

## Dermal Cell Damage Induced by Topical Application of Non-Steroidal Anti-Inflammatory Drugs is Suppressed by Trehalose Co-Lyophilization in *Ex Vivo* Analysis

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**ABSTRACT.** Topical administration of non-steroidal anti-inflammatory drugs (NSAIDs) is generally considered safer than oral administration, although the former can occasionally induce cutaneous irritation. We hypothesized that the cutaneous irritation by topical NSAIDs might be suppressed by trehalose, which has protective effects on biological membranes. Using the three-dimensional cultured human skin model, Living Skin Equivalent-high, we found that cutaneous damage due to NSAIDs was reduced by concomitant use of trehalose and that this effect of trehalose was reinforced by co-lyophilization of NSAIDs with trehalose. The anti-inflammatory effect of co-lyophilized NSAIDs with trehalose was comparable to that seen with NSAIDs alone in a rat model. Our results suggest that co-lyophilization of NSAIDs with trehalose might be a novel procedure that can help prevent NSAIDs-induced skin irritation.

**KEY WORDS:** cutaneous damage, NSAIDs, topical application, trehalose.

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Non-steroidal anti-inflammatory drugs (NSAIDs) are usually used as an oral formulation. However, they can also be applied topically, such as in forms of gels and creams, which is considered safer route of administration [7]. Topical application of NSAIDs has been applied for the treatment of acute soft tissue trauma, inflammatory and degenerative musculoskeletal disorders and some inflammatory skin diseases in humans [6]. In domestic animals, topical application of NSAIDs is routinely used [2, 8, 11, 19]. Topical NSAIDs have the advantage of being easy to apply: they also deliver a high local drug concentration with low systemic absorption. Recently, however, reports of adverse effects are increasing [1, 14, 15].

When the barrier function of the skin is disrupted for any reason, irritant contact dermatitis, a skin inflammation characterized by rash and dry skin, can occur with subsequent epidermal cellular damage. Because most NSAIDs are acidic, they are inherent irritants. Topical diclofenac, which is one of the most common topical NSAIDs used in clinical

trials for the treatment of osteoarthritis, has been reported to cause mild irritant contact dermatitis in some patients [1, 15]. In addition, previous reports have shown that several NSAIDs have direct cytotoxic actions. These are caused by permeabilization of biological membranes and the subsequent increase in the concentration of intracellular calcium ions [18, 20]. In response to direct toxicity of a drug or chemical, skin irritation is triggered by a reversible inflammatory reaction induced by the arachidonic acid cascade and pro-inflammatory cytokines in skin cells [9]. Additionally, allergic and photoallergic contact dermatitis is also known to be provoked by several NSAIDs [14].

Trehalose is a non-reducing disaccharide with multiple functions including cellular protection against stresses, such as desiccation, heat and oxidation [4]. We previously reported that trehalose suppressed postsurgical adhesion, several complications after subarachnoid hemorrhage and oral dryness during dental treatment in preclinical or clinical researches [3, 5, 12]. We also reported that co-lyophilization of aspirin with trehalose clearly reduced the gastric mucosal damage induced by aspirin [10]. In previous papers, we have hypothesized that trehalose confers protective effects on cellular components, in particular, the cell membrane and thereby suppressed these pathologies [1, 3, 7, 10].

In this study, we expected that indomethacin, diclofenac, ibuprofen and piroxicam would induce skin damage and that co-lyophilizing these NSAIDs with trehalose would reduce the damage. We assessed the effects of co-lyophilized indomethacin, diclofenac, ibuprofen and piroxicam with trehalose (here after called co-lyophilized indomethacin/trehalose, co-lyophilized diclofenac/trehalose, co-lyophilized ibuprofen/

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trehalose and co-lyophilized piroxicam/trehalose) on skin damage using an *ex vivo* 3D cultured human skin model, TESTSKIN™ Living Skin Equivalent-high kit (LSE-high). LSE-high is an organotypic co-culture consisting of human dermal fibroblast in a collagen-containing matrix overlaid with human keratinocytes [21]. There have been several reports showing that MTT assays using LSE-high are useful in evaluating levels of skin irritation due to pharmaceutical and cosmetic materials [17, 21].

Indomethacin, diclofenac, ibuprofen, piroxicam, trehalose and  $\lambda$  carrageenan were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hydrophilic ointment was purchased from Mylan Inc. (Canonsburg, PA, U.S.A.).

Molar ratios of co-lyophilized indomethacin, diclofenac, ibuprofen and piroxicam to trehalose were 1:25.3, 1:15.7, 1:1.1 and 1:23.3, respectively. These four drugs were separately dissolved in ethanol, and the trehalose was dissolved in distilled water. Each drug and trehalose solution were mixed together in a glass flask. After freezing in liquid nitrogen, the mixture was lyophilized over 3 days using a freeze-dryer (FDU-1100; EYELA, Tokyo, Japan).

The concentrations (% w/w) of indomethacin, diclofenac, ibuprofen and piroxicam in the hydrophilic ointment were 1.0, 1.0, 3.0 and 3.0, respectively, regardless of the trehalose content. Hydrophilic ointments containing saline, trehalose, indomethacin, diclofenac, ibuprofen, piroxicam, co-lyophilized indomethacin/trehalose, co-lyophilized diclofenac/trehalose, co-lyophilized ibuprofen/trehalose, co-lyophilized piroxicam/trehalose or the corresponding mixture were applied to LSE-high or rats.

LSE-high and its culture media were purchased from TOYOBO Co., Ltd. (Osaka, Japan). LSE-high was incubated at 37°C/5% CO<sub>2</sub> in a humidified incubator. The extent of skin damage was evaluated in LSE-high using colorimetric MTT assays following the application of 100 mg of ointment with or without drug, according to the manufacturer's instructions for LSE-high. At 20 hr after the application of ointment to the epidermal side of LSE-high, the ointment was removed. The MTT solution (0.33 mg/ml) was applied and incubated for 3 hr at 37°C. A portion of LSE-high was transferred to a test tube containing 0.04 M HCl in isopropanol and immersed for 16 hr at room temperature in the dark. The absorbance of the solution was measured at 570 nm using a spectrophotometer (V-670; JASCO, Tokyo, Japan).

Male Wistar rats weighing 200–250 g were obtained from SLC Japan (Shizuoka, Japan) and maintained in a 12-hr light/dark cycle (lights on at 09:00) at constant temperature 22–24°C. Rats were fed standard laboratory chow and tap water. Animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals, School of Engineering, the University of Tokyo.

Hydrophilic ointment (100 mg) containing diclofenac, indomethacin, co-lyophilized diclofenac/trehalose or co-lyophilized indomethacin/trehalose was applied to the hind paws of rats anesthetized with pentobarbital every 2 hr for 4 hr. Following this topical application, 0.1 ml of 1% (w/v) carrageenan in saline was injected into the right footpad of rats. As a control, 0.1 ml of saline was injected into the left

footpad. The hydrophilic ointments were applied to the paws at 0, 1, 2, 3 and 4 hr after the carrageenan injection. Concomitant with this procedure, the volume of the hind paw was measured using a Plethysmometer (MK-101P; Muromachi Kikai, Tokyo, Japan). Inhibition rates were evaluated as an increase in paw volume in the drug-treated groups compared with those in the saline-treated group.

All data were expressed as mean  $\pm$  standard deviation (SD) and were analyzed for statistical significance by analysis of variance (ANOVA), followed by Scheffe's test (Fig. 1) or unpaired Student's *t* tests (Fig. 2) using KaleidaGraph® software (HULINKS Inc., Tokyo, Japan). A *P*-value less than 0.05 was considered significant.

As shown in Fig. 1, dermal cell viability of LSE-high treated with trehalose-containing ointment was similar to that for LSE-high treated with saline-containing ointment. In contrast, the application of ointments containing diclofenac, ibuprofen or piroxicam, largely reduced dermal cell viability. For LSE-high treated with indomethacin-containing ointment, dermal injuries were relatively lower compared with LSE-high treated with other NSAIDs. Dermal cell death induced by NSAIDs, except for indomethacin, in LSE-high was reduced by mixing with trehalose. In all NSAIDs used in this study, dermal cell injuries were significantly attenuated when the drugs were co-lyophilized with trehalose. Taken together, the results suggest that concomitant use of trehalose with NSAIDs suppresses dermal damage in LSE-high. The findings also indicate that co-lyophilization of NSAID with trehalose, compared to simple mixing of the two, showed more suppressing effect of cutaneous damage.

Injection of carrageenan into the hind paw of rats caused a marked increase in its volume, consistent with edema formation. Cutaneous application of diclofenac alone reduced paw volume in the carrageenan-treated rats (Fig. 2). The anti-inflammatory effects of co-lyophilized diclofenac/trehalose were similar to that of diclofenac alone (Fig. 2). Additionally, we also found that there were no significant differences in anti-inflammatory effect between indomethacin and co-lyophilized indomethacin/trehalose (data not shown). These results suggest that the anti-inflammatory effects of diclofenac and indomethacin were not impaired by co-lyophilization with trehalose.

Two major findings were obtained from this study. First, dermal cell death caused by the topical application of NSAIDs used in this study was reduced by concomitant use of trehalose. This effect was reinforced by the co-lyophilization. Second, the anti-inflammatory effects of co-lyophilized diclofenac/trehalose and co-lyophilized indomethacin/trehalose were comparable to those seen with the NSAID alone.

LSE-high was used to evaluate NSAID-induced cutaneous damage. Results of *ex vivo* MTT assays using LSE-high were similar to those of *in vivo* assays using guinea pigs [21]. Therefore, MTT assay using LSE-high can be used as a substitute for *in vivo* analysis. However, LSE-high also tends to have higher permeability than normal full-thickness skin [21]. Generally, a cell membrane with higher permeability is thought to exhibit cell damage to a greater extent. The topical application of NSAIDs to LSE-high appeared to cause

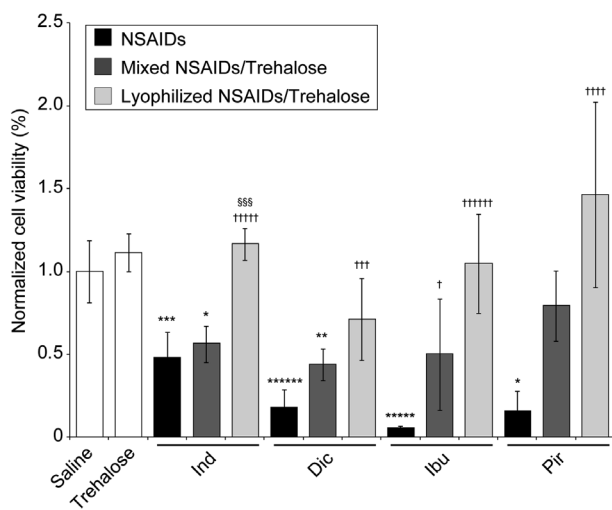


Fig. 1. Effects of co-lyophilization with trehalose on cytotoxicity induced by NSAIDs in LSE-high. LSE-high was treated with hydrophilic ointment containing saline, trehalose, a single NSAID an NSAID mixed with trehalose or an NSAID co-lyophilized with trehalose. The NSAIDs were: 1% (w/w) indomethacin (Ind); 1% (w/w) diclofenac (Dic); 3% (w/w) ibuprofen (Ibu); and 3% (w/w) piroxicam (Pir). Cell viability was evaluated by measuring the absorbance at 570 nm of extracts from drug- and saline-treated LSE-high. Data are presented as the mean  $\pm$  SD;  $n=4$  in each group. \* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.005$ , \*\*\*\* $P<0.0005$  and \*\*\*\*\* $P<0.0001$  compared with the vehicle group (ANOVA); †††† $P<0.005$ , ††††† $P<0.001$ , †††††† $P<0.0005$  and ††††††† $P<0.0001$  compared with the NSAID group (ANOVA); and §§§ $P<0.005$  compared with the mixed NSAID/trehalose group (ANOVA).

severe levels of dermal cell death in this study.

Several NSAIDs, including indomethacin, diclofenac and ibuprofen, are known to enhance membrane permeabilization [18, 20], which is thought to be one of the reasons for gastric erosion to occur after oral administration of NSAIDs. Therefore, we postulated that cutaneous damage in LSE-high could also have resulted from NSAID-induced reduction of the hydrophobic cell membrane barrier. In addition, the differences in cytotoxicity of the tested NSAIDs might be dependent on their membrane permeabilization activity, which would differ from one NSAID to another. Further studies are needed to elucidate the relationship between the NSAID structure and its cytotoxicity.

Cutaneous damage induced by the tested NSAIDs in LSE-high was suppressed by trehalose alone. Trehalose confers a protective effect to cell membrane damage caused by stressors, such as desiccation, heat and oxidation [4, 13]. This effect is thought to be involved in direct interaction of trehalose with membrane phospholipid [4, 13]. The trehalose effects seen in this study could be derived from its protective action on the cell membrane.

We also found that suppression of the cutaneous damage was enhanced when trehalose was co-lyophilized with the tested NSAID. This is consistent with our recent study that co-lyophilized aspirin/trehalose decreased the severity of

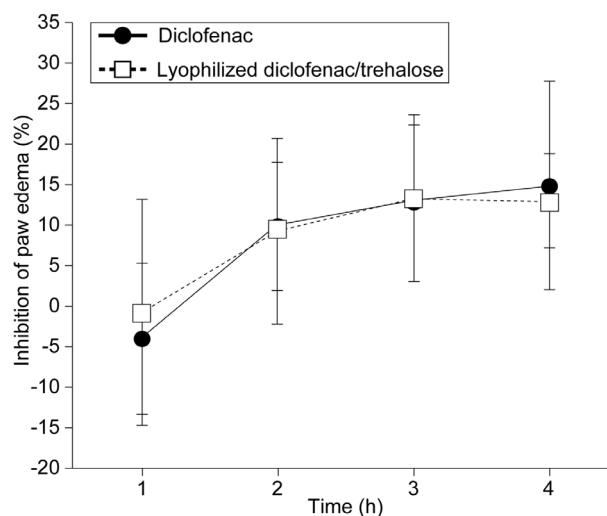


Fig. 2. Comparison of the anti-inflammatory effects of co-lyophilized NSAID/trehalose and NSAID in topical applications using a carrageenan-induced paw edema model. Hydrophilic ointments containing diclofenac or co-lyophilized diclofenac/trehalose were topically applied to the footpads of carrageenan-treated rats. The inhibition rate of paw edema was calculated from an increase in paw volume in the drug-treated groups and compared with that in the vehicle group.  $n=6$  in the vehicle group.  $n=6$  in the diclofenac group.  $n=7$  in the co-lyophilized diclofenac/trehalose group. Data are presented as the mean  $\pm$  SD.

aspirin-induced gastric ulceration in healthy dogs [10]. We hypothesize that co-lyophilization accelerates the specific interaction between the NSAIDs and trehalose and that this interaction is necessary for a marked reduction of NSAID-induced cell damage. As previously reported by the group of Sakurai, simulation analyses combined with nuclear magnetic resonance measurements showed that dehydration pockets that formed on the top of trehalose allow for the acceptance of hydrophobic molecules, such as benzene, thereby leading to a formation of a stable intermolecular complex [16]. It was speculated that indomethacin, diclofenac, ibuprofen and piroxicam all stably interacted with trehalose, as they possess a benzene ring. The simulation analyses also showed that the binding of both a hydroxyl group and a methyl group to a benzene ring affected the pattern of interaction with trehalose [16]. Therefore, the variation in molecular structure of the NSAIDs tested in this study likely creates a difference in the suppressive effects of trehalose with respect to cutaneous damage in LSE-high. Alternatively, reductions of the tested NSAIDs-induced cutaneous damage may be simply due to the inhibition of the NSAIDs penetration into the epidermis of LSE-high via its interaction with trehalose. Further studies are needed to evaluate the transdermal absorption of co-lyophilized NSAIDs/trehalose.

The carrageenan-induced paw edema model showed that topical application of co-lyophilized NSAIDs/trehalose suppressed inflammation equally as well as NSAIDs alone. Therefore, co-lyophilization with trehalose did not impair the anti-inflammatory activity of NSAIDs not only in LSE-high

but also in the rat model. We speculate that skin penetration of NSAIDs is not inhibited by co-lyophilization with trehalose. These considerations are in agreement with those from our previous study, which showed that co-lyophilization with trehalose does not alter the absorption of aspirin [10].

NSAIDs co-lyophilized with trehalose may be applied to any area of the body that is highly sensitive to irritation and cellular damage. In conclusion, co-lyophilized NSAID/trehalose could be a low-irritant formulation of NSAIDs that could be used in topical application.

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