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Buccal Mucosal Ulcer Healing Effect of rhEGF/Eudispert hv Hydrogel

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We have studied the effect of rhEGF on the buccal mucosal ulcer healing. rhEGF was rapidly degraded upon incubation with the hamster buccal mucosal homogenates; The degradation of rhEGF was significantly inhibited by sodium lauryl sulfate (SLS). Eudispert hv hydrogel and Polycarbophil 974P hydrogel were prepared for rhEGF delivery and their mucoadhesiveness was measured by the Instron® method. The mucoadhesive force of Eudispert hv was significantly greater than that of Polycarbophil 974P. Moreover, rhEGF in Eudispert hv hydrogel remained stable for about 2 months. To evaluate the ulcer healing effect of rhEGF, the buccal mucosal ulcer was induced in golden hamsters using acetic acid. At 24 h after administration of rhEGF/Eudispert hv hydrogel, the ulcerous area was decreased compared with rhEGF solution and, as a result, the curative ratio was 36.8±5.68%. By the addition of SLS (0.5%) to Eudispert hv hydrogel, the curative ratio increased 1.5 times. The mechanism of the action was probably due to a combination of protection of the drug against proteases present in mucosa and prolongation of the release of rhEGF from the formulation at the site of action.

Key words: rhEGF, Eudipert hv hydrogels, Buccal mucosa, Ulcer healing effect

INTRODUCTION

rhEGF is a single-chain polypeptide containing 53 amino acid residues (MW: 6045) and three disulfide bridges (Dibiase and Rhodes, 1991). It has been reported that rhEGF stimulates the proliferation and differentiation of epithelial tissues such as intestinal mucosa, corneal epithelial tissue, and lung and trachea epithelia (Carpenter and Cohen, 1979). Moreover, rhEGF is able to inhibit gastric acid secretion (Bower *et al.*, 1975; Elder *et al.*, 1975; Gregory, 1975; Konturek *et al.*, 1984; Carpenter *et al.*, 1984) and protect gastroduodenal mucosa against tissue injury induced by ulcerogenic agents (Gregory *et al.*, 1978; Konturek *et al.*, 1981a; Konturek *et al.*, 1981b; Kirkegaard *et al.*, 1983).

In order to use rhEGF as a therapeutic agent, it is necessary to develop a physically and chemically stable formulation during storage because of potential instability of the protein in an aqueous solution (Senderoff *et al.*, 1994; Son and Kwon, 1995). Moreover, because poor membrane permeability and susceptibility of proteolytic breakdown may lead to a insufficient delivery of the protein after a systemic administration (Han *et al.*, 1998a), a local delivery may achieve a faster and improved healing of wounds and burns.

As a dosage form for local delivery of the drug, gel formulation (Morimoto *et al.*, 1984; Morimoto *et al.*, 1987) is an efficient form because it offers a proper balance between retention at the site of administration and the release rate of the drug. It was reported that Polycarbophil 974P has the highest binding affinity to human mucosal epithelial cells (Lehr *et al.*, 1990). In addition, Kim *et al.* (1992) reported that the gel preparations of Eudispert, a block copolymer of methacrylic acid and methyl methacrylate, showed excellent staying and bioadhesive effects in the lower part of the rectum in rats. We have previously reported that oral bioadhesive gels containing rhEGF were effective against induced acute and chronic gastric ulcers in rats (Han *et al.*, 1998b).

Buccal mucosal ulcers often induced by bacteria, viruses, local physical trauma and etc. For treatment of buccal mucosal ulcers, mouth rinses, topical corticosteroids, anti-

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biotics, immunomodulators and others are used. Currently, the possibility of rhEGF in the treatment of ulcers in buccal mucosal has not been studied in the literature. Because bioavailability is generally low for proteins, it appears reasonable to design a local delivery system for rhEGF for the treatment. However, even when rhEGF is administered locally, the poor bioavailability of the protein due to instability of rhEGF in the tissue is expected. The aim of this study, therefore, was to evaluate the effect of rhEGF on buccal mucosal ulcer healing. We were particularly interested in the possibility of inclusion of protease inhibitors in the formulation, since the inhibitor may potentially improve the healing effect. Therefore, we have evaluated the wound healing effect of rhEGF in Eudispert hv hydrogels, a bioadhesive formulation, containing a protease inhibitor. In this study, hamster buccal pouch was used as a primary experimental system because the ulcer in the tissue may be readily induced without a complicated surgical procedure.

MATERIALS AND METHODS

Materials

rhEGF was kindly provided by Daewoong Pharm. Co. (Seoul, Korea). Bovine serum albumin (Sigma Chemical Co., U.S.A.), a dye binding assay kit (Bio-rad Laboratories, U.S.A.), Eudispert hv (Röhm Pharma, Germany) and Polycarbophil 974P (Röhm Pharma, Germany) were used. All other materials were analytical grade. Syrian golden hamsters (male, 100~130 g) were purchased from Ansung Animal Center (Kyeonggi, Korea).

Collection of mucosa

Hamsters were sacrificed by inhalation of an overdose of ether vapor for approximately 10 min in a closed chamber. The cheek pouch was everted by carefully sliding a closed forceps into the pouch until reaching the distal end of the pouch, and slowly drawing the cheek pouch out of the mouth. The pouch was then removed by cutting at its base, rinsed in chilled isotonic saline, and placed in borosilicate glass vials.

Preparation of buccal mucosal homogenates of hamster

After collection of mucosa, the tissue was immediately homogenized in an isotonic phosphate buffer cooled with an ice bag (homogenizer : Ultra Turex T 25, Janke & Hunkel GmbH, Germany). The homogenates were centrifuged at 3020×g in a refrigerated (4°C) centrifuge (Himac CR 15D, Hitachi Co. Ltd., Japan) for 15 min. The protein concentration of the supernatant was determined using dye binding assay (Braford, 1976) using bovine serum albumin as the standard.

Enzymatic degradation of rhEGF in mucosal homogenates

Tissue supernatant (200 μL ; 2.5, 5.0 and 10.0 mg protein/mL) was preincubated for 15 min. After the addition of 200 μL of rhEGF solution (200 μg/mL), the mixture was incubated for 3 h at 37°C in a shaker water bath. We also tested the stabilizers such as hydroxypropyl-β-Cyclodextrin (HP-β-CyD), hydroxypropylmethyl cellulose (HPMC), and sodium lauryl sulfate (SLS) on the degradation of rhEGF in buccal mucosal homogenate. HP-β-CyD (0.5%), HPMC (0.5%) or SLS (0.1, 0.25 and 0.5%) was added before starting incubation of rhEGF in mucosal homogenates. Subsequently the incubation mixture was sampled at predetermined time intervals. The reaction was stopped by addition of acetonitrile and vortexed for 10 seconds. After centrifugation for 10 min at 10,000×g, the supernatant was evaporated under a stream of nitrogen. The residues were redissolved with 100 μL of mobile phase for HPLC analysis.

Separation of rhEGF by HPLC

Each experimental and control sample was subjected to a Vydac® protein and peptide C_{18} column interfaced with a Hitachi HPLC system. The mobile phase was a mixture of acetonitrile and 0.22% triethylamine in deionized water (22:78, pH 6.5 adjusted with H_3PO_4). The flow rate was 0.8 mL/min. Detection was monitored at 214 nm. Ketoprofen was used as the internal standard.

Preparations of Eudispert hv and Polycarbophil 974P hydrogel

Three formulations were prepared for Eudispert hv hydrogel and the preparation of Polycarbophil 974P hydrogel replaced Eudispert hv by Polycarbophil 974P in Rx 2 (Table I). Eudispert hv powder was suspended in distilled water and a half amount of 2.5 N NaOH solution was poured. It was mixed in motar and then the rhEGF solution was added. Finally, the remaining NaOH solution was added and mixed. The gel preparations were stored at 4°C. The pH of the gel preparation was also measured after 24 h when the pH of gels reached a steady state. The mucoadhesiveness of the gel preparations was measured by the Instron® (M 4400, Instron Co., U.S.A.) method (Park and Park, 1990).

Table I. Preparations of Eudispert hv hydrogel containing rhEGF

	Rx 1	Rx 2	Rx 3
Eudispert hv	50 mg	100 mg	150 mg
Distilled Water	700 μL	530 μL	350 μL
NaOH (2.5 N)	150 μL	270 μL	400 μL
rhEGF(1 mg/mL)	100 μL	100 μL	100 μL
Total	1 g	1 g	1 g

Release of rhEGF from Eudispert hv hydrogel

The rhEGF release study was performed using the Franz cell. A nitrocellulose membrane filter (pore size : 0.8 μm, diameter : 25 mm) was inserted between the donor and receiver cells. 5.3 mL of phosphate buffer (pH 6.2) was added to the receiver cell and equilibrated at 37°C. 200 mg of Eudispert hv hydrogel (10%) containing rhEGF (0.01%) was put into the donor side. 200 μL samples were removed from the receiver side at predetermined time intervals and replaced with a drug-free buffer, and the drug concentration in the sample was assayed by the enzyme immunoassay (ELISA) method using Quantikine® kits obtained from R&D Systems Inc (Minneapolis, MN, U.S.A.).

Assessment of mucoadhesive force

The buccal mucosa was obtained from golden hamsters immediately after inhalation of an overdose of ether vapor. The underlying connective tissues were subsequently removed to isolate the buccal mucosal membrane. For mucoadhesive tests, the buccal mucosal membranes (3.14 cm^2) were glued onto two acrylate plates using cyanoacrylate adhesive. One tissue plate was then secured onto a holder stage and the other was in the lower part. Hydrogels were placed between two buccal mucosal plates. The standard contact time was 10 min and the initial pressure was 0.5 N. The platform attached to the road cell transducer was raised at a constant speed of 5 mm/min by a precisely geared motor and the force required for the fracture of mucoadhesive bond was recorded.

Induction of buccal mucosal ulcer by acetic acid

The buccal mucosal ulcer was induced by acetic acid according to the modified method of Okabe and Pfeiffer (1972) Male golden hamsters weighing 100~130 g were used. Hamsters were anesthetized by injection of 7:3 mixture of ketamine and xylazine. Immediately after anesthetizing the hamsters, the cheek pouches were drawn carefully by closed forceps. 10 μL of acetic acid (30%) was spread on the mucosal side of the cheek pouches. The cheek pouches were pushed in the mouth and all hamsters were maintained normally on Purina Laboratory Chow and water ad lib. Five days after treatment, the ulcer areas were measured.

Estimation of buccal mucosal ulcer healing effect of rhEGF

The healing of acetic acid induced buccal mucosal ulcer was estimated 1 day after treatment with Eudispert hv hydrogel containing rhEGF. The areas of ulcer were measured (mm^2) and the curative ratio was calculated from the ulcer area using the following equation. To estimate the ulcer healing effect of rhEGF, 20 μL of rhEGF solution (1 mg/mL) was spread on buccal mucosa of hamsters under anesthesia. 200 mg of Eudispert hv hydrogel containing 0.01% rhEGF (20 μg as rhEGF) was administered to hamsters using a plastic syringe and sutured.

Curative ratio (%) =
(area of ulcer without treatment – area of ulcer after treatment)
/area of ulcer without treatment × 100

Histological examination

Eudispert hv hydrogel with SLS (0.5%) (no rhEGF) was applied on buccal mucosa to examine the irritation by SLS. At 24 h after administration of Eudispert hv hydrogel, hamsters were sacrificed and the pouches were then removed by cutting at their base. The tissues were fixed in 10% neutral formalin fixative. They were dehydrated through increasing concentrations of ethyl alcohol (70, 80, 90 and 100%), and embedded in the paraffin wax with an embedding machine (Histocentre 2, Leica, Germany). The tissue blocks were cut 4 μm in thickness by a rotary microtome (Histocut 820, Leica, Germany), stained with hematoxylin and eosin (H&E), and observed under a light microscope (U-MDOB, Olympus, Germany).

RESULTS AND DISCUSSION

Enzymatic degradation of rhEGF in mucosal homogenates

The degradation of rhEGF after incubation with the buccal mucosal homogenates was determined by measuring the loss of rhEGF using HPLC analysis. rhEGF was rapidly degraded in the buccal mucosal homogenates, and the degradation rate accelerated with an increase in protein concentration of supernatants (Fig. 1). This observation suggests that the buccal administration route

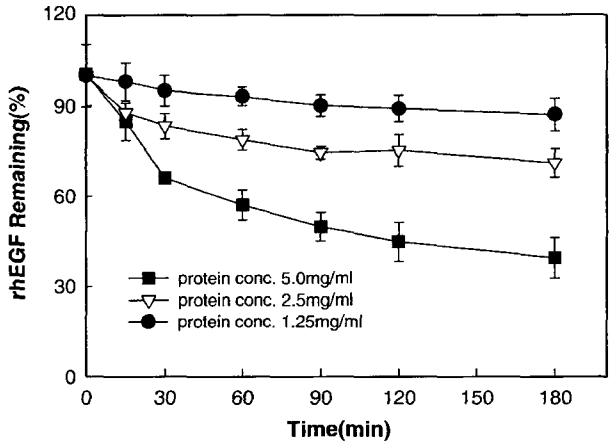


Fig. 1. Proteolysis profile of rhEGF in buccal mucosal homogenate of golden hamster. Each point represents the mean ± S.D. (n=3).

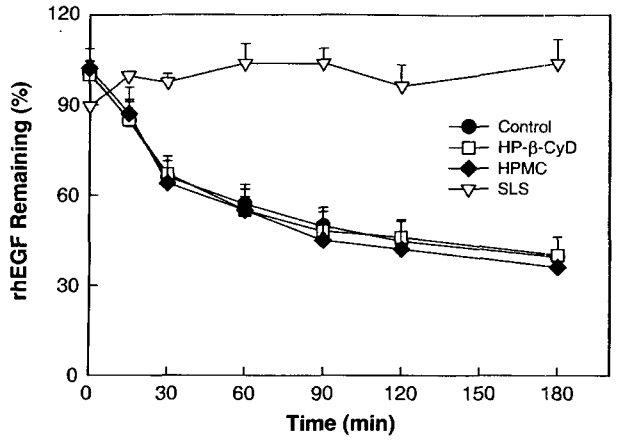


Fig. 2. Effect of HP-β-CyD, HPMC and SLS on the degradation of rhEGF in buccal mucosal homogenate of golden hamster. The tissue protein concentration is 5.0 mg/mL. Each point represents the mean ± S.D. (n=3).

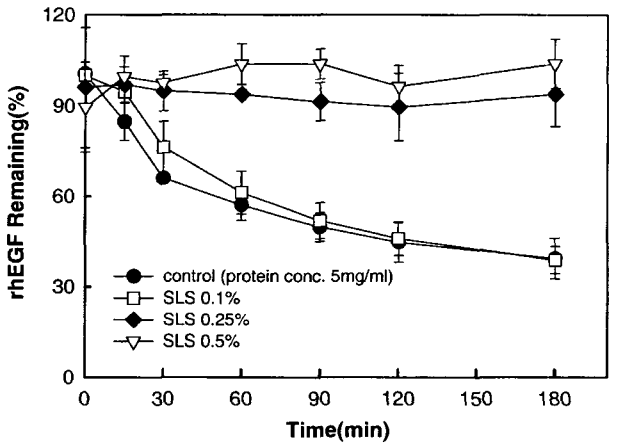


Fig. 3. Inhibitory effects of SLS on rhEGF proteolysis in buccal mucosal homogenates. The tissue protein concentration was 5.0 mg/mL. Each point represents the mean±S.D. (n=3).

needs protective means against enzymatic degradation. The degradation of rhEGF was significantly inhibited by the addition of SLS. However, the degradation was not inhibited by HP-β-CyD or HPMC (Fig. 2). When the concentrations of SLS were 0.25 (%) and 0.5 (%), the degradation of rhEGF almost completely inhibited (Fig. 3), and SLS may also used as a stabilizer in aqueous solution (data not shown).

rhEGF release from Eudispert hv hydrogel

The release of rhEGF from Eudispert hv hydrogel (10%) was measured by the ELISA method using Quantikine® kit. Fig. 4 shows the release profile of rhEGF (100 μg/g) from Eudispert hv hydrogel. The release profile of rhEGF from Eudispert hv hydrogel had a relatively large time lag, followed by a linear release as a function of time. The

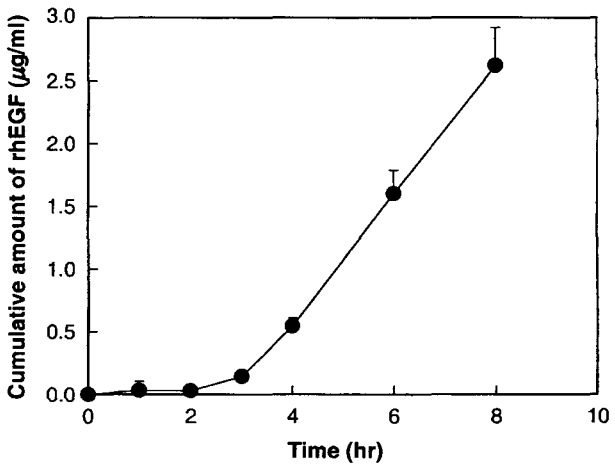


Fig. 4. The cumulative amount of rhEGF released through the nitrocellulose membrane filter (0.8 μm) in pH 6.2 phosphate buffer at 37°C. Each point represents the mean ± S.D. (n=3).

Table II. pH and mucoadhesiveness of Eudispert hv and Polycarbophil 974P Hydrogel

Formulation	Eudispert hv		Polycarbophil 974P	
(polymer content)	pH	mucoadhesiveness ($\times 10^{-3}$ N)	pH	mucoadhesiveness ($\times 10^{-3}$ N)
Rx 1 (5%)	6.97	0.35 ± 0.06	-	-
Rx 2 (10%)	6.85	0.58 ± 0.01	5.67	0.14 ± 0.03
Rx 3 (15%)	6.85	1.37 ± 0.05	-	-

transported amount of rhEGF across the membrane filter was about 2.6 μg/mL up to 8 h. The initial large time lag might be caused by the time for wetting and swelling of Eudispert hv hydrogels before the release of rhEGF. Therefore, the rate limiting step is considered as detachment of rhEGF from hydrogel following hydration by influx of dissolution medium.

Determination of mucoadhesive force of polymer hydrogels

The mucoadhesive force of polymer hydrogel was shown in Table 2. The mucoadhesive force increased as the polymer content of the hydrogels increased within the investigated polymer concentration range. And the mucoadhesive force of Eudispert hv (10%) hydrogel was 4.1 times greater than that of Polycarbophil 974P (10%) hydrogel.

Buccal mucosal ulcer healing effect

By treatment with 10 μL of 30% acetic acid, relatively large and clearly demarcated ulcers were produced in all animals on the fifth day after treatment (Fig. 7(a)). The ulcer floor was moderately covered with necrotic debris and adhesion of the ulcer base with each other was not observed compared with 25 μL of 30% acetic acid.

Table III. Ulcer area (mm^2) and curative ratio (%) at 24 h after administration of Eudispert hv hydrogel containing rhEGF to golden hamster

	Initial ulcer area	Ulcer area (1st day after treatment)	Curative ratio (%)
Control	29.00 ± 5.00	27.50 ± 4.79	5.63 ± 3.29
rhEGF solution	28.00 ± 5.16	23.00 ± 3.42	15.42 ± 5.42
rhEGF/Eudispert hv	26.50 ± 4.99	17.50 ± 4.99	36.83 ± 5.68
rhEGF/SLS/Eudispert hv	29.33 ± 2.31	12.67 ± 1.53	56.94 ± 3.18

*Initial ulcer area was measured the ulcer area on 5th day after ulcer inducing by Okabe *et al.* Method.

We chose Eudispert hv hydrogel as a buccal mucosal delivery system of rhEGF because Eudispert hv hydrogel showed markedly better mucoadhesive properties compared with Polycarbophil 974P. The ulcer healing effect of rhEGF after buccal administration of Eudispert hv hydrogel was shown in Table 3. At 24 h after administration of rhEGF using this hydrogel, the ulcer area was decreased 1.3 times compared with solution and the curative ratio was 36.83 ± 5.68%. Eudispert hv hydrogel containing rhEGF showed 2.4 times the curative ratio compared with the rhEGF solution (36.83 ± 5.68 vs 15.42 ± 5.42). This may be due to bioadhesiveness of Eudispert hv hydrogel and protection of rhEGF in polymer networks (Lueβen *et al.*, 1994). In fact, rhEGF was not degraded for almost 2 months in Eudispert hv hydrogel, which was different from the aqueous solution (Fig. 5). And by addition of SLS (0.5%) to Eudispert hv hydrogel containing rhEGF, the curative ratio increased 1.5 times compared with unadded control. Son and Kwon (1995) reported that surfactants, Triton X-100 and Tween 20 inhibited aggregation of hEGF in aqueous solution while the biological activity of hEGF remained constant 102 (%) and 98 (%), respectively at 37°C for 2 weeks. Kiyohara *et al.* (1991) reported that protease inhibitor accelerated wound repair when EGF

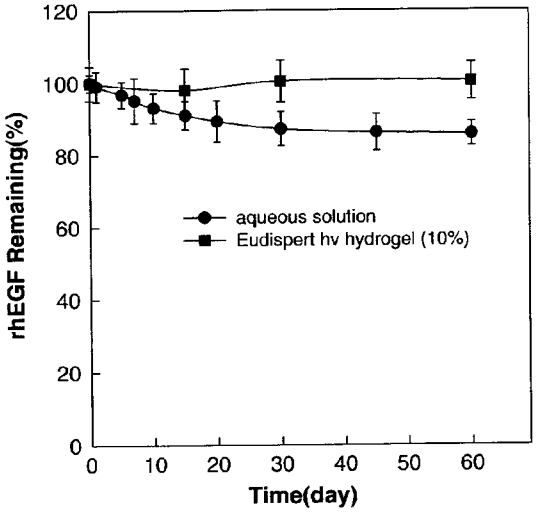


Fig. 5. Remaining amount of rhEGF in aqueous solution and in Eudispert hv hydrogel after two months at 4°C. Each point represents the mean ± S.D. (n=3).

was applied to open wound sites in the rat. Therefore, these results suggest that SLS may enhance the efficacy of rhEGF by inhibition of self aggregation and/or enzymatic degradation. Fig. 6 shows the light micrograph of buccal mucosa of a hamster untreated (a) and treated (b) with SLS (0.5%). After 24 h of treatment with Eudispert hv hydrogel (no rhEGF) containing SLS, the epidermis was slightly inflamed (Fig. 6(b)). But the morphological changes were not observed in the epithelium and connective tissue. We also didn't find any differences between normal mucosa and SLS treated mucosa to physical eyes. In irritant patch testing, Patil *et al.* (1994) reported that the concentration of SLS influences to a larger degree than the exposure time. However, further studies are needed about the cumulative effect of SLS with repeated administration.

Fig. 7 shows representative photographs of buccal mucosal ulcers induced by the acetic acid (a) and the

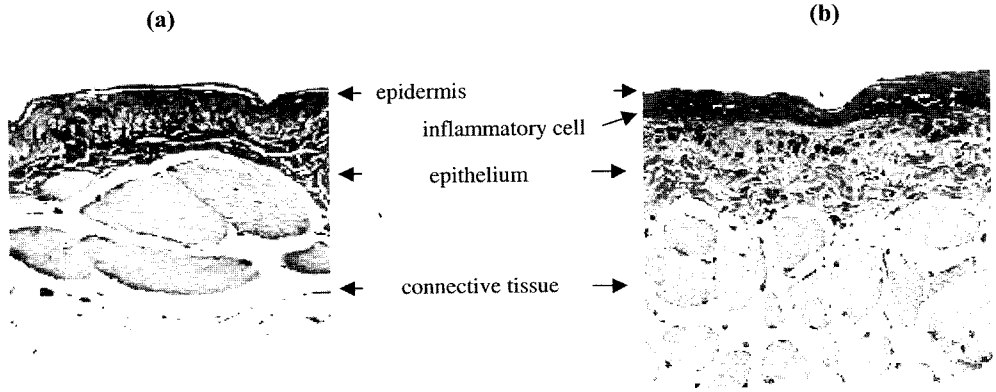
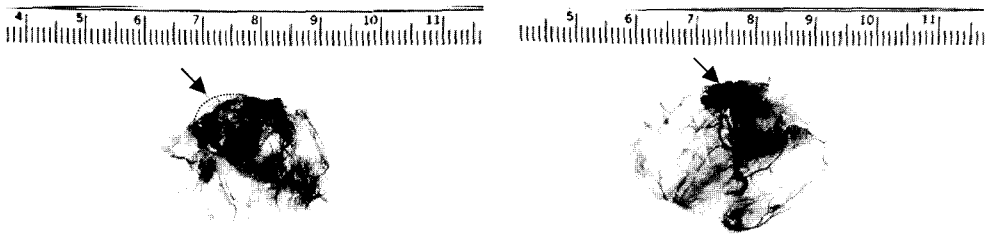


Fig. 6. Photomicrographs in the buccal mucosa of hamster untreated (a) and treated (b) with SLS (0.5%). After 24 h treatment with Eudispsrt hv hydrogel (no rhEGF) containing SLS (b), there were observed slight inflammation in the mucosal membrane and no morphological changes in the connective tissue. H & E, ×200.



(a) initial ulcer (b) after treatment of hydrogel

Fig. 7. Representative photographs of buccal mucosal ulcers (a) and the healing effect at 24 h after administration of Eudispert hv hydrogel containing rhEGF (b). Arrows indicate ulcer area.

healing effect after a single administration of Eudispert hv hydrogel containing 20 μg of rhEGF (b). Although rhEGF rapidly degraded in buccal mucosal homogenates (Fig. 1), the ulcer healing effect of rhEGF by Eudispert hv hydrogel preparation was very effective compared with the rhEGF solution. Moreover, by addition of SLS (0.5%) to Eudispert hv hydrogel, the curative ratio of rhEGF was increased significantly compared with the unadded control. These results may be attributed to the fact that Eudispert hv hydrogel may prolong the contact time between the dosage form and absorption site and SLS inhibits the proteolytic degradation of peptide in buccal mucosa.

CONCLUSIONS

At 24 h after administration of rhEGF/Eudispert hv hydrogel, the ulcer area was decreased 1.3 times compared with solution and the curative ratio was 36.83 ± 5.68%. The ulcer healing effect caused by hydrogel was 2.4 times greater than that caused by the rhEGF solution. By addition of SLS (0.5%) to Eudispert hv hydrogel, the curative ratio of rhEGF was increased significantly compared with the control. This result may be attributed to the fact that SLS inhibit the proteolytic degradation of peptide in buccal mucosa (Fig. 2). Moreover, Eudispert hv hydrogel may prolonged the contact time of a dosage form at the absorption site. Therefore, the Eudispert hv hydrogel formulation containing SLS would be a promising topical preparation for rhEGF in the treatment of buccal ulcer.

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