 1 day after the injection and was accompanied by hemorrhage, inflammatory cell infiltration, and edema around the necrotic area (Fig. 1A). The necrosis and

Table 2. Histopathological appearance of subcutaneous tissue after injection of OC-108 in rats

Test solution		OC-108								
Days after injection		1	3	7	14	28	56	84	112	140
Number of animals		8	8	8	8	8	8	8	8	8
Necrosis	–	2	0	0	0	0	3	2	5	5
	+	5	4	0	5	7	5	6	3	2
	++	1	4	4	3	1	0	0	0	1
	+++	0	0	4	0	0	0	0	0	0
Hemorrhage	–	4	4	6	7	8	8	8	8	8
	+	4	4	2	1	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Edema	–	0	7	8	8	8	8	8	8	8
	+	7	1	0	0	0	0	0	0	0
	++	1	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Inflammatory cell infiltration	–	0	0	0	0	0	1	1	1	2
	+	8	2	1	4	8	7	7	7	6
	++	0	6	5	4	0	0	0	0	0
	+++	0	0	2	0	0	0	0	0	0
PCNA stain	–	1	0	0	0	0	1	0	0	0
	+	5	6	0	0	2	3	4	3	8
	++	1	2	3	4	1	4	4	4	0
	+++	1	0	5	4	5	0	0	1	0
Neovascularization	–	7	7	4	8	8	8	8	7	8
	+	1	1	4	0	0	0	0	1	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Days after injection		1	3	7	14	28	56	84	112	140
Number of animals		8	8	8	8	8	8	8	8	8
Epithelioid cell granuloma	–	8	8	4	0	0	0	0	0	0
	+	0	0	4	0	2	7	6	5	7
	++	0	0	0	6	6	1	2	2	1
	+++	0	0	0	2	0	0	0	1	0
Fibrosis	–	8	8	4	0	0	0	0	0	0
	+	0	0	4	4	0	5	1	5	3
	++	0	0	0	4	7	3	6	2	3
	+++	0	0	0	0	1	0	1	1	2
Cavitation	–	0	0	0	8	8	8	8	8	8
	+	4	2	5	0	0	0	0	0	0
	++	4	4	3	0	0	0	0	0	0
	+++	0	2	0	0	0	0	0	0	0
Vacuoles	–	8	8	8	8	8	8	8	8	8
	+	0	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Aluminum stain (collagen fibers)	–	2	4	0	2	5	6	8	7	8
	+	2	4	7	4	3	2	0	0	0
	++	4	0	1	2	0	0	0	1	0
	+++	0	0	0	0	0	0	0	0	0
Aluminum stain (macrophages)	–	0	7	0	0	0	0	8	0	8
	+	5	1	8	8	8	8	0	8	0
	++	3	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0

inflammatory cell infiltration became more severe over time and were most severe 7 days after the injection. From about that time, an epithelioid cell granuloma, constituted mainly by macrophages, and fibrosis were observed around the necrotic area. The size of granuloma was maximal 14 days after the injection and thereafter, gradually shrank until 56 days. On the other hand, fibrosis developed with the granuloma and reached maximum at 28 days. The degree of the fibrosis tended to increase as the granuloma shrank. The granuloma with fibrosis remained small in size from 56 to 140 days (Fig. 1: C and D). PCNA-positive cells, denoting growth activity, increased mainly in mesenchymal cells in the granuloma (Fig. 2B) and lasted until 140 days. Aluminum was detected mainly around the necrotic area, collagen fibers, and in the macrophages. Over a long period, necrotic debris, and aluminum-positive macrophages were observed in the granuloma (Figs. 1C and 3C).

Table 3 shows histopathological findings after injection of PAO. By PAO treatment, necrosis, hemorrhage, inflammatory cell infiltration, and edema were observed in the subcutaneous tissues around the pouch 1 day after the injection (Fig. 1E). These changes diminished or disappeared 7 to 14 days after the injection. On the other hand, a residue of oil, probably the PAO base, was macroscopically confirmed in the pouch during the observation period and histopathological examination revealed a formation of vacuoles and cavities (Fig. 1: F and G). Components of the oil decomposed progressively and a large number of vacuoles remained even 140 days after the injection. Fibrosis was observed around the cavities or the vacuoles 14 days or later (Fig. 1: F – H). The degree of fibrosis was mild compared with that in the OC-108-treatment group and was stable until 140 days.

Table 4 shows histopathological findings after injection of saline. By saline treatment, necrosis, edema, and inflammatory cell infiltration were observed slightly in the subcutaneous tissues around the pouch 1 day after

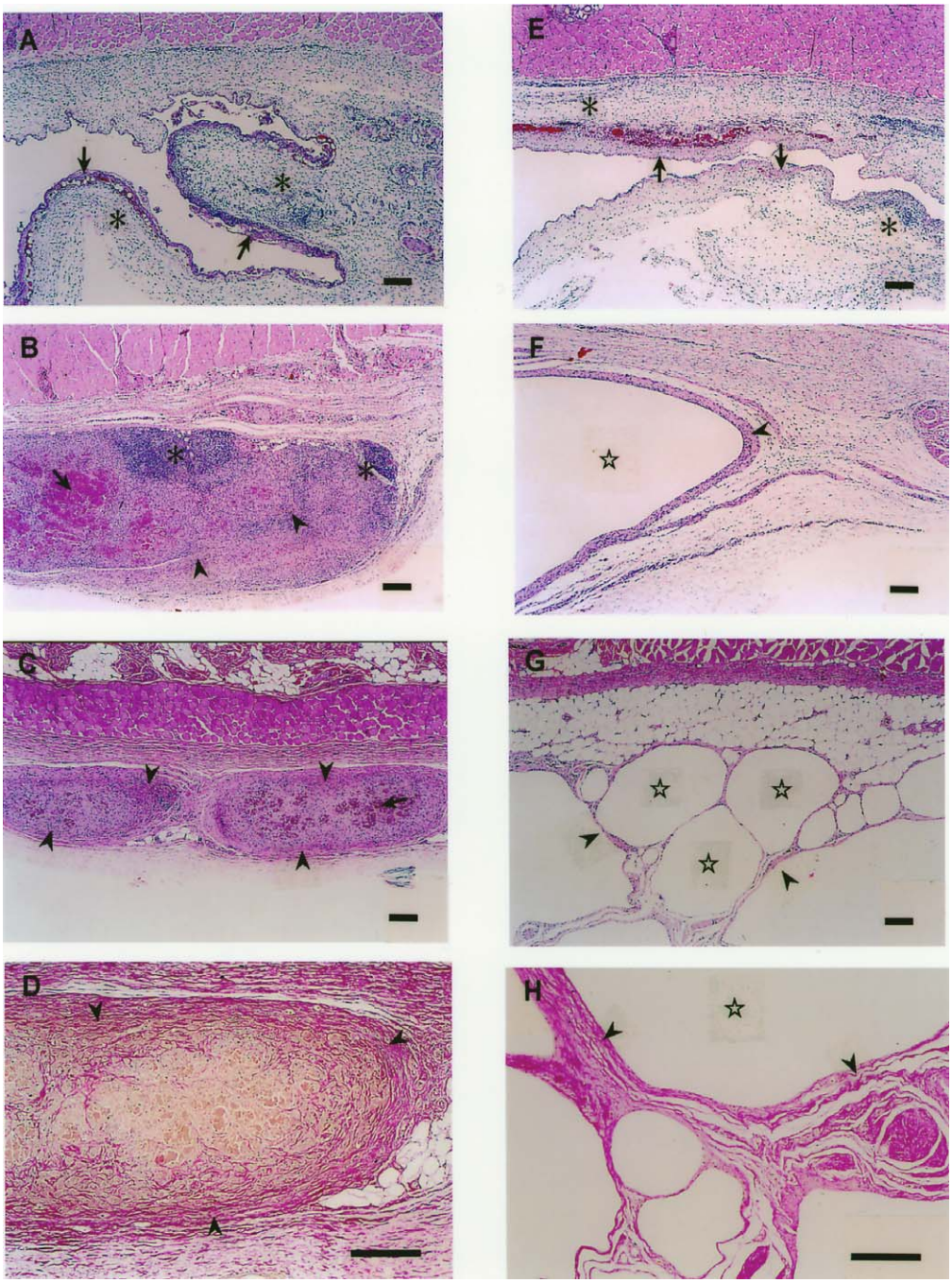

Fig. 1. Histological appearance of subcutaneous tissue after injection of OC-108 and PAO in rats. A: 1 day after injection of OC-108. Note necrosis (arrows), edema, hemorrhage, and inflammatory cell infiltration (asterisks) around the pouch. B: 14 days after injection of OC-108. Note marked epithelioid cell granuloma with necrotic substances (arrow), inflammatory cell infiltration (asterisks), and fibrosis (arrowheads). C, D: 140 days after injection of OC-108. Note shrunken granuloma with intense fibrosis (arrowheads) and slight necrosis (arrow). E: 1 day after injection of PAO. Note necrosis (arrows), edema, hemorrhage, and inflammatory cell infiltration (asterisks) around the pouch. F: 14 days after injection of PAO. Note marked cavity (star) surrounded by slight inflammatory cell infiltration and fibrosis (arrowhead). G, H: 140 days after injection of PAO. Note numerous vacuoles (stars) surrounded by fibrosis (arrowheads). Sections were stained with hematoxylin-eosin (A – C, E – G) and elastica-van Gieson (D, H). Bar equals 200 μm.

the injection. These changes diminished progressively and no noticeable findings were confirmed from 7 days after the injection.

Figure 4 summarizes the profiles of histological

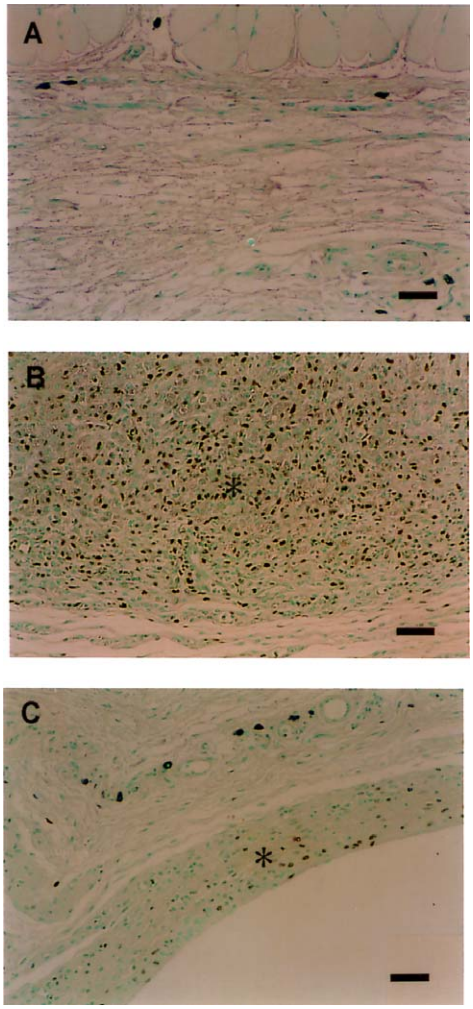

Fig. 2. Immunohistochemical staining for PCNA in subcutaneous tissue 14 days after injection of saline, OC-108, and PAO. A: Saline injection. B: OC-108 injection. Note numerous PCNA-positive mesenchymal cells (asterisk) in the granuloma. C: PAO injection. Note a few PCNA-positive cells (asterisk) around the cavity. Bar equals 50 μm.

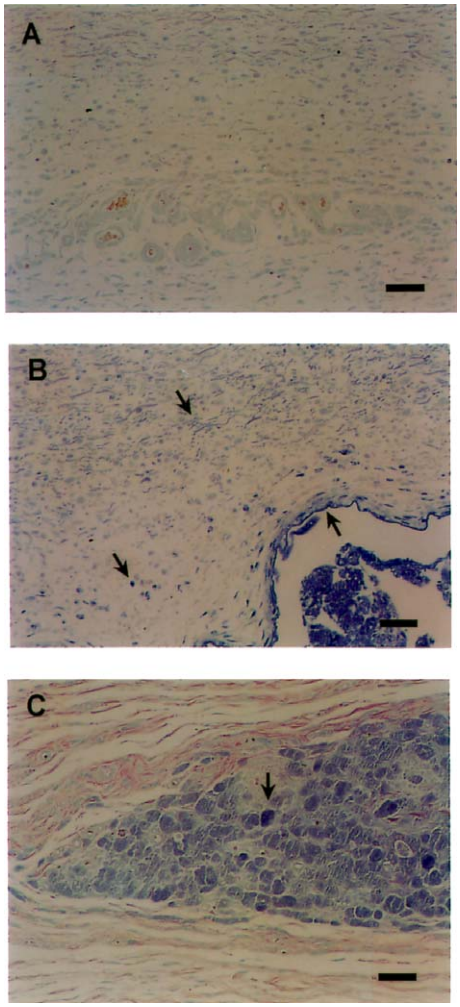

Fig. 3. Histochemical staining of aluminum in subcutaneous tissue after injection of saline and OC-108. A: 1 day after saline injection. B: 1 day after OC-108 injection. Note aluminum localization (arrows) around necrotic area, collagen fibers, and in the macrophages. C: 112 days after OC-108 injection. Note aluminum remaining in large macrophages (arrow) in the granuloma. Sections were stained with naphthochrome green. Bar equals 50 μm.

change after injection of the test solutions by scoring the findings.

Inflammation inducing action of active components in OC-108

OC-108 and 20 mg/ml alum (equivalent to OC-108) significantly increased vascular permeability, leukocyte infiltration, and granuloma formation compared with saline. The effects of alum in inducing vascular permeability and leukocyte infiltration were greater than those of OC-108, while no significant difference between the two groups was observed in inducing granuloma formation. Tannic acid at 0.75 mg/ml (equivalent to OC-108) showed no significant effect (Figs. 5A, 6A, and 7A). Alum also significantly increased vascular permeability and leukocyte infiltration at 10 mg/ml or more and produced a granuloma at 5 mg/ml or more (Figs. 5B, 6B, and 7B). These effects of alum were significantly dose-dependent.

Effects of tannic acid on inflammation inducing action of alum

To confirm the effect of tannic acid on the inflammation inducing action of alum, leukocyte infiltration and granuloma formation by alum only were compared with those by alum in combination with tannic acid (not OC-108) in the condition free from excipients because the excipients could affect the reaction.

Table 3. Histopathological appearance of subcutaneous tissue after injection of PAO in rats

Test solution		PAO								
Days after injection		1	3	7	14	28	56	84	112	140
Number of animals		8	8	8	6[a]	8	8	7[b]	8	8
Necrosis	–	7	6	8	6	8	8	7	8	8
	+	1	2	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Hemorrhage	–	2	7	7	6	8	8	7	8	8
	+	6	1	1	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Edema	–	0	0	5	6	8	8	7	8	8
	+	2	4	3	0	0	0	0	0	0
	++	6	4	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Inflammatory cell infiltration	–	0	0	0	3	7	5	3	7	6
	+	7	6	7	3	1	3	4	1	2
	++	1	2	1	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
PCNA stain	–	0	2	3	1	7	4	0	3	4
	+	7	6	4	5	1	4	7	5	4
	++	1	0	1	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Neovascularization	–	6	5	3	4	7	7	7	8	7
	+	2	3	5	2	1	1	0	0	1
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Epithelioid cell granuloma	–	8	8	8	6	8	8	7	8	8
	+	0	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Fibrosis	–	8	8	8	1	0	2	0	1	1
	+	0	0	0	5	7	5	5	6	4
	++	0	0	0	0	1	1	2	1	3
	+++	0	0	0	0	0	0	0	0	0
Cavitation	–	0	0	1	0	0	3	6	3	7
	+	2	1	1	0	0	0	0	0	0
	++	3	4	3	2	0	1	0	0	0
	+++	3	3	3	4	8	4	1	5	1
Vacuoles	–	6	0	4	2	6	2	1	3	1
	+	2	6	4	4	1	1	0	1	0
	++	0	0	0	0	1	0	0	0	2
	+++	0	2	0	0	0	5	6	4	5

[a]Two rats died after the injection. [b]One rat died after the injection.

Alum at 20 mg/ml (equivalent to OC-108) facilitated infiltration of leukocytes, and the effect peaked 3 days after the administration. Tannic acid at 0.75 mg/ml (equivalent to OC-108), in combination with alum, significantly inhibited the infiltration of leukocytes 3 days after the administration (Fig. 8A).

Alum at 20 mg/ml induced granuloma formation, and the effect was maximal 14 days after the injection and lasted 7 to 35 days. Tannic acid at 0.75 mg/ml, in combination with alum, significantly but marginally facilitated granuloma formation 14 days after the injection, but not at 7, 21, and 35 days (Fig. 8B). In the saline-treatment group, no granuloma was macroscopically confirmed at any time point.

Discussion

Since hemorrhoids are human-specific, to date there is no experimental animal model for the disease. Moreover, injection into rectal submucosa, identified as the development site of human hemorrhoids, is rather difficult technically in experimental animals. On the other hand, in a preliminary study, inflammation followed by fibrosis at the injection site was observed histopathologically when PAO, a current sclerosing agent for hemorrhoids, was injected into an air pouch created in rat subcutaneous tissue. Thus the conventional air pouch method made it possible to evaluate the efficacy of sclerosing agents for hemorrhoids. Therefore, in this study, OC-108 was initially injected s.c. into an air pouch in rats, and the sclerosing and retracting effects of the agent were estimated on the basis of histopathological findings.

With OC-108 treatment, inflammation with necrosis was observed in the early stage after the injection, and subsequently granuloma and fibrosis, comprising the restoration process, were also observed. The granuloma reached maximum size 14 days after the injection and thereafter shrank progressively over time. The fibrosis, accompanied by granuloma formation, reached maximum later than when the granuloma reached its maximum size. This suggests that the fibrosis is largely involved in shrinking the granuloma and that OC-108 has sclerosing and retracting activity. Hemorrhoids have been regarded as varicosities or sliding down of submucosal vascular cushions in the anorectal region, exhibiting the cardinal symptoms of bleeding and prolapse; veins are enlarged in advanced hemorrhoids (1).

Table 4. Histopathological appearance of subcutaneous tissue after injection of saline in rats

Test solution		Saline								
Days after injection		1	3	7	14	28	56	84	112	140
Number of animals		8	8	8	8	8	8	8	8	8
Necrosis	–	4	8	8	8	8	8	8	8	8
	+	4	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Hemorrhage	–	8	8	7	8	8	8	8	8	8
	+	0	0	1	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Edema	–	2	4	8	8	8	8	8	8	8
	+	5	3	0	0	0	0	0	0	0
	++	1	1	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Inflammatory cell infiltration	–	0	5	8	8	7	8	8	8	8
	+	8	3	0	0	1	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
PCNA stain	–	2	7	8	8	8	8	8	8	8
	+	4	1	0	0	0	0	0	0	0
	++	2	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Neovascularization	–	6	8	8	8	8	8	8	8	8
	+	2	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Days after injection		1	3	7	14	28	56	84	112	140
Number of animals		8	8	8	8	8	8	8	8	8
Epithelioid cell granuloma	–	8	8	8	8	8	8	8	8	8
	+	0	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Fibrosis	–	8	8	8	8	8	8	8	8	8
	+	0	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Cavitation	–	2	7	8	8	8	8	8	8	8
	+	1	1	0	0	0	0	0	0	0
	++	2	0	0	0	0	0	0	0	0
	+++	3	0	0	0	0	0	0	0	0
Vacuoles	–	8	8	8	8	8	8	8	8	8
	+	0	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Aluminum stain (collagen fibers)	–	8	8	8	8	8	8	8	8	8
	+	0	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0
Aluminum stain (macrophages)	–	8	8	8	8	8	8	8	8	8
	+	0	0	0	0	0	0	0	0	0
	++	0	0	0	0	0	0	0	0	0
	+++	0	0	0	0	0	0	0	0	0

On the basis of our experimental results and the pathological anatomy of the disease, the probable mechanisms for the effectiveness of OC-108 are deduced as follows. By the four-step injection of OC-108 in the interstitium of hemorrhoids (6), fibrosis associated with granulomatous inflammation will surround and constrict the dilated veins and reduce their size, and it will also increase the fixation of the displaced hemorrhoids to the underlying muscular coat. The consequence of these changes will reduce the symptoms of prolapse and bleeding.

Microscopically, necrotic debris was observed in the granuloma's center, and aluminum was detected around the necrosis and in the macrophages forming the granuloma. Considering that alum has been known for its astringent property to precipitate proteins (11), the necrosis observed in the early stage after injection of OC-108 is probably caused by the astringency of alum. The mobilization of macrophages and the formation of granuloma constituted by macrophages are considered to result from a host reaction against necrotic debris containing aluminum. Macrophages are thought to play a key role in a transition process of inflammation to restoration by providing a continuing source of cytokines and growth factors necessary to stimulate new extracellular matrix production and fibrosis (12, 13).

The present study demonstrated that both OC-108 and PAO could induce fibrosis at the injection site. However, in the PAO-treatment group, the main finding was fibrosis accompanied by a residue of oil (the base) at the injection site, and no granuloma formation was observed. These results show that the fibrosis development process by PAO treatment is different from that by OC-108. In addition, the degree of the fibrosis induced by OC-108 treatment was greater than that by PAO. This is considered attributable to the high effectiveness of OC-108 on the prolapse of severe internal hemorrhoids in the clinical studies (5, 6), while the effectiveness of PAO on prolapse is reportedly insufficient (1, 2). Furthermore, owing to histologically

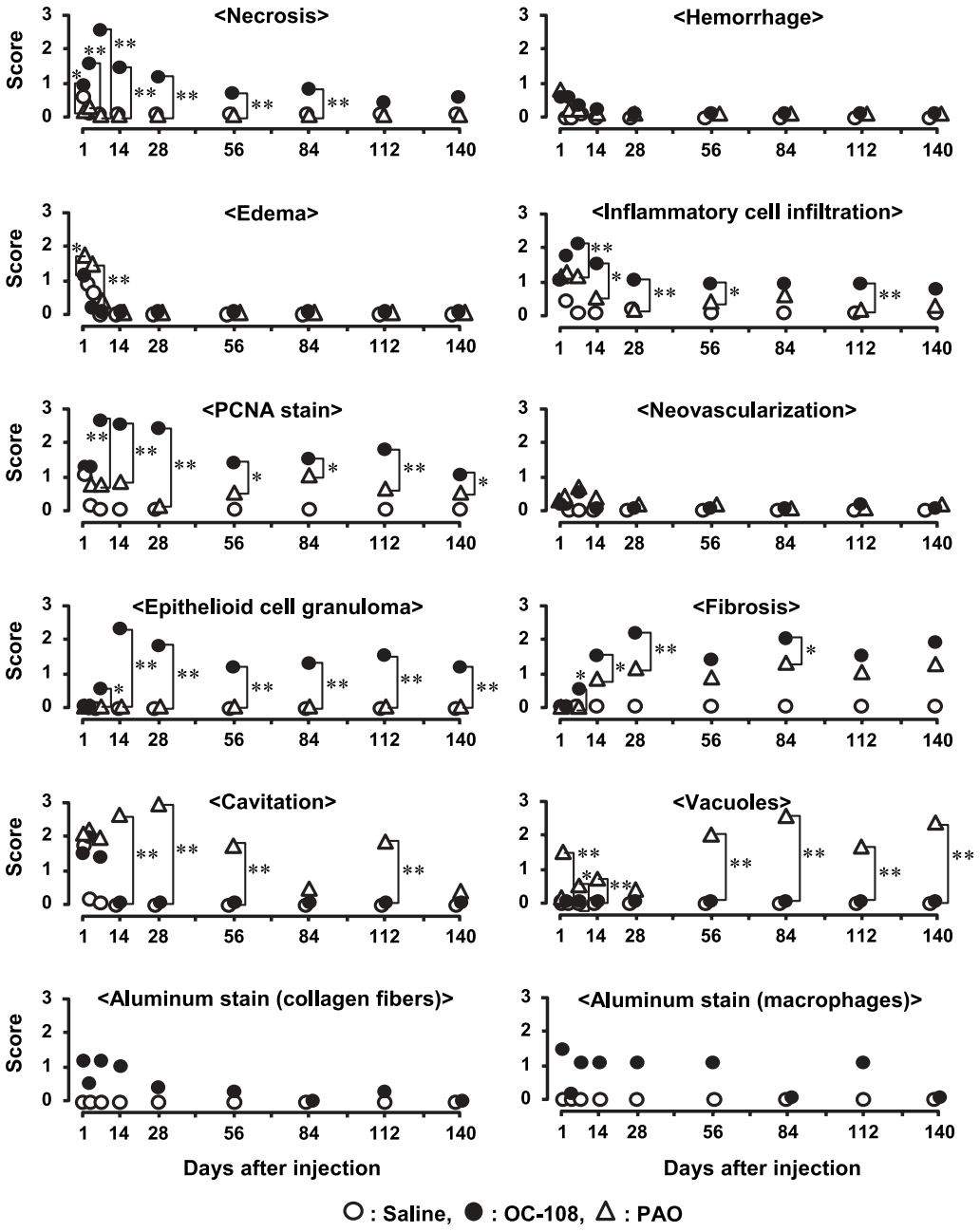

Fig. 4. Histological profiles after injection of saline, OC-108, and PAO in rats. Grades of histopathological findings, –, +, ++, and +++, were replaced by the scores of 0, 1, 2, and 3, respectively. Data are expressed as mean values. Each group consisted of 8 rats, except 6 in 14-day PAO group and 7 in 84-day PAO. **P*<0.05 and ***P*<0.01, compared between OC-108 and PAO groups (Wilcoxon rank-sum test).

characteristic changes by PAO treatment such as formation of cavities and vacuoles, the interstitium at the injection site exhibited a spongy structure. These findings imply that the interstitium of hemorrhoids treated with PAO is easily stretched and disrupted if repeatedly subjected to downward force during defecation, and this is supposedly related to the fact that PAO provides only short-term benefits in the majority of patients (2). On the other hand, the interstitium at the injection site of OC-108 exhibited a dense structure by forming a granuloma with fibrosis over a long time, which probably contributes to the long-lasting therapeutic effect of OC-108 (6).

Next, to clarify the main active components in OC-108, inflammation inducing effects of OC-108, alum, and tannic acid at the injection site were examined

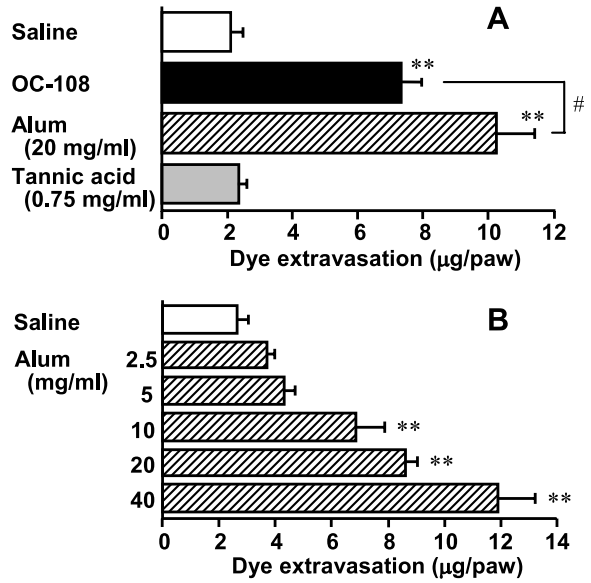

Fig. 5. Vascular permeability for 5 min immediately after s.c. injection of the test solutions in mice. A: Effects of saline, OC-108, alum, and tannic acid. B: Dose-response of alum. Data are expressed as means ± S.E.M. (n = 8). ***P*<0.01, compared with saline group (Dunnett's multiple comparison test); #*P*<0.05, compared between OC-108 and alum groups (Student's *t*-test).

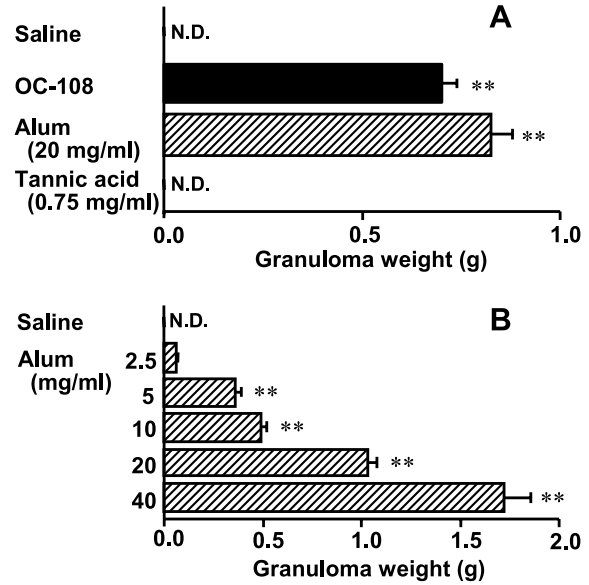

Fig. 7. Granuloma formation 14 days after s.c. injection of the test solutions in rats. A: Effects of saline, OC-108, alum, and tannic acid. B: Dose-response of alum. Data are expressed as means ± S.E.M. (n = 8). N.D., no granuloma macroscopically detected. ***P*<0.01, compared with saline group (Dunnett's multiple comparison test). No significant difference was observed between OC-108 and alum groups (Student's *t*-test).

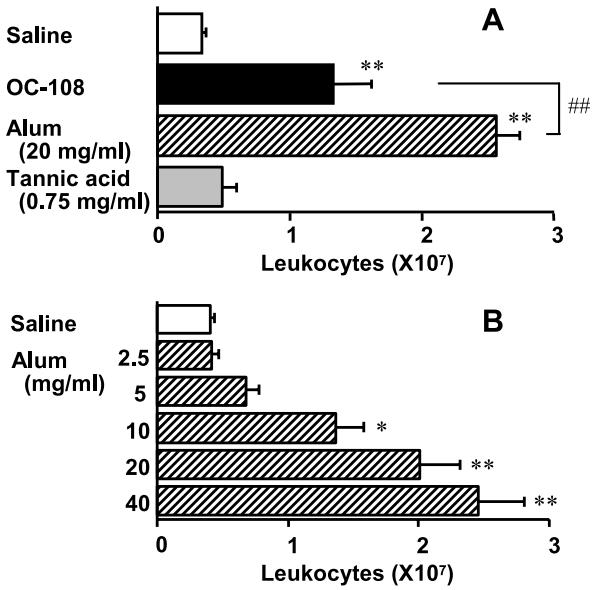

Fig. 6. Leukocyte infiltration 3 days after s.c. implantation of sponges containing the test solutions in rats. A: Effects of saline, OC-108, alum, and tannic acid. B: Dose-response of alum. Data are expressed as means ± S.E.M. (n = 8). **P*<0.05 and ***P*<0.01, compared with saline group (Dunnett's multiple comparison test); ##*P*<0.01, compared between OC-108 and alum groups (Student's *t*-test).

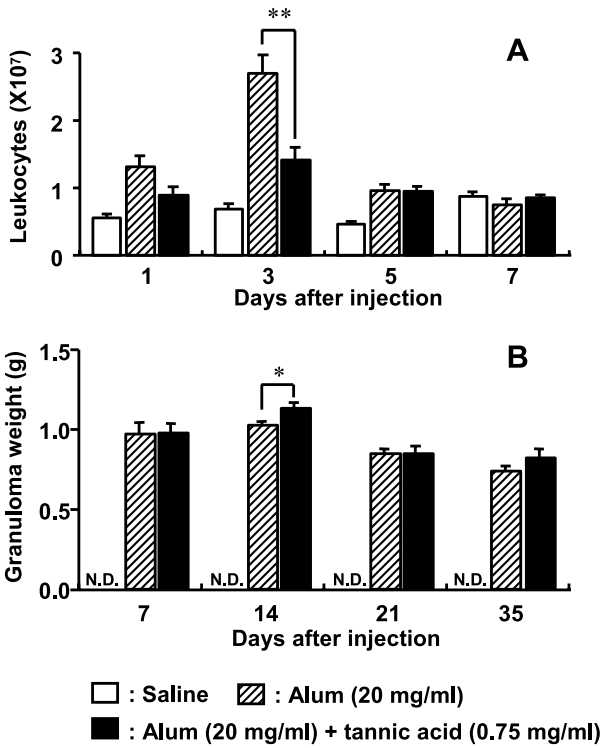

Fig. 8. Effects of tannic acid on inflammation inducing action of alum. A: Leukocyte infiltration after s.c. implantation of sponges containing the test solutions in rats. B: Granuloma formation after s.c. injection of the test solutions in rats. Data are expressed as means ± S.E.M. (n = 8). N.D., no granuloma macroscopically detected. **P*<0.05 and ***P*<0.01, compared between alum group and tannic acid combined group (Student's *t*-test).

using the methods that are useful for quantitative evaluation of inflammatory reactions. Alum, as well as OC-108, showed inflammation-inducing actions (increase in vascular permeability, infiltration of leukocyte, and formation of granuloma) but tannic acid did not. The results demonstrate that alum is the main active ingredient of OC-108.

In addition, the influence of tannic acid on inflammatory reactions induced by alum was examined to elucidate the significance of tannic acid in formulating OC-108. Tannic acid inhibited the leukocyte infiltration caused by alum at the peak reaction time but did not inhibit the granuloma formation. Leukocytes infiltrating into tissues are thought to contribute to the tissue injury by releasing reactive oxygen species, various proteases, and arachidonate metabolites, and so on (14). Taking this characteristic of leukocytes into account, the results obtained in the present study suggest that tannic acid, as an adjunct ingredient, reduces excessive acute inflammatory reactions that are possibly related to tissue injury, without inhibiting the tissue sclerosing effect of alum. The precise mechanism(s) responsible for the effect remains to be determined; however, it may be related to the binding of aluminum with tannic acid (15).

In conclusion, it is indicated that OC-108 causes sclerosis and retraction of hemorrhoids through fibrosis associated with granulomatous chronic inflammation induced by the main active ingredient alum and that the adjunct ingredient tannic acid reduces excessive acute inflammation induced by alum.

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