# Inhibitory effects on fibroblast proliferation of Eppi-ka-jutsu-to, Ephedrae Herba and constituents of Ephedra

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**Abstract**

Concerning Eppi-ka-jutsu-to (EJ) known as possessing an anti-inflammatory effect, the inhibitory effects of its component drugs on the proliferation of fibroblasts (Balb/c-3T3, clone A31) were investigated. EJ, Ephedrae Herba, and the preparations combined with Ephedrae Herba suppressed fibroblast proliferation remarkably. Examination of *l*·ephedrine, *d*·pseudoephedrine, *l*·N·methylephedrine, *d*·N·methylpseudoephedrine and *l*·norephedrine, which are alkaloid constituents of Ephedrae Herba, proved that *d*·pseudoephedrine (250, 25 μg/ml) and *l*·norephedrine (250, 25 μg/ml) showed a significantly suppressive effect on the proliferation. Therefore, the anti-inflammatory effect of EJ at the chronic stage of inflammation was thought to be exerted mainly by Ephedrae Herba, and in addition, *d*·pseudoephedrine, *l*·norephedrine was considered to be an active component.

**Key words** anti-inflammatory effect, Ephedrae Herba, *l*·ephedrine, Eppi-ka-jutsu-to, fibroblast proliferation, *l*·norephedrine, *d*·pseudoephedrine.

**Abbreviations** EJ, Eppi-ka-jutsu-to (Yue-Bi-Jia-Shu-Tang), 越婢加朮湯 ; DMEM, Dulbecco's modified Eagles medium ; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid ; MES, 2-(N-morpholino) ethanesulfonic acid, monohydrate.

## Introduction

We have reported previously that Kampo medicines, which are used clinically for chronic inflammatory diseases such as rheumatoid arthritis, suppressed both the amount of granulation tissue and total collagen hydroxyproline contents in carrageenin airpouch inflammation in rats.[1] One of the mechanisms of suppression at the chronic stage of inflammation was thought to be the inhibitory effect on the proliferation of fibroblasts.[1] The present study was undertaken in an attempt to examine the antiinflammatory activity of EJ and its component drugs, by focusing on the inhibitory effects on the proliferation of fibroblasts.

## Materials and Methods

*Preparation of Kampo extracts* : EJ was chosen as the experimental prescription from the previous report.[1] The constitution of EJ is listed in Table I. Medicines contained in EJ such as Ephedra (Mao), Gypsum (Sekko), and the preparations with one medicine removed such as EJ excluded Ephedra (EJ-Mao), EJ excluded Gypsum (EJ-Sekko) were also used as test drugs. All the medicines were supplied by Tochimoto Tenkaido Co., Ltd. (Osaka). Each decoction was made from Kampo medicines after gently boiling for 60 min with 400 ml water to exactly 300 ml. The decoction was divided into adequate amounts (20-30 ml), kept in a freezer at -40℃, and used for the

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Table I Constitution of Eppi-ka-jutsu-to (EJ).

| | | |
|---|---|---|
| Mao (麻黄), Ephedrae Herba, *Ephedra intermedia* SCHRENK et MAYER* | 6.0g | from China |
| Sekko (石膏), Gypsum Fibrosum, *gypsum* $CaSO_4$ $2H_2O$ | 8.0 | China |
| Shokyo (生姜), Zingiberis Rhizoma, *Zingiber officinale* ROSCOE. | 1.0 | China |
| Taiso (大棗), Zizyphi Fructus, *Zizyphus jujuba* MILL. var. *inermis* REHD. | 3.0 | China |
| Kanzo (甘草), Glycyrrhizae Radix, *Glycyrrhiza glabra* L. var. *glandulifera* REG. et HERD. | 2.0 | China |
| Byakujutsu (白朮), Atractylodis Rhizoma, *Atractylodes macrocephala* KOIDZUMI | 4.0 | China |

Each crude drug was mixed with 400 ml of water and boiled gently to 300 ml.
*Contents of Ephedra alkaloids determined by HPLC were 0.42% of ephedrine, 0.70% of pseudoephedrine and 0.045% of methylephedrine. Norephedrine was undetectable in this dried Ephedra species.

investigation after dissolving.

*Materials* : Balb/c-3T3, clone A31 mouse embryo fibroblasts were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka). Cell wells 25820MP (24-well plates) were from Corning Glass Works (N.Y., U.S.A.). and Millex-HA filter units were from Japan Millipore Ltd. (Tokyo). Fetal bovine serum (FBS) was from Whittaker Bioproducts Inc. (Md., U.S.A). Dulbecco's modified Eagles medium (DMEM) was purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo). N-2-hydroxyethylpiperazine - N'- 2 - ethanesulfonic acid (HEPES) and 2 -(N - morpholino) ethanesulfonic acid, monohydrate (MES), were from Dojin Chemical Laboratory Co., Ltd. (Kumamoto). *l* · ephedrine, *d* · pseudoephedrine hydrochloride, *d* · N · methylpseudo-ephedrine, *l* · N · methylephedrine, and *l* · norephedrine were from Aldrich Chemical Co. (Wis., U.S.A.). Dexamethasone was from Nacalai Tesque, Inc. (Kyoto) and indomethacin from Sigma Chemical Co. (Mo., U.S.A). All other reagents, including sodium bicarbonate, penicillin G potassium, streptomycin sulfate, crystal violet, glutaraldehyde solution and acetic acid were purchased from Wako Pure Chemical Inc., Ltd. (Osaka).

*Cell cultures* : Balb/c-3T3, cloned A31 cells were chosen as fibroblasts. They were cultured at 37℃ in 95% air and 5% $CO_2$ humidified atmosphere in a $CO_2$ incubator, Forma MIP3193 (Sanyo Electric Tokki Co., Ltd., Tokyo). Subculture was prepared using 10% (v/v) FBS containing medium as the basic medium. Constituents of 1000 ml of the medium were 10.00 g DMEM, 3.698 g sodium bicarbonate, 5.96 g HEPES, 0.1 g penicillin G potassium, and 0.1 g streptomycin sulfate.

For the cell growth assay, the cells were dispersed from 70% confluent cultures into 24-well plates at a density of $5\times10^3$ cells (0.5 ml) per well. On the next day (Day 0), the medium was exchanged with 1 ml of medium containing 5% FBS and 0.25%, 1% or 4% decoction (water-soluble part : sterilized by filtration with Millex-HA 0.45 $\mu$m filter). The experimental cultures were continued for 3 days, and cell number measurements were carried out every day by crystal violet staining.[2,3]

*Determination of cell numbers by crystal violet staining* : Cells were fixed by the addition of 100 $\mu$l of 11% glutaraldehyde solution to 1 ml of medium. After being shaken for 15 min, the plates were washed three times with deionized water. The plates were then air-dried and stained by the addition of 1 ml of 0.1% solution of crystal violet dissolved in 200 mM MES (pH6.0). After leaving for 30 min at room temperature, excess dye was removed by extensive washing with deionized water and the plates were air-dried prior to bound dye solubilization in 1 ml of 10% acetic acid. The optical density (O.D.) of dye extracts was measured using a UV-visible recording spectrophotometer UV-265FS (Shimadzu Corporation, Kyoto). The wavelength selected was 590 nm. Cell numbers were estimated from the standard line between O.D. and cell numbers :

$$X=16807.2Y-1969.46$$

X : cell number per well

Y : O.D. at 590nm

$r=0.998$, $0<p<0.002$

*Statistical analysis* : Statistical transactions were undertaken with the Medical plan II ver.

3.0 (Dataform Co., Kyoto). The results, expressed as mean±S.E.M., were subjected to statistical analysis according to the unpaired *t*-test or Wilcoxon test.

## Results

The inhibitory effect of EJ on fibroblast proliferation is shown in Fig. 1. EJ both at 4% and 1% from day 1, and at 0.25% from day 2 suppressed the cell number of the proliferation significantly.

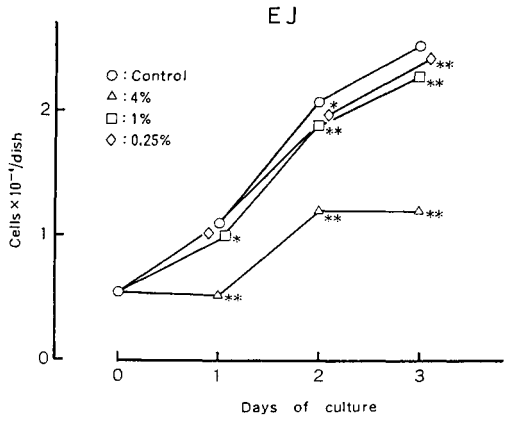


Fig. 1 Inhibitory effect of EJ on the proliferation of fibroblasts (Balb/c-3T3, clone A31) in culture.
Each point represents the number of cells per dish, and is the mean value±S.E.M. (vertical bar) of 6 determinations. Vertical bars within the size of the symbol used are omitted. Values are statistically significant *versus* corresponding controls ; $*p<0.05$ ; and $**p<0.01$.

In order to confirm that this suppressive effect was not due to cell damage or drug toxicity because of the test drug being added directly to the medium, the following experiments were performed. During this EJ treatment procedure, the number of nonadherent cells floating in the medium on day 1 or day 2 was determined. There was no significant difference from the control. On day 1, control : $7.8\pm1.3\times10^2$ cells/ml, 4%EJ : $7.8\pm0.7\times10^2$ cells/ml, 1%EJ : $8.3\pm1.7\times10^2$ cells/ml, 0.25%EJ : $7.8\pm1.6\times10^2$ cells/ml, and on day 2, control : $9.4\pm1.6\times10^2$ cells/ml, 4% EJ : $9.4\pm3.1\times10^2$ cells/ml, 1%EJ : $9.4\pm3.9\times10^2$ cells/ml, 0.25%EJ : $9.4\pm3.1\times10^2$ cells/ml. In addition, exchanging the medium on day 1 or day 2 revealed that the cell number recovered to the level of control after two days (Table II). Moreover, no change occurred in pH of the medium when the drug or decoction was added to the medium (data not shown).

The effects of both constituents of EJ (eg. "Mao" : Ephedra) and one constituent-removed EJ (eg. "EJ-Mao" : EJ without Ephedra) on fibroblast proliferation were then investigated (Fig. 2). "Mao" inhibited fibroblast proliferation from day 1 by 1%, and from day 2 by 0.25%. However, "EJ-Mao" did not exert any influence upon fibroblast proliferation on any day. The effects

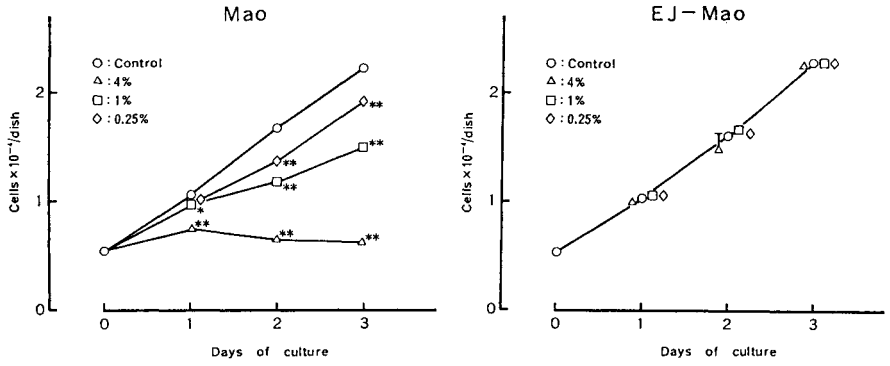


Fig. 2 Effects of "Mao" (Ephedra) and "EJ-Mao" (EJ without Ephedra) on fibroblast proliferation.
Each point represents the number of cells per dish, and is the mean value±S.E.M. (vertical bar) of 6 determinations. Vertical bars within the size of the symbol used are omitted. Values are statistically significant *versus* corresponding controls ; $*p<0.05$ ; and $**p<0.01$.

Table II Cell number ($\times 10^{-3}$) cultured with EJ containing medium.

| | Control | 4% EJ | 1% EJ | 0.25% EJ |
|---|---|---|---|---|
| Day 0 | 5.5±0.1 | —— | —— | —— |
| Day 1 | 11.0±0.2 | 5.2±0.2 | 10.0±0.2 | 10.2±0.4 |
| Day 2 | 20.8±0.2 | 12.1±0.2 | 19.0±0.2 | 19.6±0.1 |
| Day 3 | 25.3±0.1 | 12.1±0.4 | 22.8±0.2 | 24.1±0.1 |
| Day 1 + 2* | 26.2±0.1 | 21.1±0.5 | 25.2±0.2 | 25.5±0.2 |
| Day 2 + 2* | 26.2±0.1 | 23.0±0.4 | 25.6±0.5 | 26.0±0.2 |

*Medium was exchanged with EJ-free, 10%FBS-containing medium on Day 1 or Day 2, and 2 days after the procedure, cell numbers were determined as described in Materials and Methods.

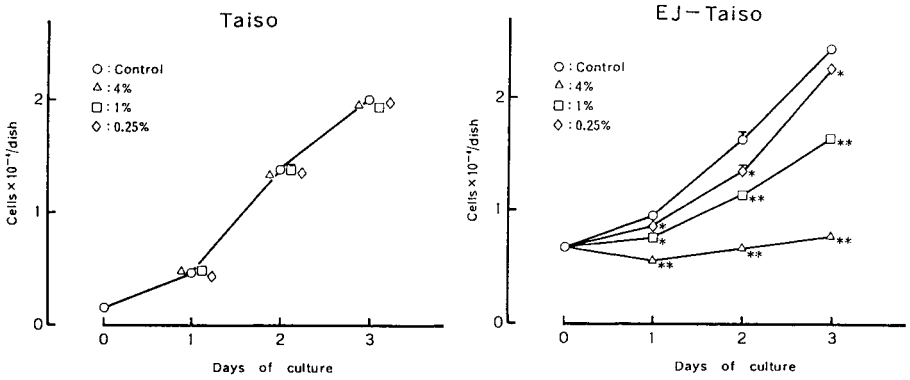


Fig. 3 Effects of "Taiso" (Zizyphus) and "EJ-Taiso" (EJ without Zizyphus) on fibroblast proliferation.

Each point represents the number of cells per dish, and is the mean value±S.E.M. (vertical bar) of 6 determinations. Vertical bars within the size of the symbol used are omitted. Values are statistically significant *versus* corresponding controls ; $*p<0.05$ ; and $**p<0.01$.

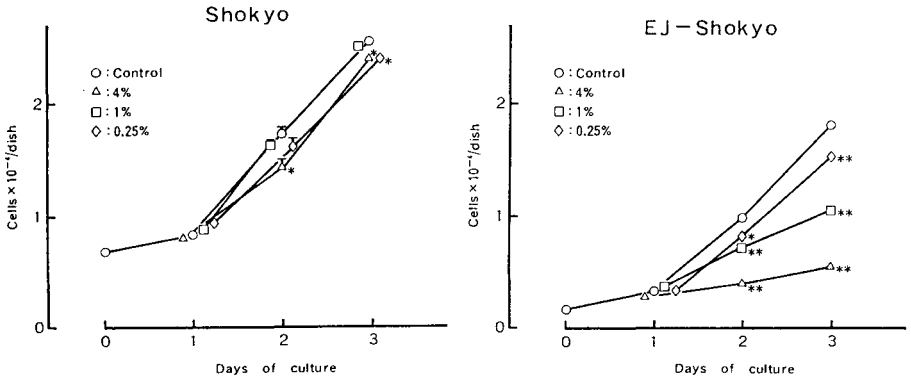


Fig. 4 Effects of "Shokyo" (Zingiber) and "EJ-Shokyo" (EJ without Zingiber) on fibroblast proliferation.

Each point represents the number of cells per dish, and is the mean value±S.E.M. (vertical bar) of 6 determinations. Vertical bars within the size of the symbol used are omitted. Values are statistically significant *versus* corresponding controls ; $*p<0.05$ ; and $**p<0.01$.

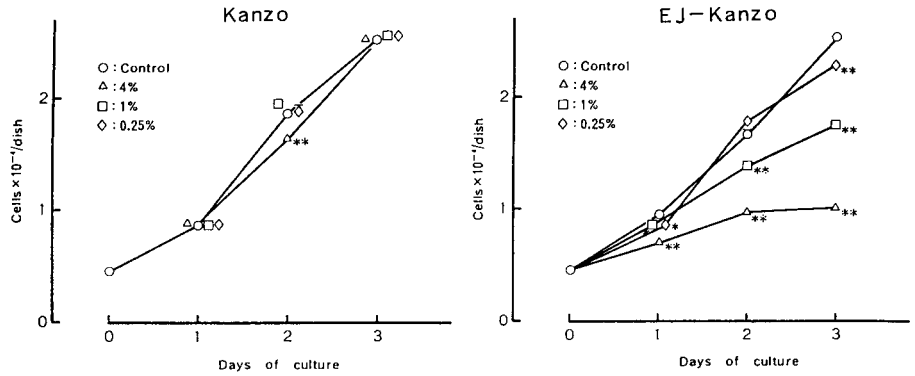


Fig. 5 Effects of "Kanzo" (Glycyrrhiza) and "EJ-Kanzo" (EJ without Glycyrrhiza) on fibroblast proliferation.
Each point represents the number of cells per dish, and is the mean value±S.E.M. (vertical bar) of 6 determinations. Vertical bars within the size of the symbol used are omitted. Values are statistically significant *versus* corresponding controls ; $*p<0.05$ ; and $**p<0.01$.

of "Taiso" and "EJ-Taiso" are shown in Fig. 3. Although "Taiso" did not suppress proliferation, "EJ-Taiso" above 0.25% suppressed proliferation significantly from day 1. Concerning the effects of "Sekko", "Byakujutsu" and "EJ-Sekko", "EJ-Byakujutsu" had results similar to those of "Taiso" and "EJ-Taiso" (data not shown). Generally, among the drugs comprising EJ and EJ minus one of these, Mao-containing exhibited strong suppression and Mao-excluded showed weak or non-suppression. "Shokyo" and "Kanzo" were recognized as having weak suppression (Fig. 4,5).

The examination of alkaloid extracts of Ephedra (Mao)———*l*・ephedrine, *d*・pseudoephedrine, *l*・N・methylephedrine, *d*・N・methylpseudoephe-

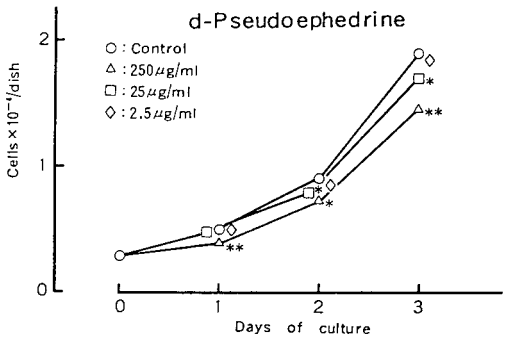


Fig. 6 Inhibitory effect of *d*-pseudoephedrine on fibroblast proliferation.
Each point represents the number of cells per dish, and is the mean value±S.E.M. (vertical bar) of 6 determinations. Vertical bars within the size of the symbol used are omitted. Values are statistically significant *versus* corresponding controls ; $*p<0.05$ ; and $**p<0.01$.

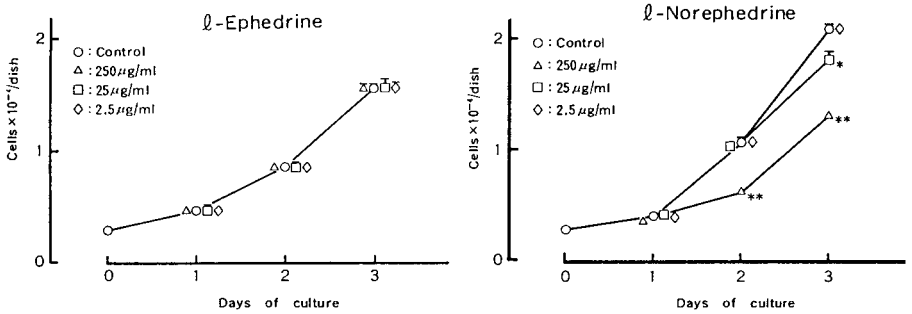


Fig. 7 Effects of *l*-ephedrine and *l*-norephedrine on fibroblast proliferation.
Each point represents the number of cells per dish, and is the mean value±S.E.M. (vertical bar) of 6 determinations. Vertical bars within the size of the symbol used are omitted. Values are statistically significant *versus* corresponding controls ; $*p<0.05$ ; and $**p<0.01$.

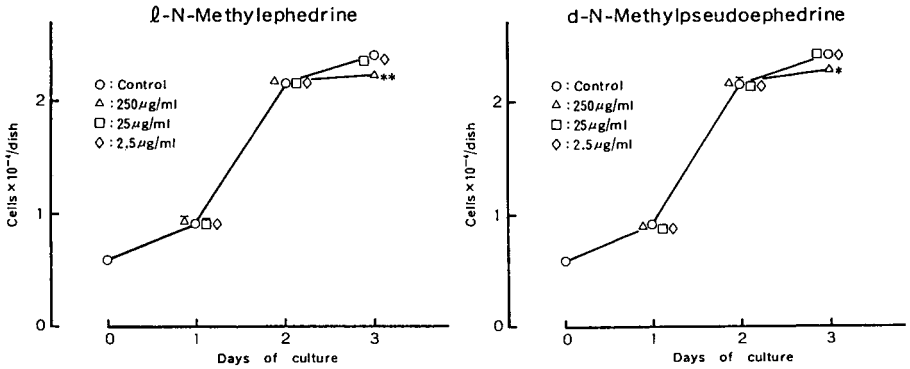


Fig. 8 Effects of *l*-N-methylephedrine and *d*-N-methylpseudoephedrine on fibroblast proliferation.

Each point represents the number of cells per dish, and is the mean value±S.E.M. (vertical bar) of 6 determinations. Vertical bars within the size of the symbol used are omitted. Values are statistically significant *versus* corresponding controls ; $*p<0.05$ ; and $**p<0.01$.

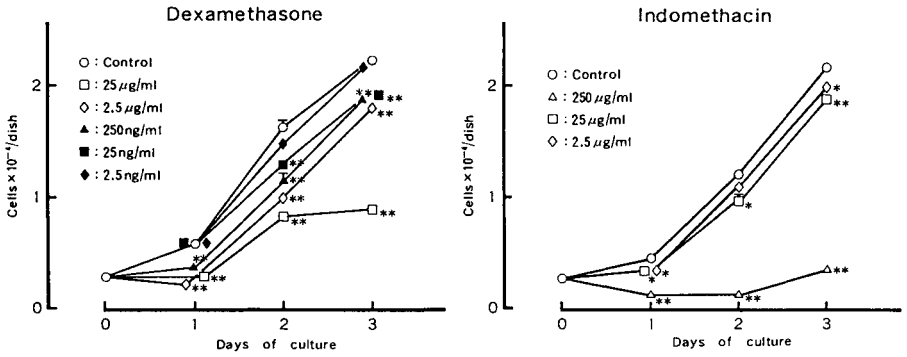


Fig. 9 Effects of dexamethasone and indomethacin on fibroblast proliferation.

Each point represents the number of cells per dish, and is the mean value±S.E.M. (vertical bar) of 6 determinations. Vertical bars within the size of the symbol used are omitted. Values are statistically significant *versus* corresponding controls ; $*p<0.05$ ; and $**p<0.01$.

drine and *l*·norephedrine (each 250, 25, 2.5μg/ml) ——proved that *d*·pseudoephedrine (250, 25 μg/ml) and *l*·norephedrine (250, 25 μg/ml) suppressed proliferation significantly, though *l*·ephedrine did not (Fig. 6,7). *l*·N·methylephedrine and *d*·N·methylpseudoephedrine showed a suppressive effect at high concentration (250 μg/ml) only at Day 3 (Fig. 8).

The effects of dexamethasone (25, 2.5 μg/ml, 250, 25, 2.5 ng/ml) and indomethacin (250, 25, 2.5 μg/ml) are shown in Fig. 9. Dexamethasone from 25 ng/ml, and indomethacin from 2.5 μg/ml inhibited proliferation.

## Discussion

EJ is a prescription originating from Chapter five of Kinki-Yoryaku (Chin-Kuei-Yao-Lueh) (Prescriptions from the Golden Chamber), Chufu-Rekisetsu-Byo (Chung Feng Li Chieh V) (Apoplexy and Arthritis), and currently it is used in the treatment of nephritis, nephrosis, beriberi, rheumatoid arthritis, enuresis and eczema.[4] This prescription is often used in inflammatory diseases, and we have proved that it suppresses the third stage of inflammation in carrageenin air-pouch inflammation in rats.[1] The present study was undertaken in an attempt to focus on and

elucidate the anti-inflammatory activity of EJ among its many properties, and to investigate which components of EJ contributed to the anti-inflammatory activity at the chronic stage of inflammation.

Anti-inflammatory activities of EJ and its component drugs were investigated in terms of their inhibitory effect on fibroblast proliferation. Ephedra and the preparations combined with Ephedra suppressed fibroblast proliferation remarkably. Examination of the constituent alkaloids of Ephedra proved that *d*・pseudoephedrine and *l*・norephedrine possess the activity of anti-inflammation, whereas *l*・ephedrine does not.

Several reports[5-11] have stated that Ephedra and its alkaloids suppressed acute inflammation at the first and second stage. Extracts of Ephedra and its whole alkaloid showed anti-inflammatory action by the Whittle method (AcOH-induced peritonitis), dextran induced paw edema and carrageenin induced paw edema.[5-9] It has been reported that this effect is mainly due to *d*・pseudoephedrine.[5,7,10] In addition, regarding its mechanism, it has been suggested that inhibition of prostaglandin biosynthesis is partly related,[10-11] and that it is also partly concerned with the sympathetic nervous system.[9-10]

In this study, Ephedrae Herba and some of its alkaloids were confirmed to possess anti-inflammatory activity, by using experiments to demonstrate their inhibitory function on fibroblast proliferation[12-14] for the first time. Our results are in agreement with the report by Hikino *et al*.[7] While the latter dealt with only *d*・pseudoephedrine and *l*・ephedrine as Ephedra alkaloids, our results included the minor anti-inflammatory activities of *l*・N・methylephedrine, *d*・N・methylpseudoephedrine and *l*・norephedrine.

It is of interest that the ratio of *l*・ephedrine to *d*・pseudoephedrine is fluctuating in the order of more than 10 times according to Ephedra species.[8,15] It is known that Ephedra intermedia, which was used in the present study, contains rather much *d*・pseudoephedrine (0.6-0.7%) and a comparatively small amount of *l*・ephedrine (0.1-0.2%).[16,17] Hikino *et al*.[16] concluded that *d*・pseudoephedrine is the anti-inflammatory component of Ephedra, because the relation between the amount of *d*・pseudoephedrine in Ephedra and anti-inflammatory activity (Whittle method) is parallel. *l*・Norephedrine, which exerted a remarkable inhibitory effect in our study, is usually undetectable or its content is very little (0.0005%) in the Ephedra intermedia.[17-19] Actually, by the determination of Ephedra alkaloids with HPLC, it was proved that *l*・norephedrine could not be detected in Ephedra used in this study (Table I). In addition, both *l*・N・methylephedrine and *d*・N・methylpseudoephedrine showed very little anti-inflammatory activity. Therefore, the anti-inflammatory principle of Ephedra is thought to be dependent upon *d*・pseudoephedrine even at the chronic stage of inflammation.

As for some possible difficulties concerning the present experiments, it might be supposed that the inhibitory effect is due to cell damage or drug toxicity because the test drug was added directly to the medium. However, we can practically rule out this possibility because of the following : A. After exchanging the medium with usual medium at the stage of the drug-induced proliferation suppression, the cell number soon recovered (Table II). B. No difference was found in the number of wafting or dead cells in the medium. C. The pH of the medium did not change with the addition of the drug. D. Proliferative curves were not downward but rather upward even in the case of suppression (eg. Fig. 1).

It might also be supposed that $Ca^{++}$ in the medium with EJ exerts any influence upon fibroblast proliferation because EJ contains Gypsum ($CaSO_4 \cdot 2H_2O$). However, "Sekko" did not affect fibroblast proliferation on any day, and "EJ-Sekko" had the same result as that of "EJ". It is reported that solubility of Gypsum in water is very low.[20]

It may also seem reasonable to consider the fact that what occurs inside the living body will not be accurately reflected because the drugs were added directly to the medium. We would like to emphasize that serum Ephedra alkaloid is sufficiently detectable in humans after Kampo medicines, which include Ephedra, are administered. Concretely, Yamamoto[21] showed human

blood concentrations of ephedrine and pseudoephedrine to be 15 ng/ml and 5 ng/ml, respectively, 2 hours after taking 2 grams of Kampo extract containing 3.5 mg and 1.2 mg each of the alkaloid. As concerns alkaloid concentration in the medium in our study, for instance, *d*-pseudoephedrine is calculated to be 1.4 μg/ml and 5.6 μg/ml in the medium with 1% "Mao" and 4% "Mao" respectively, which both showed inhibitory effects on fibroblast proliferation. In fact, in the experiment using pure alkaloid, at least 25 μg/ml of *d*-pseudoephedrine were needed to be an effective concentration. It seems that this concentration level is similar to experimental animal blood levels.

With regard to the mechanism of the anti-inflammatory effects of Ephedra alkaloid, some investigators have reported that it is concerned with an inhibitory effect on the production of arachidonic acid due to suppressed arachidonate release at the acute stage of inflammation.[11] Moreover, Ephedra was reported to inhibit the conversion from arachidonic acid to prostaglandin $E_2$.[22] In our study, indomethacin which is used as a positive control, suppressed fibroblast proliferation at more than $10^{5}$ M, so it is likely that arachidonic acid metabolism is also involved in the chronic stage of inflammation. However, even at the acute stage of inflammation, Ephedra alkaloids suppress paw-edema reaction caused by histamine, serotonin and prostaglandin $E_1$,[10] and this seems not to be due to a suppressive effect on arachidonate metabolism only as has been reported.[11]

## Acknowlegements

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## 和文抄録

抗炎症作用のある越婢加朮湯（EJ）について，その構成生薬による線維芽細胞（Balb/c-3T3, clone A31）の増殖抑制作用を検討した。EJ，麻黄，およびEJ去一味のうち麻黄が含まれる場合に強い増殖抑制作用を認めた。次に麻黄のアルカロイド成分である*l*-ephedrine, *d*-pseudoephedrine, *l*-N-methylephedrine, *d*-N-methylpseudoephedrine, *l*-norephedrineで検討したところ，*d*-pseudoephedrine（250, 25 μg/ml）および*l*-norephedrine（250, 25 μg/ml）で有意な増殖抑制がみられた。以上の結果から，EJの慢性期抗炎症効果は，主として麻黄に由来し，その抗炎症活性物質として*d*-pseudoephedrineの他*l*-norephedrineも想定された。

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