Amino Acids (2002) 22: 187–198

Amino
Acids
© Springer-Verlag 2002
Printed in Austria

An investigation on skin wound healing in mice with a taurine-chitosan gel formulation

Z. Değim[1], **N. Çelebi**[1], **H. Sayan**[2], **A. Babül**[2], **D. Erdoğan**[3], and **G. Take**[3]

[1] Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, Ankara,
[2] Department of Physiology, Faculty of Medicine, Gazi University, Ankara, and
[3] Department of Histology, Faculty of Medicine, Gazi University, Ankara, Turkey

Accepted June 4, 2001

Summary. The process of wound healing begins immediately following surface lesions or just after exposure to radiation, chemical agents or extreme temperatures.

Taurine (2-aminoethane sulfonic acid), an amino acid containing sulfur, is found in almost all tissues in mammals, playing various important physiological roles in each organ. Taurine exhibits an antioxidant effect and is also known to have effects on cell proliferation, inflammation and collagenogenesis. Many antioxidants have been used to eliminate the negative effects of oxygen free radicals on wound healing.

The objective of the present study was to examine the wound healing effect in mice of taurine-chitosan gel, which releases taurine slowly over a long time period. Fifty mM of taurine in 1.5% chitosan polymer (TAU-GEL) and 1.5% chitosan polymer (CHI-GEL) were applied to full thickness skin wounds of mice once a day for seven days. After seven days of treatment, lipid peroxide formation-malondialdehyde (MDA) and hydroxyproline (HPX) levels and the tensile strength of wound tissues were measured. All results were compared with those of the untreated control group (CONT). The structural alterations in the skin layers were also histologically investigated.

It was found that locally administered TAU-GEL form significantly increased wound tensile strength by decreasing the MDA and increasing HPX levels. These results were supported by histological findings. All observations suggest that taurine gel may be effective in wound healing.

Keywords: Amino acids – Chitosan – Gel – Hydroxyproline – Malondialdehyde – Taurine – Tensile strength – Wound healing

Introduction

Wound healing is a localized biological event that progresses through three general phases of inflammation: wound cell migration, mitosis, and extracellu-

lar matrix production and remodelling. The process of wound healing begins immediately following surface lesions or when skin proteins become exposed to radiation, chemical damage or extreme temperatures (Schultz et al., 1991; Dijke and Iwata, 1989).

It has been reported previously that free radicals might affect wound healing and play an important role in collagen damage. Many antioxidants are used to eliminate the negative effects of oxygen free radicals on wound healing (Foschi et al., 1988; White and Heckler, 1990).

Taurine (2-aminoethane sulfonic acid), an amino acid containing sulfur, is found in all tissues in mammals and biological fluids in other animals, and is often present in high (mM) concentrations in the intercellular region. Taurine has some antioxidant effects on cell proliferation, inflammation and collagenogenesis. Taurine has also been used to prevent oxidant damage in many tissues and on incisional skin wounds (Stewart et al., 1989; Högstrom, 1987; Bergren et al., 1988; Foschi et al., 1988). The effect on skin wound healing of taurine – chitosan gel, which releases taurine slowly, has not been investigated before.

Recent reports have shown that chitosan, a polyglucosamine, suppresses or decreases the amount of fibroplasias when applied to fresh wounds. It has been reported that chitosan reduces exophytic callus in bone repair and allows the growth of vascular grafts. It permits regeneration of normal tissue elements in skin wounds with minimal scar formation (Bartone and Adickes, 1988; Malette et al., 1983).

The purpose of this study was to prepare a TAU-GEL dosage form that releases taurine slowly and to compare its effect on the skin wound healing with the CHI-GEL and CONT groups. Another aim was to determine the role of taurine on incisional skin wounds, by investigating the effect of taurine on lipid peroxide formation (MDA), hydroxyproline levels and wound tensile strength.

Materials and methods

Materials

Taurine (2-aminoethane sulfonic acid) was purchased from ICN Biomedicals Inc., USA. Chitosan – H was kindly provided from Dainichiseika Color & Chemicals Mfg, Co. Ltd., Japan. Ketamine HCl was purchased from Eczacibaşi ilaç San., Turkey.

Methods

Preparation of TAU-GEL: 50 mM taurine gel dosage form was prepared in a chitosan gel (1.5%). Preparation of the gel dosage form of chitosan (CHI-GEL) (1.5%) was carried out as follows. A weighed amount of polymer was carefully added to the required amount of 1% acetic acid. It was gently stirred for 1 minute and then it was kept at room temperature overnight before application. The pH of the gel was 4.5.

The TAU-GEL dosage form was prepared by adding taurine (50 mM) to the gel form. These dosage forms were divided into small portions and stored at 4°C before daily applications.

In vivo experiments: This study was performed on 22 male Swiss Albino mice*, weighing 30.0 ± 1.0 g. They were fasted for 18 hours before operations; however, during the experiment they consumed water and food adlibitum. The mice were anesthetized by an intraperitoneal injection of ketamine HCl (35 mg/kg). Their backs were shaved and cleaned with merbromine solution. Two linear, full thickness surgical wounds 2.5 cm long were produced on both sides of the spine (parallel to each other, 1.5 cm from the spine). The wounds were closed with discontinuous silk sutures (5/0) at 0.5 cm intervals.

The mice were divided into three groups:

a) Chitosan-gel group [CHI-GEL]: 20 μL of chitosan gel without taurine was locally applied to the skin incision wounds, once a day for 7 days.
b) Experimental group (Taurine-chitosan gel group) [TAU-GEL]: 50 mM taurine in 1.5% chitosan gel (20 μL) was locally applied to full – thickness skin wounds, once a day for 7 days.
c) Control group [CONT]: Untreated mice.

The animals were sacrificed with an overdose of anesthetic on the 7th day of the study. Tissue specimens from the wounds were excised and an aliquot specimen was used to test wound tear strength. The other part was frozen at −40°C for measurement of malondialdehyde (MDA) and hydroxyproline (HPX) levels.

Measurement of wound tensile strength: Full thickness skill flaps (5 × 5 mm portions) were dissected for the mechanical tests. The wound tear strength was determined with a grass model 7 Polygraph (Grass force displacement transducer FT 03) (Çelebi et al., 1994).

Malondialdehyde content of the skin: Tissue MDA levels were estimated by the method of Casini (Casini et al., 1986). The tissue samples were weighed and sealed in test tubes and 10% ice-cold trichloroacetic acid was added (1 g tissue plus 10 mL TCA 10%). This mixture was homogenized and centrifuged for 15 minutes at 3,000 g. 1.5 mL of the supernatant was put into microcentrifuge tubes and centrifuged at 3,000 g for 8 minutes at room temperature. 750 μL supernatant was taken and added to an equal volume of 0.67% (w/v) thiobarbituric acid. It was boiled for 15 minutes and the absorbance was recorded at 535 nm for each sample. Lipid peroxidation levels were expressed in terms of MDA by the extinction coefficient of $1.5 \times 10^5\,mol^{-1}\,cm^{-1}$.

Measurement of hydroxyproline (HPX) levels: HPX levels were measured by the method described by Woessner (Woessner et al., 1961). The weighed samples were sealed in small Pyrex test tubes and hydrolyzed for 3 hours at 130°C by adding 5 mL of 6 N HCL. The hydrolysates were neutralized by 2.5 N NaOH. HPX oxidation was initiated by adding 1 mL chloramine T and the tube contents were kept at room temperature for 20 minutes. Chloramine T was then destroyed by adding 1 mL 3.15 M perchloric acid. After 5 minutes, 1 mL of Ehrlich's reagent was finally added; the mixture was shaken, and the tubes were placed in a 60°C water bath for 20 minutes. They were then cooled in tap water for 5 minutes. The absorbance values of the solutions were determined at 557 nm. The HPX values were calculated from the L – hydroxyproline standard curve.

Histological investigations: In order to compare the histological effects on the TAU-GEL, CHI-GEL and CONT groups, structural changes in the wound areas were examined by transmission electron microscope (TEM) (EM900 Carl Zeiss). Tissues were fixed in phosphate-buffered containing 2.5% gluteraldehyde for 2 hours; then they were post-fixed in 1% osmium tetraoxide (OsO_4) and dehydrated in a series of graded alcohols.

---

*The study protocol describing how the animals were to be used was approved by the Ethics Committee for Care and Use of Laboratory Animals of Gazi University Medical School.

After passing through propylene oxide, the specimens were embedded in Araldyt CY212, 2-dodecen-1-yl succinic anhydride (DDSA) and benzyldimethyl amine (BDMA). Semithin sections were cut perpendicular to the superficial surface of the wound skin and stained with toluidin blue and examined with a BH_2 Olympus light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an EM900 Carl Zeiss transmission electron microscope (TEM).

Statistics: All data were expressed as mean ± standard deviation. The differences between groups were analyzed by one way ANOVA test. $p < 0.05$ was considered to be significant.

Results

Figure 1 shows the tensile strength, which was found to be highest in the TAU-GEL group ($p < 0.05$).

Tissue MDA and HPX levels are shown in Figs. 2 and 3, respectively.

Tissue MDA levels in the TAU-GEL group were significantly lower than those in the CHI-GEL and CONT groups ($p < 0.05$).

HPX levels in the wounds of the TAU-GEL group was found to be higher than those of the CHI-GEL and CONT groups ($p < 0.05$).

Histological findings

In the CHI-GEL group, epithelial regeneration was observed around the incision when wound tissue was investigated by semithin sectional preparation under a light microscope seven days after application.

In particular, the basal layer epithelium was observed to have proliferated and scar tissue was identified at the surface (Fig. 4a). The TAU-GEL group was investigated by semithin sectional preparation under a light microscope. It was noted that epithelium development was completed and a regular structure was observed in the dermis (Fig. 4b).

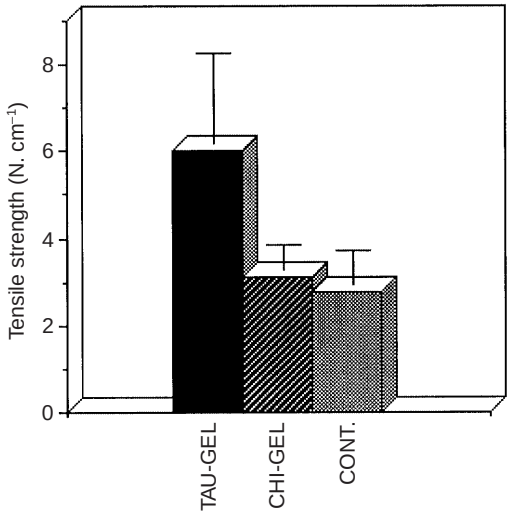

Fig. 1. Tensile strength (X ± SD) of the skin wounds of the TAU-GEL group (n = 11), CHI-GEL group (n = 11) and CONT group (n = 9); $p < 0.05$

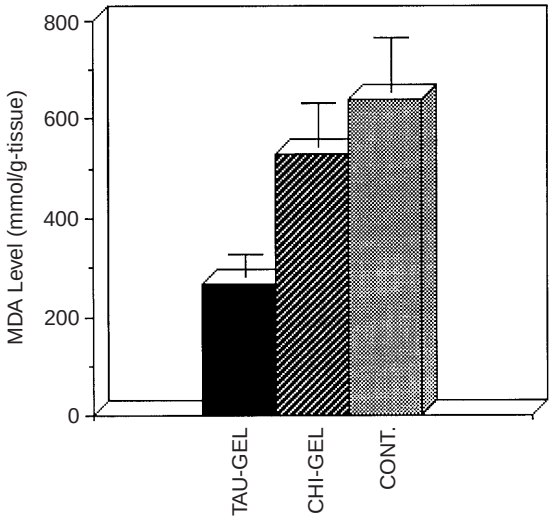

Fig. 2. MDA levels (X ± SD) of the skin wounds of the TAU-GEL group (n = 14), CHI-GEL group (n = 16) and CONT (n = 16); $p < 0.05$

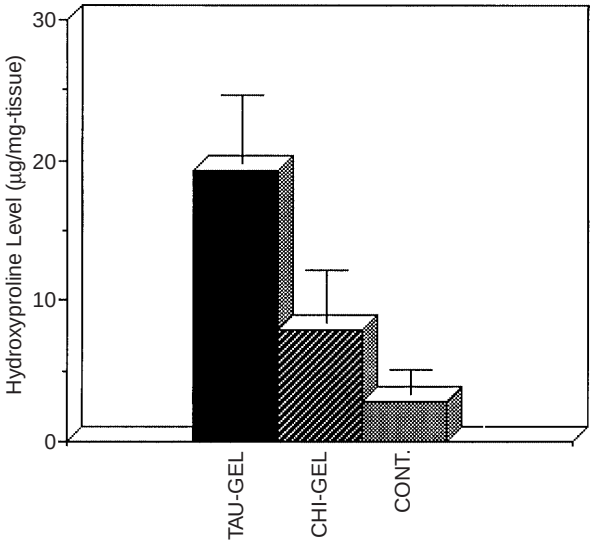

Fig. 3. Hydroxyproline levels (X ± SD) of the skin wounds of the TAU-GEL group (n = 14), CHI-GEL group (n = 16) and CONT group (n = 16); $p < 0.05$

When wound tissue was investigated by ultrathin sectional preparation under a transmission electron microscope seven days after CHI-GEL application in the same area, the basal cell layer was observed to be intensely colored and the nuclei were found to be heterochromatic. An abundance of free ribosomes was also noted. Weakly colored spinosum layer cells were identified. There were some free ribosomes and tonofilaments in the cell cytoplasm, and some desmosomes at the sides of cell walls were also observed. The basal lamina appeared as a thin line. Fibroblasts were identified and their nuclei

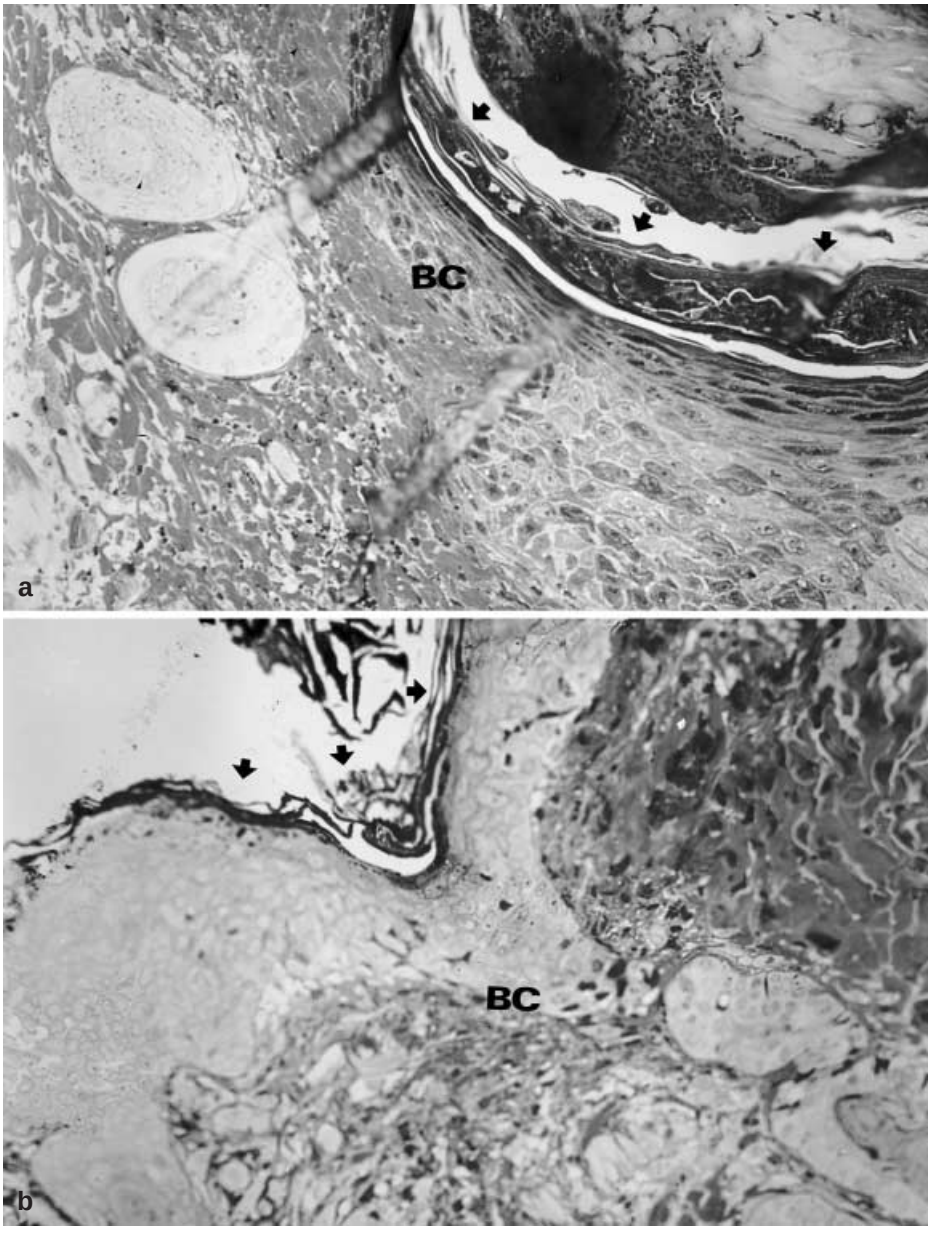

Fig. 4. **a** Semithin sectional image of the CHI-GEL group seven days after application under light microscope: Wound area (arrows), basal layer cells (*BC*). (Toluidine blue × 100). **b** Semithin sectional image of the TAU-GEL group 7 days treatment under light microscope: Wound area (*arrows*), basal layer cells (*BC*). (Toluidine blue × 100)

were observed to be intensely colored. Some young and irregular collagenous fibers were also significant (Fig. 5a,b).

When the TAU-GEL group was investigated under a transmission electron microscope, the basal dermis was observed to be more differentiated than the dermis of the other groups. The nuclei, organelles and tonofilaments of the basal cells were found to be normal, while the basal lamina was ob-

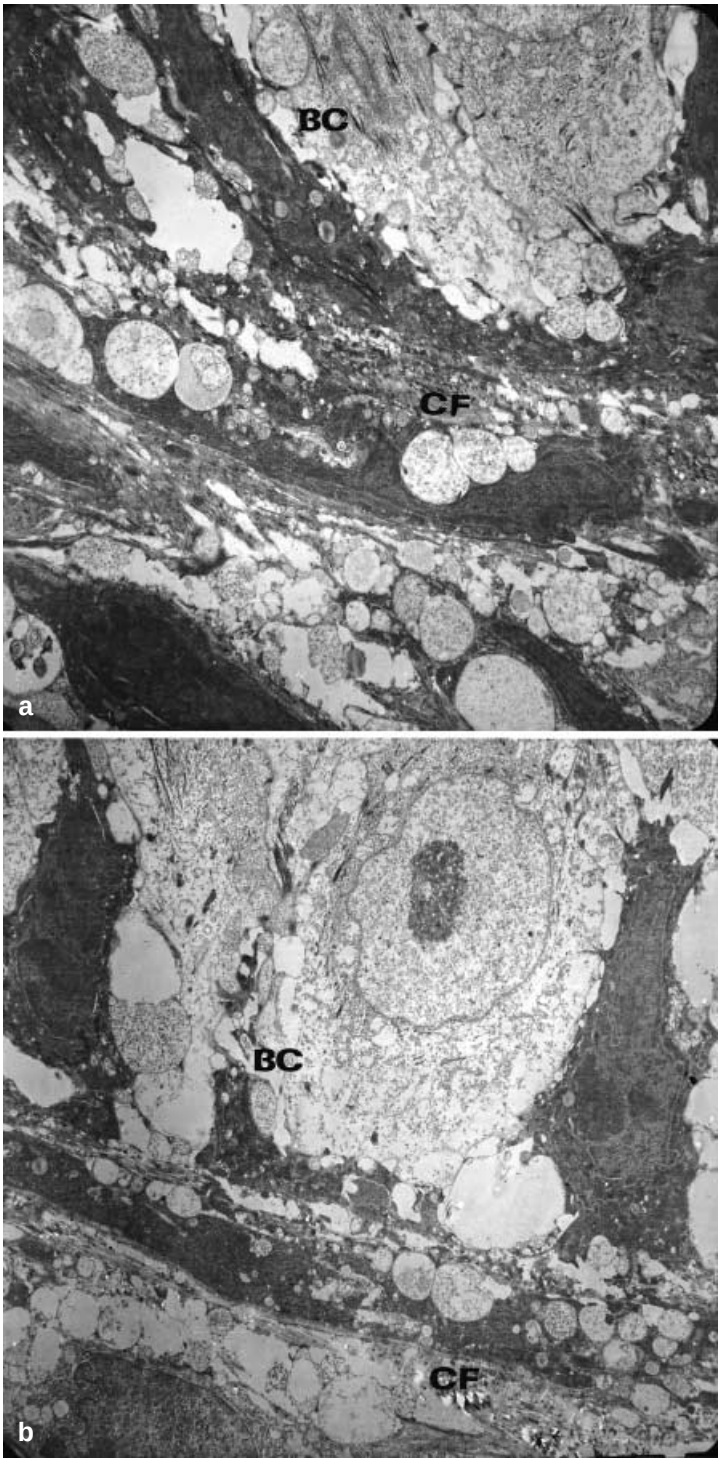

Fig. 5. **a** Image of the wound area (CHI-GEL group) under transmission electron microscope: Basal layer cells (*BC*), collagenous fibers (*CF*). (Uranyl acetate and lead citrate × 9,000). **b** Another image of the same group (CHI-GEL group) under transmission electron microscope: Basal layer cells (*BC*), some collagenous fibers in dermis (*CF*). (Uranyl acetate and lead citrate × 9,000)

served to be well developed. In fibroblasts, GER tubules were observed larger. It was observed that synthesis of collagenous fibers had started at the edge of the fibroblasts, but these fibers seemed to be irregular in appearance (Fig. 6a,b)

The results show that the wound tensile strength of mice was significantly higher on the 7th day of treatment in the TAU-GEL group than in the CHI-GEL and CONT groups. The mean MDA level in TAU-GEL group was significantly lower than that of the CHI-GEL and CONT groups. We also observed that the HPX levels in the TAU-GEL group were higher than those in the CHI-GEL and CONT groups. Tensile strength, MDA and HPX findings were supported by these histological observations. There were significant differences between the tensile strength, the MDA and HPX levels in TAU-GEL group compared with those in the CHI-GEL and CONT groups ($p < 0.05$).

Discussion

It is suggested that taurine acts as a general detoxifier for oxidizing compounds, reacting with and removing chemically active free radical species. These species are generated at high rates in certain organs and cells, such as the heart, retina and neutrophils, and can cause extensive chemical damage to lipids, proteins, carbohydrates, and nucleic acids. Taurine is present in high concentrations in these cells and tissues (Laidlaw et al., 1989) and is considered to play important physiological roles in each organ.

It has been reported that taurine enhances cell proliferation (Hunter, 1985) and viability (Pasantes-Morales et al., 1984) and that this in turn affects inflammation and collagenogenesis (Gordon et al., 1986; Wang et al., 1989). It has also been shown that taurine has antioxidant effects (Alvarez et al., 1983; Pasantes-Morales et al., 1985). The inflammatory cells produce oxygen free radicals in many tissues. It has been shown that oxygen free radicals cause serious cellular damage by inducing lipid peroxidation. These compounds, acting directly or indirectly through derived noxious agents such as hydrogen peroxide, hypochloric acid or the singlet oxygen, induce tissue necrosis (Foschi et al., 1988).

The effects of free radicals on wound healing have been investigated and clarified (White and Heckler, 1990). These studies suggest that free radicals play an important role in collagen damage and in the early reduction of wound margin strength (Högstrom, 1987; Bergren et al., 1988; Foschi et al., 1988). Some compounds, such as histamine, that destroy membrane fluidity and integrity may be released by free radicals. As a direct antioxidant, taurine significantly reduces lipid peroxidation, and as an indirect antioxidant, it acts to stabilize the plasma membrane (Nakashima et al., 1982; Pasantes-Morales and Cruz, 1985). In the present study, the effect of taurine on lipid peroxidation in wound tissue was determined with MDA production. In the literature it was reported that MDA is a biochemical marker of lipid peroxidation (Huxtable, 1992). It was found that taurine treatment caused a

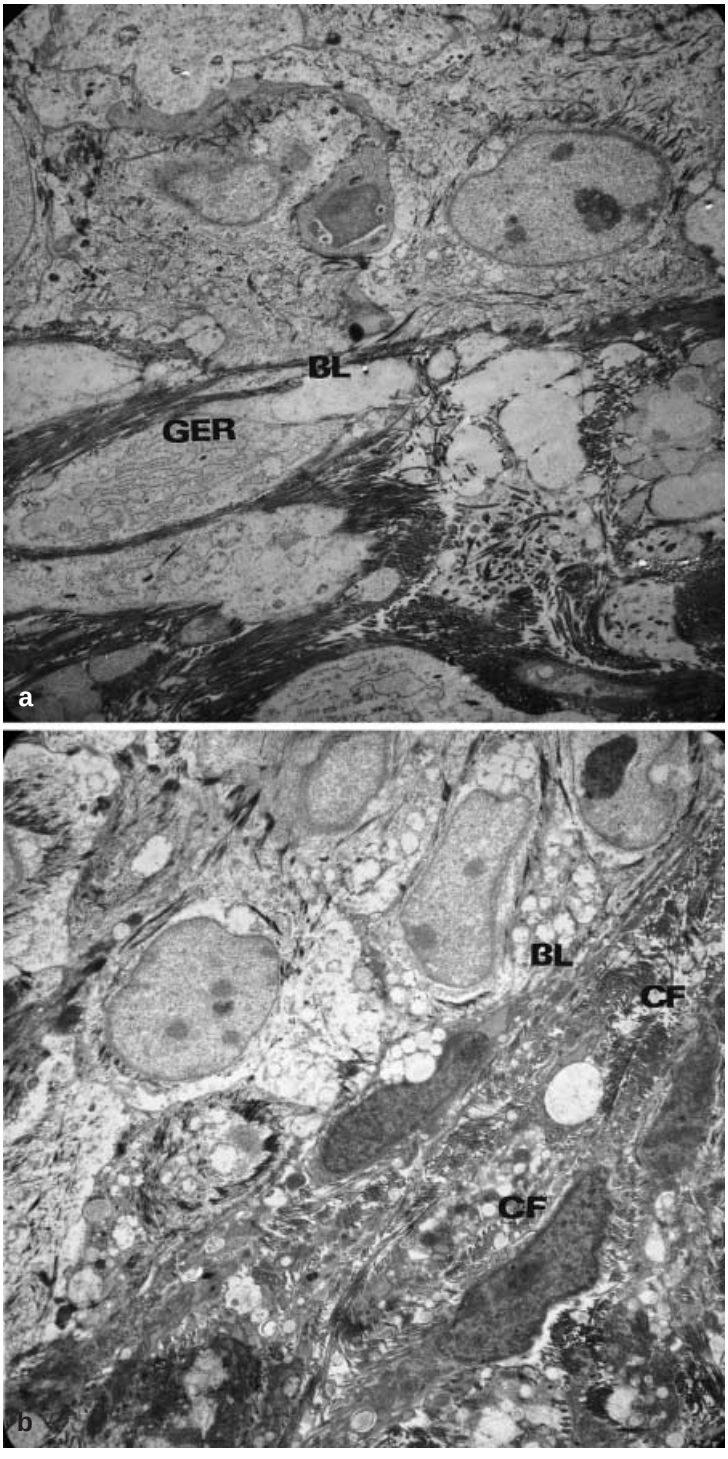

Fig. 6. a Image of the wound area (TAU-GEL group) under transmission electron microscope: Basal lamina (*BL*), GER tubules in fibroblast at dermis (*GER*), collagenous fibers (*CF*). (Uranyl acetate and lead citrate × 9,000). **b** Another image of the same group (TAU-GEL group) under transmission electron microscope: Basal lamina (*BL*), collagenous fibers (*CF*). (Uranyl acetate and lead citrate × 9,000)

significant decrease in MDA levels ($p < 0.05$). This finding is supported by previous studies (Nakashima et al., 1982; Pasantes-Morales and Cruz, 1985). In the case of taurine deficiency, there is a significant increase in MDA levels (Harada et al., 1990). Decreased MDA levels in the TAU-GEL group may be a consequence of the effect of taurine on the inhibition of lipid peroxidation in mast cells and other cells. Dinçer et al. reported that locally administered taurine significantly increased wound tensile strength by decreasing the MDA and histamine levels and prevented the degranulation of the mast cells (Dinçer et al., 1996). On the other hand, isolated rat serosal mast cells exposed to free radical generating systems increased the release of histamine in accordance with the MDA production. Moreover, the secretory granules of rat mast cells have been shown to be one of the subcellular sources of superoxide production (Henderson and Kaliner, 1978).

It has been reported that the increased ion and water permeability of the membrane due to the membrane damage caused by lipid peroxidation (Pasantes-Morales and Cruz, 1985) was prevented by taurine, possibly caused by a calcium dependent mechanism (Harada et al., 1990). It was reported that this mechanism stabilized the membrane (Huxtable, 1992).

We also examined the effect of taurine on wound healing by measuring HPX content. On day 7 the wound tensile strength and HPX level in the wounds of the TAU-GEL group were found to be higher than those in the CHI-GEL and CONT groups. It is generally accepted that the mechanical strength of soft tissues and the HPX level in the tissue depend mainly on collagen, whose synthesis is an essential feature of wound healing. Some studies have shown that taurine had a regulatory role in collagenogenesis (Gordon et al., 1986). In our study, the gel form was found to be essential for taurine administration, since taurine is released slowly from the gel. Taurine-chitosan gel makes a thin film layer in the wound area and permits taurine release. As long as the gel is present in the wound area, it continues to release taurine, and this method has been found to be more efficient.

Chitosan was used as a polymer in this study. Chitosan itself has several biological properties, e.g., it is biodegradable and it reduces blood cholesterol levels, stimulates the immune system and accelerates wound healing (Conti et al., 2000). The releasing mechanism of chitosan appears to be diffusion controlled (Prasad et al., 1991). Recently, it has been reported that chitosan permits regeneration of tissue elements in skin wounds and causes no adverse effects on wound healing (Bartone et al., 1988). Chitosan exhibits many advantages for topical therapy, including good flow, non-irritancy, and a potential for a suitable release rate of the dosage form. TAU-GEL dosage form also decreases the period of wound healing or it accelerates the wound healing process.

In this study, tensile strength, MDA and HPX levels and histological findings together suggested that TAU-GEL could be effective for rapid collagen production.

In conclusion, taurine slow release formulation (TAU-GEL dosage form) has been shown to enhance the re-epithelization and tensile strength of wounds.

References

Alvarez JG, Storey BT (1983) Taurine, hypotaurine and albumin inhibit lipid peroxidation in rabbit spermatozoa and protect against loss of motility. Biol Repro 29: 548–555

Bartone FF, Adickes E (1988) Chitosan: effects on wound healing in urogenital tissue: preliminary report. J Urology 140: 1134–1137

Bergren ST, Bodzin JH, Cortes JA (1988) Improved survival using oxygen free radical scavengers in the presence of ischemic bowel anastomosis. Am Surg 54: 333–336

Casini A, Ferrali M, Pompella A, Maellaro E, Comsarti M (1986) Lipid peroxidation and cellular damage in extrahepatic tissue of bromo benzene in toxicated mice. Ann J Pathol 123: 520–531

Corti B, Giunchedi P, Genta I, Conte U (2000) The preparation and in vivo evaluation of the wound-healing properties of chitosan microspheres. STP Pharm Sci 10: 101–104

Çelebi N, Erden N, Gönül B, Koz M (1994) Effect of epidermal growth factor dosage forms on dermal wound strength in mice. J Pharm Pharmacol 46: 386–387

Dijke P, Iwata KK (1989) Growth factors for wound healing. Biotechnology 7: 793–798

Dinçer S, Babül A, Erdo an D, Özoḡul C, Dinçer SL (1996) Effect of taurine on wound healing. Amino Acids 10: 59–71

Foschi D, Trabucchi E, Musozzi M, Castoldi L, Di Mattia D, Rodaelli E, Marazzi M, Franzini P, Berlusconi A (1988) The effects of oxygen free radicals on wound healing. Int J Tissue React 10: 373–379

Gordon RE, Shaked AA, Solano DF (1986) Taurine protects hamster bronchioles from acute NO_2 induced alterations. Am J Pathol 125: 585–600

Harada H, Cusack BJ, Olson RD, Strou W, Azumo J, Hamaguchi T, Schaffer SW (1990) Taurine deficiency and doxorubicin: interaction with the cardiac sarcolemmal calcium pump. Biochem Pharmacol 39: 745–751

Henderson WR, Koliner M (1978) Immunologic and nonimmunologic generation of superoxide from mast cells on basophils. Clin Invest 61: 187–196

Högstrom H (1987) Mechanism and prevention of decrease in wound margin strength. Acta Chir Scand [Suppl] 539: 5–63

Hunter EG (1985) Adult ventricular myocytes isolated from CHF 146 and CHF 147 cardiomyopathic hamsters. Can J Physiol Pharmacol 64: 1503–1506

Huxtable RJ (1992) Physiological actions of taurine. Physiol Rev 72: 101–163

Kopple JD (1989) Antimutagenic effect of taurine in a bacterial assay system. Cancer Res 49: 6600–6604

Laidlaw SA, Dietrich MF, Lamtenzan MP, Vargas HI, Block JB, Kopple JD (1989) Antimutagenic effects of taurine in a bacterial assay system. Cancer Res 49: 6600–6604

Malette WG, Quigley HJ, Gaines RD, Johnson ND, Rainer WG (1983) Chitosan: a new hemostatic. Ann Thor Surg 36: 55

Nakashima T, Taniko T, Kriyama K (1982) Therapeutic effect of taurine administration on carbon tetrachloride-induced hepatic injury. Jpn J Pharmacol 32: 583–589

Pasantes-Morales H, Cruz C (1985) Taurine and hypotaurine inhibit light-induced lipid peroxidation and protect rod outer segment structure. Brain Res 330: 154–157

Pasantes-Morales H, Wright CE, Gaull GE (1984) Protective effect of taurine, zinc and tocopherol on retinol-induced damage in human lymphoblastoid cells. J Nutr 114: 2256–2261

Pasantes-Morales H, Wright CE, Gaull GE (1985) Taurine protection of lymphoblastoid cells from iron-ascorbate induced damage. Biochem Pharmacol 34: 2205–2206

Prasad SA, Sanford M (1991) Evaluation of chitosan citrate complexes as matrices for controlled release formulation using a 3^2 full factorial design. Drug Dev Ind Pharm 17/14: 1931–1945

Schultz G, Rotari DS, Clark W (1991) EGF and TGF- in wound healing and repair. J Cell Biochem 45: 346–352

Wang O, Giri SN, Dallas MH, Nokashima JM (1989) Effects of taurine on bleomycin-induced lung fibrosis in hamsters. Proc Soc Exp Biol Med 190: 330–338
White MJ, Heckler FR (1990) Oxygen free radicals and wound healing. Clin Plast Surg 17: 473–484
Woessner JF (1961) The determination of hydroxyproline in tissue and protein samples containing small proportion of this imino acid. Arch Biochem Biophys 93: 440–447

Authors' address: N. Çelebi, Department of Pharmaceutical Technology, Faculty of Pharmacy, Gazi University, Etiler 06330 Ankara, Turkey

Received January 15, 2001