[Chem. Pharm. Bull. 36(2) 757—762 (1988)]

Anti-inflammatory Activity of Ointments of Indomethacin and Its Calcium Salt Applied to Abdominal Skin of Rat

TARO OGISO,* YOSHIMASA ITO, MASAHIRO IWAKI and HIDEHIKO ATAGO

Faculty of Pharmaceutical Sciences, Kinki University, 4–1 Kowakae 3-chome, Higashi-osaka 577, Japan

(Received July 27, 1987)

Anti-inflammatory activities of ointments containing indomethacin (IND) and its calcium salt (IND-Ca), applied to abdominal skin, were investigated in rats with carrageenin-induced paw edema and adjuvant arthritis, in addition to estimation of the irritant effect of the formulations on rabbit skin. The IND and IND-Ca gel ointments (1%, 0.14 g, 1 cm^2) applied to skin produced potent inhibitory effects on the paw edema and adjuvant arthritis, nearly equal to that after oral dosing (2.5 and 5.0 mg/kg) of IND. The 0.5% drug ointments also exerted a marked inhibitory effect on carrageenin-induced paw edema. These ointments produced only slight irritation on rabbit skin. The irritancy of IND-Ca dissolved in diethylene glycol monoethyl ether was almost negligible, while IND solution prevented hair growth at the application sites for several days. The results suggest that IND-Ca ointment is suitable for clinical application.

Keywords——anti-edema effect; indomethacin ointment; indomethacin-calcium ointment; carrageenin-induced edema; adjuvant arthritis; ointment irritation; systemic anti-inflammatory effect; ointment

Indomethacin (IND), a potent nonsteroidal anti-inflammatory drug, is generally administered orally or rectally in the treatment of various inflammatory diseases.[1-5] Technical advances in drug-delivery systems have allowed increased utilization of transdermal formulations for therapeutic purposes. Thus, an IND gel ointment has been developed and used for topical application.[6,7] It has been shown that this preparation is very effective for external diseases, such as arthritis deformans, arthralgia, muscle ache, tenositis, and tenosynovitis, but is less effective for internal diseases, *e.g.*, inflammation of lumbar vertebra and hip joint.[8]

Our previous studies on IND-calcium (IND-Ca) gel ointment containing Azone®(AZ) suggested that the salt of IND was significantly absorbed through rat skin and that therapeutically effective concentrations as an anti-inflammatory drug were maintained for 48 h.[9] Therefore, the IND-Ca gel ointment is useful as a topical dosage form aimed at delivering a systemic effect of the anti-inflammatory drug, and may be effectively used for internal inflammatory diseases and other inflammation. To demonstrate the efficacy of the formulation, we tested the effects of the ointment applied locally on experimental inflammatory models. In addition, the irritancy of the formulation was estimated on the rabbit skin from the viewpoint of practical application.

Materials and Methods

Reagents and Animals——IND (J.P. grade) and AZ were generous gifts of Sumitomo Seiyaku Co., Ltd. and Nelson Research and Development Co., respectively. Hiviswako 104® and λ-carrageenin were purchased from Wako Pure Chemical Industries, Ltd. *Mycobacterium butyricum* (killed and dried *Mycobacterium butyricum*) was obtained from Difco Lab. Diethylene glycol monoethyl ether (carbitol, Kishida Chemical Co.) was used as a solvent. All other chemicals used were of reagent grade. Healthy male Wister rats weighing 140—160 g and male Japanese white rabbits weighing 2.2—3.5 kg were used. The animals had free access to MF or RC4 diet (Oriental Yeast Co., Ltd.) and water before the experiment.

TABLE I. Composition of IND and IND-Ca Ointments

Component	Concentration (%, w/w)				
	Rp. 1	Rp. 2 (IND)	Rp. 3 (IND-Ca)	Rp. 4 (IND)	Rp. 5 (IND-Ca)
Drug	—	1.0	1.05	0.5	0.53
DMSO[a)]	10.0	10.0	10.0	10.0	10.0
Ethanol	20.0	20.0	20.0	20.0	20.0
Azone®	6.0	6.0	6.0	6.0	6.0
Solbitan monooleate	6.0	6.0	6.0	6.0	6.0
Hiviswako 104®	1.0	1.0	1.0	1.0	1.0
Diisopropanolamine	1.1	1.1	1.1	1.1	1.1
Diisopropyl adipate	2.0	2.0	2.0	2.0	2.0
Water	53.9	52.9	52.85	53.4	53.37

a) Dimethylsulfoxide.

Preparation of Ointments——IND or IND-Ca was dissolved in dimethylsulfoxide and mixed with an ointment base (Hiviswako 104®) containing water, ethanol, diisopropyl adipate, diisopropanolamine and absorption enhancers (AZ and sorbitan monooleate). Details of the ointment composition are listed in Table I.

***In Vivo* Percutaneous Absorption Experiment**——The *in vivo* percutaneous absorption experiment was carried out by the same method as reported previously.[9)] The ointment (0.14 g) was applied over the shaved abdominal skin (1 cm^2) without anesthesia and the rats were fixed in Bollman cages for 36 h.

Carrageenin-Induced Paw Edema and Application of Ointment——Groups of 6 rats were used and animals were not anesthetized throughout the experiment. On the day before the experiment, the hair of the abdominal area was carefully removed. On the next day, 0.14 g (0.7 or 1.4 mg as IND/rat) of ointment was uniformly spread over the shaved skin (1 cm^2, designated by attaching an adhesive tape with a cut-out area) and immediately occluded with a sheet of aluminum foil and adhesive tape. Six hours later, carrageenin (1% w/v, 0.1 ml) was injected subcutaneously into the sole of the left hind paw. Thereafter, the volumes of the right and treated (left) paws were measured at intervals of 1 h until 6 h after injection of carrageenin, using a plethysmometer. Edema weight was calculated from the difference between the injected and non-injected paws. The control groups were treated with the placebo ointment and carrageenin in the same manner as above.

Adjuvant Arthritis and Application of Ointment——*Mycobacterium butyricum* suspended in liquid paraffin (6 mg/ml, 0.05 ml) was injected into the sole of the left hind paw of rats under light pentobarbital (32 mg/kg, intraperitoneal) anesthesia to induce adjuvant arthritis. The rats with established adjuvant arthritis were taken 11 d after the injection of adjuvant (6 rats in each group) and the ointment (0.14 g, 1 cm^2, 1.4 mg as IND/rat) was applied to the shaved abdominal skin for 24 h each on days 11, 13 and 15 under occlusion in the Bollman cages. The shaving of hair was done on days 10 and 14. The placebo ointment was applied to other rats with adjuvant arthritis. The unabsorbed ointment was wiped off with absorbent cotton soaked in warm water after the 24-h application. The left hind paw volume of these treated and control rats was measured on day 16 by the same method as used in the carrageenin edema experiment.

Assessment of Irritation of Ointment and Solution Containing Drugs——On the day before the experiment, the hair of the back of a male rabbit was carefully removed with an electric clipper. On the next day, 1 g (10 mg as IND/rabbit) of ointment was applied to the back skin (3 × 3 cm area) for 24 h under occlusion. The unabsorbed ointment was wiped off after the application. Twentyfour, 48 and 72 h after the cessation of application, the edema and erythema intensities were observed and the skin irritation was evaluated according to the primany irritation score.[10)] Separately, the 5% drug (IND equivalent) carbitol solution (1.5 ml) was applied to the back skin for 24 h, using a glass chamber (20 mm i.d.), and the skin irritation was also estimated by means of the irritation score.[10)]

Analytical Method——IND in plasma samples was determined by the gas-liquid chromatographic method of Guissou *et al.*[11)] with slight modifications (diethyl ether as the extraction solvent and trisilyldiazomethane as the methylating agent were used).

Pharmacokinetic and Statistical Analyses——The area under the plasma drug concentration–time curve (*AUC*) after topical application of ointment was calculated by the trapezoidal method up to the last determined point. The absolute bioavailability was calculated by using the following equation:

$$\text{bioavailability } (\%) = \frac{AUC_{\text{p.c.}} \cdot \text{dose}_{\text{i.v.}}}{AUC_{\text{i.v.}} \cdot \text{dose}_{\text{p.c.}}} \times 100$$

where $AUC_{p.c.}$ and $AUC_{i.v.}$ are AUC after percutaneous and intravenous administrations, respectively.

The means of all data are presented with their standard deviation (S.D.). Statistical analysis was performed using the non-paired Student's t-test, and a p value of 0.05 or less was considered to be significant.

Results

Plasma IND Concentration after Percutaneous Application of Ointment

The plasma IND concentrations after percutaneous application (0.14 g, 1 cm^2, 1.4 mg as IND/rat, 36 h) of ointments in unanesthetized rats are shown in Fig. 1. Our results demonstrate that both IND and IND-Ca were readily absorbed through the skin, maintaining anti-inflammatory plasma concentrations (0.5—3 μg/ml[12]) during 36 h. The absorption of IND-Ca from the ointment (Rp. 3) was faster than that of IND (Rp. 2), as shown by an earlier time to peak concentration, and the peak plasma concentration (C_{max}, 2.11 ± 0.31 μg/ml) was significantly higher than that (1.61 ± 0.30 μg/ml) after the corresponding IND ointment ($p < 0.025$). The bioavailability ($36.7 \pm 3.5\%$) for IND-Ca ointment was also increased compared with that ($28.7 \pm 6.7\%$) for IND ointment. The AUC_{0-36} value after intravenous injection (4 mg/kg) of IND was 59.1 μg·h/ml.

Inhibitory Effect of Ointment on Carrageenin-Induced Edema

Figure 2 shows the inhibitory effects of IND and IND-Ca ointments on the carrageenin-

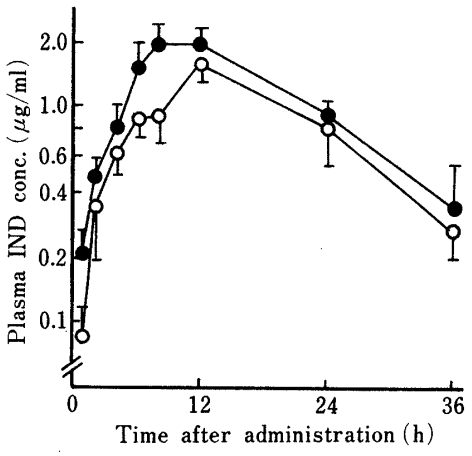

Fig. 1. Plasma Concentration of IND after Single Percutaneous Administration of IND or IND-Ca Ointment

Each point represents the mean ± S.D. of 3 rats. The applied dose of each ointment was 0.14 g/cm^2. ○, IND gel ointment (Rp. 2); ●, IND-Ca gel ointment (Rp. 3).

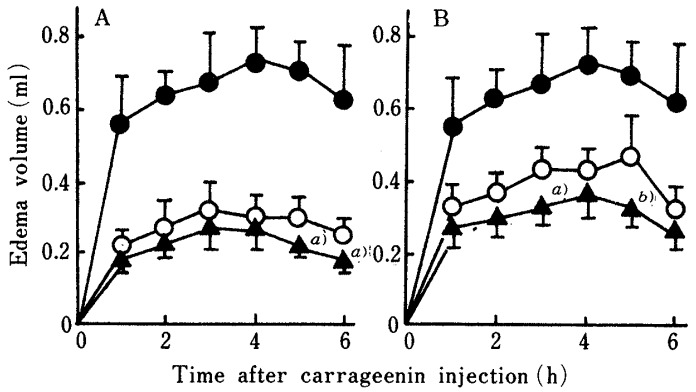

Fig. 2. Effect on Carrageenin-Induced Paw Edema of IND or IND-Ca Ointment Applied to Abdominal Skin

Each point represents the mean ± S.D. of 6 rats. The applied dose of 1% ointment (A) or 0.5% ointment (B) was 0.14 g/cm^2.

●, placebo ointment; ○, IND ointment (Rp. 2 or 4); ▲, IND-Ca ointment (Rp. 3 or 5). $p < 0.01$ in the placebo *vs.* IND or IND-Ca at all points. *a*) $p < 0.05$, *b*) $p < 0.01$ compared with IND.

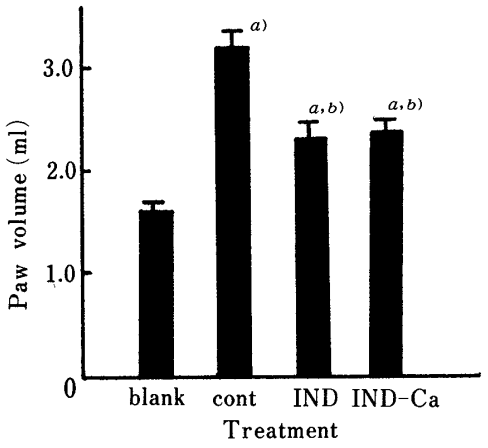

Fig. 3. Effect on Adjuvant Arthritis of IND or IND-Ca Ointment Applied to Abdominal Skin

Each bar represents the mean±S.D. of 6 rats. Blank, untreated; Cont, placebo ointment (Rp. 1); IND, IND ointment (Rp. 2); IND-Ca, IND-Ca ointment (Rp. 3). *a*) $p<0.01$ compared with the blank, *b*) $p<0.02$ compared with the control.

TABLE II. Score of Skin Reaction with IND or IND-Ca Ointment Applied to Rabbit Back Skin

Day	Placebo	IND (Rp. 2)	IND-Ca (Rp. 3)
1	4.7±0.6	2.5±0.6[a)]	2.2±0.6[a)]
2	4.3±0.6	1.8±0.5[a)]	1.8±0.5[a)]
3	3.6±0.6	1.8±0.5[a)]	1.5±0.6[a)]

Each value represents the mean±S.D. of 4 experiments. The score was expressed as the total of erythema and edema scores. The applied dose of ointment was 1 g/9 cm^2 (10 mg/rabbit). *a*) $p<0.01$ compared with the placebo.

induced paw edema. The 1% drug ointments produced significant inhibitory effects, with a 50—60% inhibition at maximum. At 0.5%, IND-Ca ointment (Rp. 5) displayed very potent inhibitory effects compared with those of IND ointment (Rp. 4). In this experiment, the placebo ointment had no effect on carrageenin-induced edema. The area under the edema volume–time curve (*AUVC*) values (1.30±0.13 and 1.77±0.15 ml·h for Rp. 3 and 5, respectively) for both IND-Ca ointments were also significantly smaller than those (1.62±0.28 and 2.23±0.25 ml·h for Rp. 2 and 4, respectively) for IND ointments ($p<0.05$ or $p<0.01$).

Inhibitory Effect of Ointment on Adjuvant Arthritis

When these ointments (1%, 0.14 g, 1 cm^2, 1.4 mg as IND/rat) were applied to abdominal skin of rats with established adjuvant arthritis for 3 d every other day, significant inhibitory effects (55.4±10.5% for IND ointment and 51.0±7.8% for IND-Ca ointment) were observed (Fig. 3). These data also suggested excellent absorption of the drug through the skin and potent inhibitory effects of both ointments on the chronic inflammation.

Skin Irritation of Ointment and Solution Containing Drug

The skin irritation scores after application of ointments (1%, 1 g, 9 cm^2, 10 mg as IND/rabbit) are shown in Table II. The placebo ointment produced a weak erythema, with an irritation score of 4—5. These drug ointments produced less irritation than the placebo ointment, suggesting that the drugs in the ointments rather reduced the vehicle-induced erythema.

The irritation scores (0.3—0.7) after 5% drug (75 mg as IND/rabbit) carbitol solution were much less than those of the ointments, and consequently the irritant effects of IND and IND-Ca were nearly negligible. Carbitol used as the solvent caused no irritation to the skin after the 24-h application. Subsequently, when hair growth at the application sites was examined, normal growth was seen in the cases of IND-Ca and placebo solutions, while growth was hardly seen for 11 d after application of IND solution, indicating that some injury to viable tissue was induced with IND. From these results, it was demonstrated that IND-Ca caused much less irritation to the skin than IND.

Discussion

It has been shown that when commercial IND ointment (10 g, 100 mg as IND) is applied to human skin, the C_{max} is approximately 20 ng/ml and only 1% of the applied dose is excreted into the urine during 3 d[13] and that on repeated administration the drug level at the application site and its surroundings significantly increased, but the blood level did not.[8] Our previous studies on IND-Ca gel ointment in rat showed that effective concentrations and high bioavailability (68—70%) were obtained after application to the skin.[9,14] The present study was designed to examine the anti-inflammatory activity and systemic effect of IND-Ca ointment applied to skin, in comparison with those of IND ointment. In the present study, on the application of IND-Ca ointment (0.14 g, 1 cm^2, 1.4 mg as IND/rat) to rat abdominal skin, therapeutically effective levels were maintained during 36 h (Fig. 1) and the bioavailability was significantly greater than that obtained after IND ointment ($p<0.025$). This value (37%) of bioavailability was lower than that (68—70%) obtained with 2% ointment (0.14 g/cm^2, 1.8 or 3.6 cm^2) reported previously.[14] The lower bioavailability may be mainly due to the partial loss of ointment (a small amount of 0.14 g) by adherence to vessels on weighing and application.

The IND and IND-Ca ointments applied to abdominal skin produced significant inhibitory effects against carrageenin-induced paw edema, which is frequently used as a test model of anti-inflammatory activity. The anti-edema effects of these ointments were nearly equivalent to those observed after oral dosing of IND.[15,16] Wada *et al.* reported that the inhibitory rate of 1% IND ointment applied to the surface of carrageenin-induced paw edema is only 19% and that of 3% ointment is 39%.[7] In comparison with the results of Wada *et al.*,[7] it is evident that our preparations, even the 0.5% ointment, exerted a high anti-inflammatory effect. The IND and IND-Ca ointments were also markedly effective on adjuvant arthritis, which is an experimental model of human rheumatoid arthritis and delayed hypersensitivity reaction induced by cellular immunity.[17,18] Nonsteroidal anti-inflammatory drugs usually exert a marked inhibitory effect equally on both adjuvant primary and secondary inflammation. This study was done on the secondary inflammation only. However it is beyond doudt that our ointments exert potent inhibitory effects on both inflammations. Thus, the anti-inflammatory effect of these ointments applied to skin on reactions of other regions or systemic reaction has been fully verified. The significant anti-inflammatory effect of IND-Ca ointment is probably due to the higher lipid solubility of IND-Ca, the ease of release from the gel base and the enhancing effect of Azone® as suggested previously,[9] although the relative contributions of these factors have not been clarified. The IND-Ca ointment produced less irritation to skin. The negligible irritant effect may make the formulation acceptable for clinical use.

DMSO, used as a solvent of IND and IND-Ca in this study, was demonstrated to have some local toxicity,[19] in particular, ophthalmologic toxicity.[20] Therefore, our study should be regarded as a fundamental experiment to test the effects of these ointments.

In conclusion, the IND-Ca ointment applied locally had an excellent effect in the therapy of acute and chronic inflammations. It seems probable that the salt may be useful for topical application aimed at providing a systemic anti-inflammatory effect, based on the high absorption and low irritation to skin.

References

1) A. C. Caruso, *Sem. Med.*, **124**, 426 (1964).
2) M. Cairella and L. Vecchi, *Clin. Therap.*, **30**, 621 (1964).
3) F. Delabarre, A. Kahan, A. de Gery, and H. Brouilhet, *Eur. Med.*, **1**, 103 (1964).
4) M. Kelly, *Med. J. Aust.*, **51**, 541 (1964).
5) W. A. Charles, R. A. Edwin and S. A. Robert, *J. Pharmacol. Exp. Ther.*, **162**, 196 (1968).

6) H. Ishihama, H. Kimata and Y. Mizushima, *Experientia*, **35**, 798 (1979).
7) Y. Wada, Y. Etoh, A. Ohira, H. Kimata, T. Koide, H. Ishihara, and Y. Mizushima, *J. Pharm. Pharmacol.*, **34**, 467 (1982).
8) T. Kanno, *Medicina*, **22**, 1208 (1985).
9) T. Ogiso, Y. Ito, M. Iwaki and H. Atago, *J. Pharmacobio-Dyn.*, **9**, 517 (1986).
10) Y. Shirasu and O. Matsuoka (ed.), "Environmental Toxicology: Methods and Safety Evaluation," Soft Science, Inc., Tokyo, 1981, pp. 444—445.
11) P. Guissou, G. Cuisinaud and J. Sassard, *J. Chromatogr.*, **277**, 368 (1983).
12) P. Miskolczi and L. Vereczkey, *J. Pharm. Biomed. Anal.*, **3**, 209 (1985).
13) Y. Azuma, *J. Pract. Pharm.*, **37**, 805 (1986).
14) T. Ogiso, Y. Ito, M. Iwaki and H. Atago, *J. Pharmacobio-Dyn.*, **10**, 384 (1987).
15) S. Evangelista and A. Meil, *J. Pharm. Pharmacol.*, **35**, 250 (1982).
16) R. J. Flower and J. R. Vane, "Prostaglandin Synthetase Inhibitors," ed. by H. J. Robinson and J. R. Vane, Raven Press, New York, 1974, pp. 9—18.
17) B. H. Waksman and C. Wennersten, *Int. Arch. Allergy Appl. Immunol.*, **23**, 129 (1963).
18) F. Quagliata and J. M. Phillips-Quagliata, *Cell Immunol.*, **3**, 78 (1972).
19) G. E. Schumacher, *Drug Intell.*, **1**, 188 (1967).
20) L. Rubin and K. Barnett, *Ann. N. Y. Acad. Sci.*, **141**, 333 (1967).