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Note

The healing effect of TGF-α on gastric ulcer induced by acetylsalicylic acid in rats

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Abstract

The present study was designed to investigate the effects of microemulsion and aqueous solution containing transforming growth factor alpha (TGF-α) and/or aprotinin administered intragastrically (i.g.) on healing of acute gastric ulcers induced by acetylsalicylic acid (ASA). The microemulsion was prepared by modification of the microemulsion formulation described in our previous study. Acute gastric lesions were induced by the application of ASA (150 mg/kg in 1.5 ml of 0.2 N HCl i.g.). TGF-α in solution or microemulsion formulations were administered at a dose of 10 μg/kg per 24 h i.g. for 2 days. The effects of TGF-α on the healing was evaluated with the measurement of ulcer score, basal gastric acid secretion, total protein content of gastric fluid, gastric mucus level and histological analysis. The results indicated that the highest decrease in ulcer area was observed in group treated with microemulsion containing TGF-α plus aprotinin (TA-ME). TGF-α in microemulsion formulation was more effective than TGF-α in solution formulation in the increase of gastric mucus secretion, in the decrease of gastric acid secretions and ulcer scores. Histological evaluation of the gastric mucosa samples revealed that, best recovery was obtained in the TA-ME treated group.

Keywords: Gastric ulcer; Acetylsalicylic acid; Transforming growth factor alpha; Aprotinin; Microemulsion; Intragastric administration

1. Introduction

The exposure of the gastric mucosa to damaging agents such as non-steroidal anti-inflammatory drugs (NSAIDs), ethanol, bile salts and hyperosmolar NaCl or stress may cause extensive injury to the surface epithelium, with loss of the continuity of this layer (Konturek, 1988; Şener-Muratoğlu et al., 2001). Acetylsalicylic acid (ASA) one of the most widely used non-steroidal anti-inflammatory drugs, damages gastrointestinal mucosa by irritant action, causing alterations of mucosal permeability and/or suppression of prostaglandin synthesis (Bennett and Schultz, 1993).

Wound healing is a complex biologic process that is well characterized at the microscopic level, but its regulation is poorly understood at the molecular level (Schultz et al., 1991). Understanding of this biological process of wound healing at general phases of inflammation, wound cell migration, mitosis and remodeling is based on the synthesis and release of several specific peptide growth factors at the site of injury (Konturek et al., 1992). Epidermal growth factor (EGF) and transforming growth factor alpha

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(TGF-α) play important roles in the natural mechanism of wound healing. These growth factors are homologous peptides produced in the gastrointestinal tract and show a similar spectrum of biologic activities (Konturek et al., 1995). TGF-α promotes the proliferation of gastric epithelial cells and inhibits acute gastric mucosal lesions induced by chemicals in normal and injured gastric mucosa. It has been shown that parenteral treatment with EGF or TGF-α, decreased dose-dependently acute mucosal damage induced by various strong irritants (Bagwe et al., 2001). TGF-α, like most other protein/peptide drugs, is clinically administered parenterally. Although various routes have been examined, the oral route seems a suitable alternative. The use of microemulsions is recently suggested as a drug carrier system for peptides. Microemulsions are drug delivery systems of peptides which can be administered orally, because of their improved drug solubilization, long shelf life, and ease of preparation and administration (Ogle et al., 1985). In our previous studies, the effects of intragastric (i.g.) administration of microemulsion formulation of EGF on the healing of acute gastric ulcers, induced by cold restraint stress in rats were investigated (Türkyılmaz et al., 1998; Çelebi et al., 2002). The present study was designed: (a) to investigate the effects of microemulsion and aqueous solution, containing TGF-α administered i.g. on healing of gastric ulcers induced by ASA, (b) to examine incorporation of aprotinin, which is an enzyme inhibitor, to microemulsion formulation and (c) to observe the positive effect on TGF-α's efficacy.

2. Materials and methods

2.1. Materials

Mouse TGF-α was purchased from Sigma (USA). Aprotinin (Trasylol 6128 KIU/mg) and ASA were donated by Bayer Türk Drug Manufacturer. Labrafil M 1944 CS (unsaturated polyglycolysed glycerides) was provided by Gattefossé® (France). Arlacel 186 (glycerolmonooleate–propylene glycol) and Brij 35 (polyoxyethylene lauryl ether) were produced by ICI Pharmaceuticals® (UK). Absolute alcohol was supplied by Riedel-de Haen (Germany). All other reagents for histological evaluation were of the best quality available.

2.2. Methods

2.2.1. Preparation and physical characteristics of the microemulsion

The w/o microemulsion was prepared by modifying the microemulsion formulation, which was described in our previous study (Türkyılmaz et al., 1998). Like the previous study, surfactant:co-surfactant ratio was chosen as 2.5, but the amount of surfactant and co-surfactant was used as low as possible in this study. Labrafil M 1944 CS, Arlacel 186: Brij 35 (5:1), absolute alcohol and distilled water were used as the oil phase, surfactant, co-surfactant and aqueous phase, respectively. TGF-α (10 μg/kg) was added into the water phase, aprotinin (3000 KIU/ml) was incorporated as an enzyme inhibitor in the microemulsion formulation.

The droplet size, viscosity, density, turbidity, refractive index, phase separation and pH measurements were performed to characterize the microemulsion. All physical characteristics of the microemulsion were measured when TGF-α and aprotinin were not present in the system. The physical stability of the microemulsion was determined under different storage conditions (4, 25 and 40 °C) during 12 months.

2.2.2. In vivo studies

2.2.2.1. Study design. Male albino Wistar rats weighing 200 ± 20 g were used throughout the study. They were divided into eight major groups, including 5–10 rats. Rats were fed with a standard diet and water ad lib. Acute gastric lesions were induced by i.g. administration of acidified ASA (150 mg/kg dissolved in 0.2 N HCl) to rats, which were fasted for 24 h before the experiments, but had free access to water. The following experiments were approved by the Ethics Commitee of Gazi University Medical Faculty, for the care and use of laboratory animals. The design of the experimental animal groups is shown in Table 1.

2.2.2.2. Determination of basal gastric acid secretion. In order to test the basal gastric acidity, the rats was anaesthetized at 90 min after ASA application, the pylorus was ligated and the stomach was cannulated. Firstly, the chymus pH was assessed (first pH) and then 1 ml of physiologic saline solution (PSS) was injected into the stomach. After 30 min, gastric content was collected, pH was assessed again (second pH) and

Table 1
Design of experimental animal groups

Code	Application
C	Healthy rats, control ($N = 7$)
ASA	Acetylsalicylic acid (150 mg/kg) suspended in 1.5 ml of 0.2 N HCl was administered intragastrically ($N = 5$)
ASA + UT	Untreated rats with ASA ulcer ($N = 5$)
SF	Physiologic saline solution without TGF-α was administered intragastrically for 2 days ($N = 10$)
T-SF	PSS containing TGF-α was administered at a dose of 10 μg/(kg day) intragastrically for 2 days ($N = 10$)
ME	Microemulsion without TGF-α was administered intragastrically for 2 days ($N = 10$)
T-ME	Microemulsion containing TGF-α was administered at a dose of 10 μg/(kg day) intragastrically for 2 days ($N = 10$)
A-ME	Microemulsion containing aprotinin (3000 KIU/ml) was administered intragastrically for 2 days ($N = 10$)
TA-ME	Microemulsion containing TGF-α (10 μg/(kg day)) and aprotinin (3000 KIU/ml) was administered intragastrically for 2 days ($N = 10$)

centrifuged at 1000 × *g* for 10 min to remove residual debris. Net volume of gastric fluid was measured and total acidity was determined by titration against 0.01 N NaOH to pH 7.0, and expressed as microequivalent H^+ per 30 min (μeq. H^+/30 min).

2.2.2.3. Determination of total protein content of gastric fluid. The total protein content of gastric fluid was determined by the method of Lowry et al. (1951). The centrifuged gastric fluid was mixed with sodium carbonate, sodium hydroxide and sodium tartrate, and finally hydrated with copper(II) sulphate solution. The reaction was terminated by adding a phenol reagent, and the absorbance was measured with a spectrophotometer (Schimadzu UV-1208) at 750 nm against its reagent blank.

2.2.2.4. Determination of ulcer score. The rats were killed under anesthesia (thiopental, 50 mg/kg), the stomach was removed and opened along the greater curvature. Lesion area was determined by measuring each lesion along its greatest diameter under a dissection microscope. Five such lesions were taken as the equivalent of a 1 mm^2 ulcer lesion (Ogle et al., 1985). The ulcer score was expressed as square millimeters.

2.2.2.5. Determination of gastric mucus. The stomach was opened along the lesser curvature, washed with saline, and weighted. The measurement of gastric mucus levels bound to the epithelial surface was performed as reported previously, using a spectrophotometer (Blandizzi et al., 1997). The amount of Alcian blue extracted per gram of wet glandular stomach was calculated from the standard curves.

2.2.2.6. Histological studies. Histological evaluation was carried out to determine the effects of i.g. TGF-α on damaged gastric mucosa. Tissue samples were prepared for transmission electron microscopy (TEM). The pieces were placed in 2.5% glutaraldehyde and then with phosphate buffer saline post fixing with 1% osmium tetraoxide. The specimens were first embedded in araldite–dodeceyl succinic anhydride (DDSA-CY 212; 1:1, v/v) overnight at room temperature, then at 24 h at 40 °C and 48 h at 60 °C. Thin sections were stained with lead citrate and uranyl acetate and were photographed using Carl Zeiss EM 900 electron microscope.

2.2.2.7. Statistical analysis. Results are given as mean ± S.E.M. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $P < 0.05$ were regarded as significant. '*n*' indicates the number of experiment.

3. Results and discussion

Preliminary reports pointed out that, studies are required to determine whether alterations in TGF and EGF levels in gastric mucosa under various physiopathologic conditions correlate with the degree of mucosal damage (Konturek et al., 1992; Konturek et al., 1997). It was reported that, the gastric ulcer healing effect of a single subcutaneous (s.c.) administration of rhEGF microspheres was increased 1.44-fold compared with s.c. administration of rhEGF loaded microspheres twice a day. It may be due to optimized osmotic pressure, high encapsulation ef-

ficiency and sustained release pattern (Han et al., 2001). It was also reported that, the EGF formulation in microemulsion was more effective than the solution form in gastric ulcer healing induced by cold strain stress model (Çelebi et al., 2002; Akbulut et al., 2002).

In the present study microemulsion and aqueous solution of TGF-α and/or aprotinin were used for the treatment of gastric ulcers induced by i.g. acidified ASA application, because TGF-α can be affected by the acid pepsin of the gastric juice, causing two- to five-fold loss of biological activity (Marchbank et al., 2002).

3.1. Physical characteristics of the microemulsion

The mean droplet size of the microemulsion was 4.9 ± 1.9 nm. The results indicate that the physical characteristics, such as viscosity, density, turbidity, refractive index, phase separation, pH and droplet size of the modified microemulsion formulation did not change under different storage temperatures (4, 25 and 40 °C) during 12 months.

The functional mechanisms of chronic gastric mucosal protection includes increased mucus secretion, cell proliferation, mucosal barrier stabilization and decreased acid secretion (Szabo and German, 1987).Thus, we examined the effects of TGF-α formulation by ulcer score, basal gastric acid secretion, total protein content of gastric fluid, gastric mucus level and histological evaluation.

3.2. Effects of TGF-α on basal gastric acid secretion

Konturek et al. (1992) reported that TGF and EGF administered i.g., was found failed to affect gastric acid secretion or to prevent the mucosal damage, suggesting that these peptides are unable to influence the mucosal integrity. In the present study, the formulations of TGF-α showed different effects on gastric acid secretions.

The effects of i.g. administration of TGF-α solution or microemulsion formulations on basal gastric acid secretion of gastric ulcer induced by ASA in rats are shown in Fig. 1. The gastric acid secretions of groups which were treated by microemulsions containing aprotinin (A-ME) or TGF-α (T-ME) or TGF-α plus aprotinin (TA-ME), were significantly lower than those of the microemulsion treated group (ME). The results of those are 38.1 ± 4.6 μeq. H^+/30 min, 30.0 ± 1.7 μeq. H^+/30 min, 20.8 ± 1.3 μeq. H^+/30 min and 54.4 ± 3.5 μeq. H^+/30 min, respectively ($P < 0.01$, <0.001, <0.001). The gastric acid secretion was significantly reduced after intragastric administration of TGF-α solution (T-SF) compared to its control group (SF), at 42.9±2.6 μeq. H^+/30 min and 57.2±3.9 μeq. H^+/30 min, respectively ($P < 0.05$).

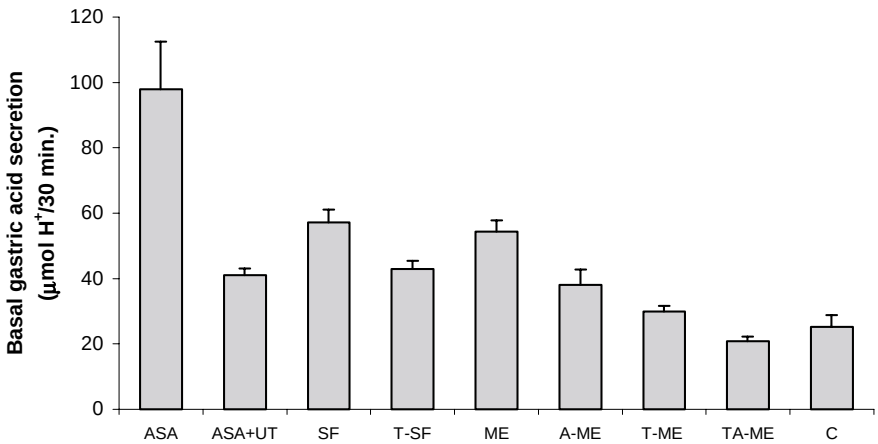

Fig. 1. Basal gastric acid secretion in different experimental groups. Data shown are mean ± S.E.M. of 5–10 rats. $P < 0.05$ for T-SF/SF, A-ME/C; $P < 0.01$ for T-ME/T-SF, T-SF/C, A-ME/ME; $P < 0.001$ for TA-ME/ASA, TA-ME/ASA-UT, TA-ME/ME, T-ME/ASA, T-ME/ME, T-SF/ASA, A-ME/ASA, A-ME/TA-ME.

The basal gastric acid secretion obtained by i.g. administration of TGF-α microemulsion was found lower than that of TGF-α solution ($P < 0.01$). Thus, the microemulsion formulation can be considered more effective than solution of TGF-α.

On the other hand, the group which was treated with the microemulsion containing TGF-α plus aprotinin (TA-ME) had the lowest basal gastric acid secretion level (20.8 ± 1.3 μeq. H^+/30 min). Only this group's basal gastric acid level decreased when compared to the untreated (ASA + UT) group's level ($P < 0.001$).

Based on the evaluation of the basal gastric secretion, the gastric pH measured (fasting pH) in the collected gastric juice, increased significantly in TAME (pH 3.65 ± 0.15) and TME groups (pH 3.55 ± 0.20), compared to the control group (pH 2.57 ± 0.28) ($P < 0.01$, <0.05).

3.3. Effects of TGF-α on total protein content of gastric fluid

The effects of TGF-α in solution or in microemulsion formulations, on total protein content of the gastric fluid with gastric ulcer induced by ASA in rats, are shown in Fig. 2. The total protein content of gastric fluid in fasted T-ME and TA-ME groups decreased remarkably, when compared with the ME group, at 2.12 ± 0.18 mg protein/ml, 1.82 ± 0.18 mg protein/ml and 3.87 ± 0.47 mg protein/ml, respectively ($P < 0.001$). In contrast, there was no significant reduction in total protein content of gastric fluid in the T-SF and A-ME groups, compared to SF and ME groups at 2.83 ± 0.14 mg protein/ml, 2.75 ± 0.26 mg protein/ml, 3.52 ± 0.31 mg protein/ml and 3.87 ± 0.47 mg protein/ml, respectively ($P > 0.05$). In addition, the total protein content of gastric fluid of TA-ME group had a remarkable reduction, compared with ASA-UT group ($P < 0.05$). On the other hand, the difference between total protein content of gastric fluid measured at T-ME and T-SF groups was not statistically significant ($P > 0.05$).

Marchbank et al. (2002) reported that TGF-α (1–50) is cleaved to TGF-α (1–43) by acid pepsin and this is the predominant form in normal gastric juice. Neutralization by i.g. or taking acid suppressants, caused the predominant form to be TGF-α (1–50). TGF-α (1–43) reduced gastric ulcer area in rat, induced with indomethacin by 19% whereas, TGF-α (1–50) reduced area by 62%.

In the present study reduced total protein content and ulcer area in TA-ME group may have relevance to the actions of new formulation.

3.4. Effects of TGF-α on ulcer score

The gastropathy associated with the ingestion of non-steroidal anti-inflammatory drugs such as aspirin, is a common side effect of this class of drugs

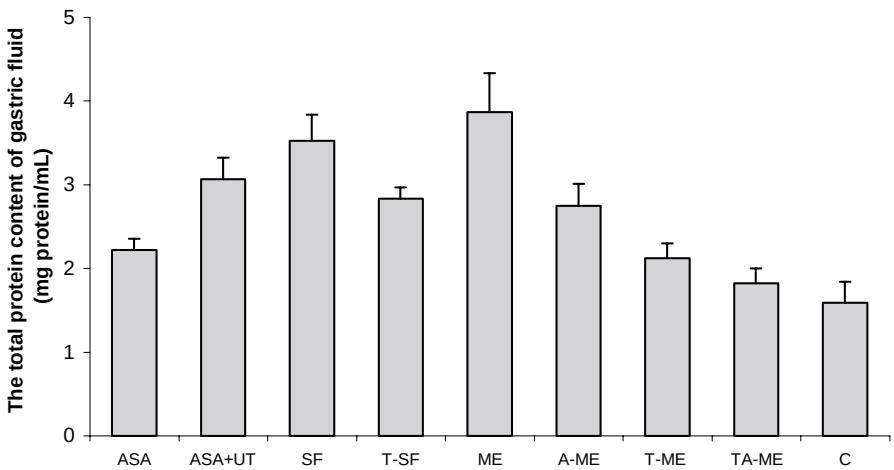

Fig. 2. The total protein content of gastric fluid in different experimental groups. Data shown are mean ± S.E.M. of 5–10 rats. $P < 0.05$ for TA-ME/ASA-UT; $P < 0.001$ for TA-ME/ME, T-ME/ME.

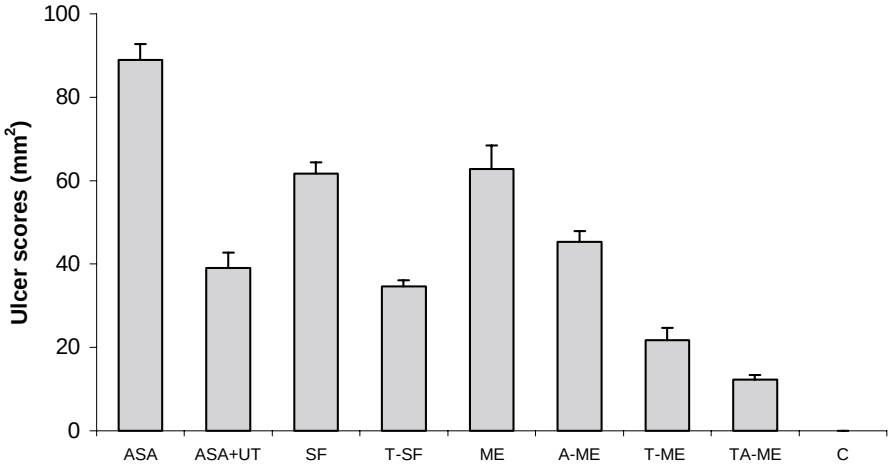

Fig. 3. Ulcer scores in different experimental groups. Data shown are mean ± S.E.M. of 5–10 rats. $P < 0.01$ for T-ME/ASA-UT, T-ME/T-SF, A-ME/ME; $P < 0.001$ for TA-ME/ASA, TA-ME/ASA-UT, TA-ME/ME, T-ME/ASA, T-ME/ME, T-SF/ASA, T-SF/SF, A-ME/ASA, A-ME/T-ME, A-ME/TA-ME.

(Konturek et al., 1994). Animal studies then confirmed the mechanisms of induction of the NSAID ulcer. These models have helped to answer key questions, which lead to our current knowledge of NSAID damage (Lee, 2000).

The effects of TGF-α in solution or microemulsion formulations on ulcer score in rats are shown in Fig. 3. The i.g. administration of ASA (150 mg/kg) produced gastric ulcers in all tested animals with an average initial area of $89.0 \pm 3.8\,mm^2$. The ulcer scores of groups treated with TGF-α and/or aprotinin in microemulsion or solution (A-ME, T-ME, TA-ME and T-SF) were calculated as $45.3 \pm 2.7\,mm^2$, $21.7 \pm 3.0\,mm^2$, $12.2 \pm 1.2\,mm^2$ and $34.7 \pm 1.5\,mm^2$, respectively. The ulcer scores of the untreated groups (ASA-UT) or treated groups with vehicles (SF and ME) were

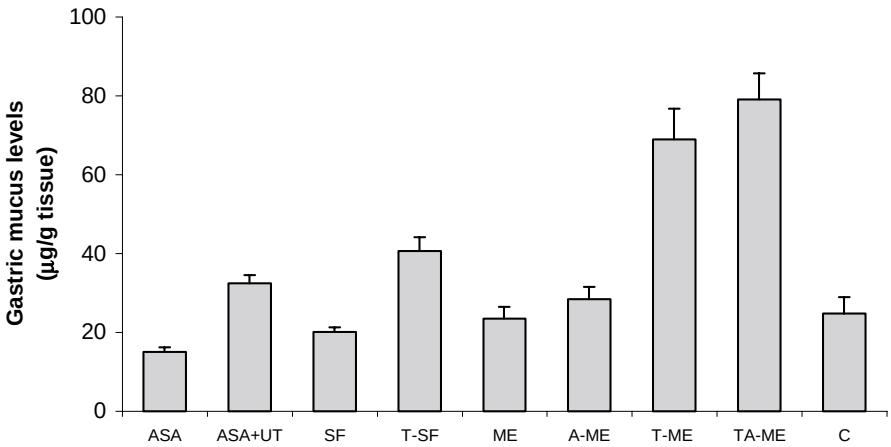

Fig. 4. Gastric mucus levels in different experimental groups. Data shown are mean ± S.E.M. of 5–10 rats. $P < 0.05$ for T-SF/SF; $P < 0.01$ for T-ME/T-SF, T-SF/ASA, A-ME/T-ME $P < 0.001$ for TA-ME/ASA, TA-ME/ASA-UT, TA-ME/ME, T-ME/ASA, T-ME/ASA-UT, T-ME/ME, A-ME/TA-ME.

determined as $39.0 \pm 3.7\,mm^2$, $61.6 \pm 2.7\,mm^2$ and $62.8 \pm 5.6\,mm^2$. As shown in results, gastric ulcer was significantly decreased in 4 days after the ulcer induction. The mean ulcer areas in all treated groups with TGF-α (T-ME, TA-ME and T-SF) reduced significantly, compared to their control groups (ME and SF) ($P < 0.001$). The results indicate that, the ulcer score decreased significantly in T-ME and TA-ME groups ($P < 0.001$), but there was no significant decrease in T-SF and A-ME groups ($P > 0.05$) compared to untreated group (ASA + UT). Furthermore, the i.g. administration of TGF-α in microemulsion was more effective than TGF-α aqueous solution in the healing of gastric ulcer ($P < 0.01$). On the other hand, the highest decrease in ulcer area was observed in group treated with the microemulsion containing TGF-α plus aprotinin.

3.5. Effects of TGF-α on gastric mucus level

Growth factors in ulcer healing has some effects on gastric secretion as decreases in acid secretion and increases in mucus levels (Szabo and Vincze, 2000). Both EGF and TGF-α exert their effects by interaction with a specific cell membrane receptor (Milani and Calabro, 2001). TGF-α appears to be the primary physiological ligand for the EGF receptor (EGFR) (Alison and Sarraf, 1994). Since both EGF and TGF-α promote cell proliferation, stimulate cell migration and inhibit gastric acid secretion, it is likely that these

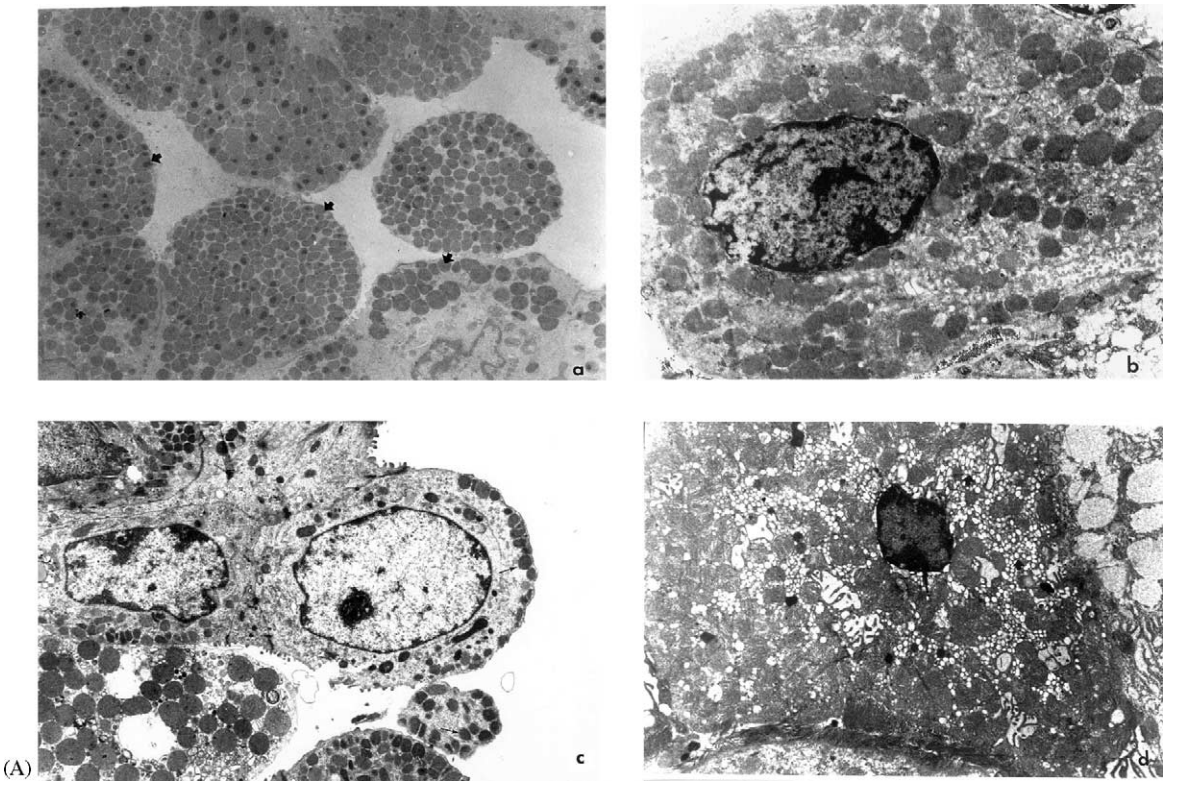

Fig. 5. (A) Transmission electron microscopic appearance of gastric mucosa in control and ASA groups (uranyl acetate–lead citrate, 9000×). Control group: (a) mucus granules (thick aurous); (b) normal appearance of parietal cells. ASA group: (c) mucus granules (thick aurous) broken intracytoplasmic junction (thick aurous); (d) active appearance of parietal cells with lots of intracytoplasmic canalicules (thick aurous). (B) Transmission electron microscopic appearance of gastric mucosa in AME, T-ME and TA-ME groups (uranyl acetate–lead citrate, 9000×). AME group: (e) mucus granules (thick aurous) broken intracytoplasmic granules (the aurous); (f) degenerative areas in cytoplasm (thick aurous). TME group: (g) normal appearance of mucus cells (thick aurous); (h) normal appearance of parietal cells (P). TAME group: (i) normal mucus cells (thick aurous); (j) normal parietal cells (P).

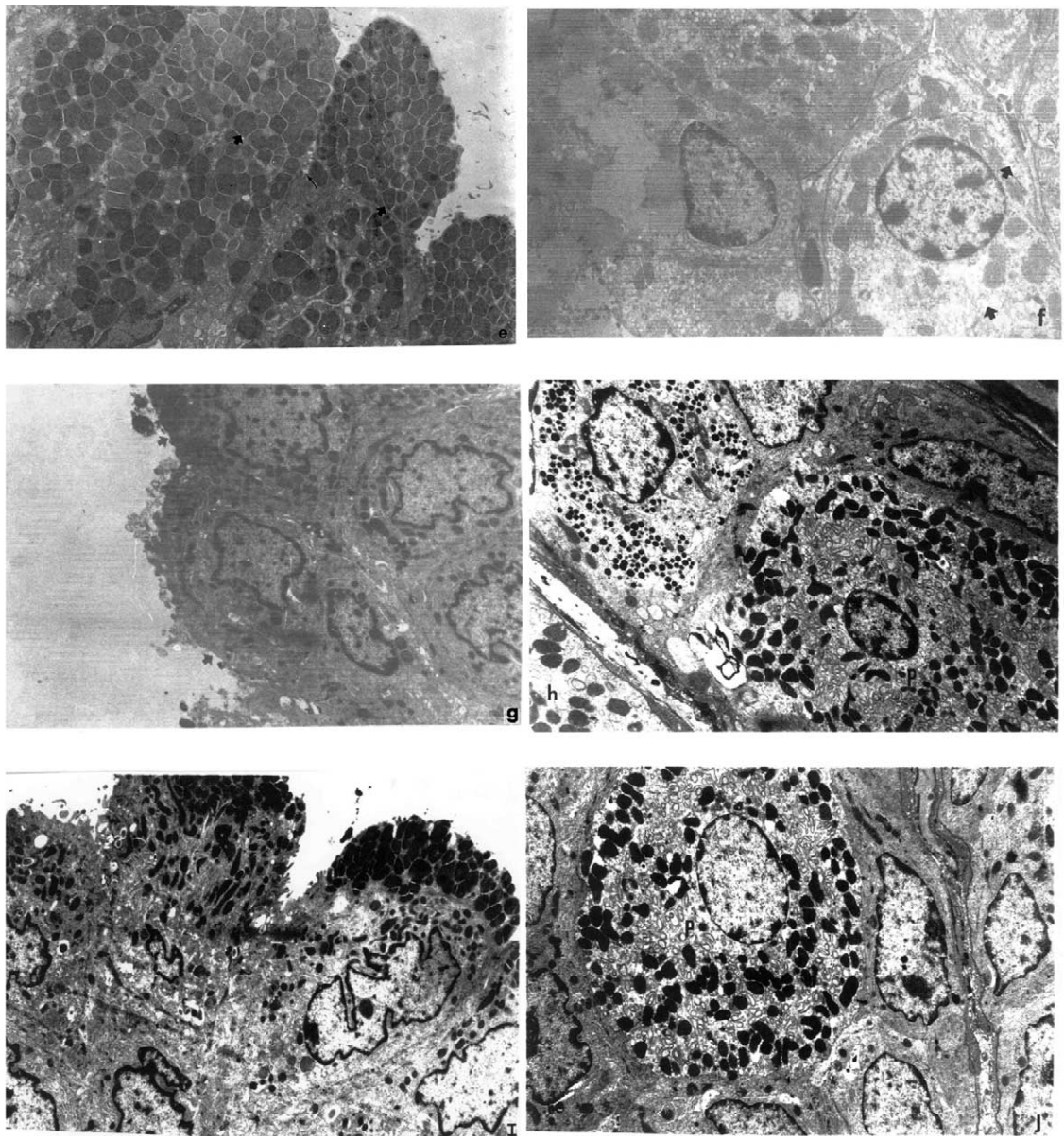

Fig. 5. (*Continued*).

two growth factors play a pivotal role in ulcer healing (Konturek et al., 1994; Alison and Sarraf, 1994). Higher mucus levels were reported in the gastric ulcer area by i.g. EGF formulation applications (Çelebi et al., 2002; Akbulut et al., 2002).

The effects of intragastric TGF-α in solution or in microemulsion formulations on gastric mucus level, gastric ulcer induced by ASA in rats are shown in Fig. 4. The gastric mucus levels increased remarkably in groups that were treated with TGF-α in microemulsion (T-ME and TA-ME), when compared to the control group (ME) ($P < 0.001$). However, gastric mucus levels did not show significant increases in the A-ME group. The mucus levels were measured as

79.1 ± 6.6 μg/g, 68.9 ± 7.8 μg/g, 28.4 ± 3.2 μg/g and 23.6 ± 3.0 μg/g tissue in TA-ME, T-ME, A-ME and ME groups, respectively. The effect of TSF group on gastric mucus secretion was significantly higher than its control group (SF) ($P < 0.05$) when compared to ASA + UT group. There was no difference between these mucus levels ($P > 0.05$). In contrary, the gastric mucus levels of TA-ME and T-ME treated groups were found to be significantly higher than ASA + UT group ($P < 0.001$).

Just like the other results in the decrease of gastric acid secretions and ulcer scores, TGF-α in microemulsion formulation was more effective than TGF-α in solution formulation in the increase of gastric mucus secretion.

3.6. Evaluation of histological studies

At the end of histological studies, surface mucous cells and parietal cells were evaluated (Fig. 5A and B). The results of TEM indicated that, exception of TME, TAME and control groups, others showed variety of degenerative changes in surface mucous epithelia which are the deformations of connected units, separation in surface cells membranes, electron pale mucous granules or their decreasing in number. Besides, enlargement in connected units of degranulation is observed. On the other hand, parietal cells showed some degenerative changes too. They had activated appearance and their intracytoplasmic canalicules are increased. Moreover, opening between the inner and outer nucleus membrane was clear. They had enlarged degenerative cytoplasmic areas and crystolysrs in the mitochandrions were also observed. As shown in Fig. 5B in TME and specially TAME groups, the cells were absolutely in normal structure as the control groups (Fig. 5B).

As a conclusion of histological experiments showed that, the microemulsion containing TGF-α stomach ulcer wounds were increasingly healed. According to the results of biochemical analysis and histological evaluation of TA-ME formulation is found to be more effective then the other formulations.

These results suggest that protease inhibitor aprotinin and ME might usefully improve the absorption and the extension of half-life of TGF-α as previously mentioned by other research groups (Trenktrog et al., 1995; Tozaki et al., 1998; Anderle et al., 2002).

4. Conclusion

These findings suggest that TGF-α incorporated with aprotinin into microemulsion formulation was the most effective one on acidified ASA induced gastric ulcer treatment.

Acknowledgements

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