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EGF containing gelatin-based wound dressings

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Abstract

In case of bulk loss of tissue or non-healing wounds such as burns, trauma, diabetic, decubitus and venous stasis ulcers, a proper wound dressing is needed to cover the wound area, protect the damaged tissue, and if possible to activate the cell proliferation and stimulate the healing process. In this study, synthesis of a novel polymeric bilayer wound dressing containing epidermal growth-factor (EGF) -loaded microspheres was aimed. For this purpose, a natural, nontoxic and biocompatible material, gelatin, was chosen as the underlying layer and various porous matrices in sponge form were prepared from gelatin by freeze-drying technique. As the external layer, elastomeric polyurethane membranes were used. Two different doses of EGF was added into the prepared gelatin sponges (1 and 15 $\mu g/cm^2$) to activate cell proliferation. EGF addition was carried out either in free form or within microspheres to achieve prolonged release of EGF for higher efficiency. The prepared systems were tested in in vivo experiments on full-thickness skin defects created on rabbits. At certain intervals, wound areas were measured and tissues from wound areas were biopsied and processed for histological examinations. The wound areas decreased upon low-dose EGF application but the difference between the affects of free EGF and microsphere loaded EGF was not so distinct. Upon increasing the dose of EGF by a factor of 15, it was observed that controlled release of EGF from microspheres provided a higher degree of reduction in the wound areas. Histological investigations showed that the prepared dressings were biocompatible and did not cause any mononuclear cell infiltration or foreign body reaction. The structure of the newly formed dermis was almost the same as that of the normal skin.

Keywords: Wound dressing; Gelatin sponge; Bilayer system; Microsphere; Tissue regeneration; Epidermal growth factor

1. Introduction

Rapid and proper healing is important in the treatment of wounds such as severe burns, trauma, diabetic, decubitus and venous stasis ulcers, and similar tissue damages. In cases of severe and large amounts of skin loss, or in the presence of difficult and non-healing wounds, immediate coverage of the wound surface with a dressing is needed. The dressing achieves the functions of the natural skin by protecting the area from the loss of fluid and proteins, preventing infection through bacterial invasion, and subsequent tissue damage. In some cases, it improves healing by providing a support for the proliferating cells. In the last 20 years there is an intense scientific activity in this area. Currently, a large number of research groups are working on the synthesis and modification of new biocompatible materials [1,2] to obtain a material that would be applicable as wound dressing, activate tissue regeneration, inhibit wound contraction and protect the wound site against unwanted external effects. In recent years, researchers have focused on bilayer wound dressings [3–8]. These types of bilayer wound dressings are constructed with an elastic external layer and a soft underlying layer. The external layer is generally made of polyurethanes or silicones which protects the wound and serves as an artificial epidermis. The underlying layer is generally made of hydrogels and provides a support or a scaffold for the proliferating cells and acts as a substitute for dermis.

One of the earliest bilayer wound dressings, consisted of a silicone membrane attached to an inner layer of collagen/chondroitin-6-sulfate sponge and was developed by Yannas et al. in the 1980s [3,9–11]. Similar

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bilayer wound dressings were later developed by Suzuki et al. [12] and Matsuda et al. [8,13] by modifying Yannas's approach. It was reported that, for the wounds on which these dressings were applied, post-operative contractions were less than that of the controls. On the other hand, addition of chondroitin 6-sulfate and dermatan sulphate reinforced the mechanical properties of collagen sponges but caused a significant decrease in the cell proliferation. Bruin and coworkers developed a synthetic skin substitute consisting of a microporous vapor-permeable polyetherurethane top layer and a biodegradable polyesterurethane elastomer network inner layer and applied to guinea pigs [7]. They reported that epidermal wound healing of partial-thickness wounds under wound coverings was accelerated compared to uncovered controls.

On the other hand, some researchers have studied the effects of growth factors in the healing process [14–22]. It is believed that, epidermal growth factor (EGF) stimulates the growth of keratinocytes in vivo, and therefore plays an important role in the process of wound healing that depends on mitosis and migration of keratinocytes. Rhinewald and Green showed, in vitro that in the presence of growth factors, higher percentage of cells leave the resting state, enter and remain in the mitotic cycle [23]. Assuming a similar effect of EGF on epidermal cells in vivo, the primary mechanism of enhanced wound healing is most likely due to increased proliferation of epidermal cells.

In vivo, EGF was applied either as ointments, mists, injections or in controlled release systems. Franklin and Lynch reported a qualitative enhancement of wound healing when mouse EGF was applied in a water-miscible ointment to full-thickness wounds in rabbit ears [24]. Niall et al. reported significant enhancement of epithelization in mice when EGF was applied topically (every 8 h) to skin defects and observed better wound closure with an increase of the dosage from 1 to 10 μg [25].

The reported results about the effects of EGF on healing were not always on the positive side. Greaves applied mouse EGF in saline to the base suction blisters on volunteers and covered these areas with sterile occlusive dressing [26]. In a similar manner, Arthurson used mouse EGF either as a topical mist in saline or as an intraperitoneal injection for treatment of epidermal wounds created on rats [27]. Thornton et al. applied mouse EGF topically as a mist once daily, and then applied Silvadene® to partial-thickness burns produced on rats [28]. In neither of these studies [26–28] healing was observed and they failed to show an acceleration of healing for partial-thickness epidermal wounds. Carpenter [29] and Buckley [30], based on in vitro results, concluded that these failures may be because of the experimental conditions that did not provide sufficient continuous exposure of residual epithelial cells to EGF. Mitogenic effect of EGF requires continuous exposure of target cells to EGF for a minimum 6–12 h.

Brown et al. mixed EGF with a topical cream [31]. The highly significant increase in healing was most likely due to the creams providing continuous exposure of residual epidermal cells to EGF, and thus increasing their mitotic activity Nanney et al. obtained similar results with continuous exposure of EGF [32].

The stimulation of wound healing by EGF has been confirmed by Laato et al. as growth of granulation tissue in sponge implants used as inductive matrices [33]. They observed, in rats, dose-dependent effect of EGF on granulation tissue formation as increased amounts of nucleic acids, and accumulation of collagen and glycosaminoglycans. Buckley et al. reported that sustained release of EGF from subcutaneous pellets accelerated process of wound repair in rats, whereas daily injections of EGF were much less effective [30]. This they measured as increase in the granulation tissue, doubling in the DNA content, increase in protein content and wet weight as compared with placebo controls. Recently, efficiency of sustained release of EGF has also been shown by various researchers [34,35].

Okumura et al. found that, an ointment containing EGF and a protease inhibitor, nafamostat, mesilate or gabexate mesilate or gelatin, accelerated the healing rate of open wounds [36]. They observed a significant increase in the dry weight of wound-side granulation tissue, uronic acid (as a measure of acid mucopolysaccharides) and hydroxyproline (as a measure of collagen). No improvement in wound healing was observed by topical application of EGF alone to open wound sites.

As mentioned previously, in many studies, high in vivo efficiency of EGF (depending on the dosage from and application method) on wound healing has been demonstrated [28–31]. The half-life of EGF in the body is, however, too short to exert the biological activity effectively when applied via injection or in free form. It is known that many proteases are activated in the injured tissue and they easily decompose EGF in the wounded or burned site of skin as soon as it is applied as an ointment [36]. Therefore, incorporation or encapsulation of EGF into a polymer matrix and its sustained release from this seems as one possible approach to enhance its in vivo efficiency.

The aim of this study is to design a novel bilayer wound dressing containing EGF (either in free form or loaded in microspheres to provide sustained release). A porous and biodegradable matrix that would serve as the host for the proliferating cells and would degrade spontaneously without creating any adverse effects while the tissue regenerates was planned to act as the underlying dermal layer. EGF was added into these sponges either in free form or in gelatin microspheres that would act as sustained release vehicles for growth factors. Thus, EGF was expected to activate cell proliferation while the

porous matrix would form the medium for these cells to adhere.

Polyurethane membranes were used as the external layer because of their biocompatibility and hemocompatibility. Besides they are highly elastomeric (extensible) and permeable to gaseous substances. They create an inert environment for the blood, control water and heat transfer through the wound area, and prevent bacterial invasion. They are mechanically strong and protect the wound from the external effects [37–42].

Gelatin was chosen as the porous soft layer material. Since it is practically more convenient than collagen and known to have no antigenicity while collagen expresses some in physiological conditions. Also, it is extremely difficult to prepare concentrated solutions of native collagen. Furthermore, gelatin is far more economical than collagen. The soft and porous gelatin sponges beneath the polyurethane films would have direct contact with the tissue and expected not to cause any damage to the wound area. Because of their high absorptive capacity they would prevent fluid accumulation. Therefore, excess water (exudate composed of wound fluids) and cell debris would be absorbed and retained inside the sponges. Tissue ingrowth would take place in the matrix and the regenerating wound tissue and implant would not be separated. The sponge is biodegradable, therefore, it would degrade and be replaced by the newly regenerated tissue. The study was concentrated on the synthesis of this matrix and on in vivo testing for its effects on wounds experimentally created on rabbits.

2. Materials and methods

2.1. Materials

EGF (human recombinant) was purchased from Sigma (USA), gelatin was obtained from Difco (USA), glutaraldehyde (50%) was from BDH (UK) and OpSite® was obtained from Smith & Nephew (UK). All other reagents were of analytical grade. All were used without any further treatment or purification.

2.2. Preparation of EGF containing microspheres

Gelatin microspheres were prepared by a modified coacervation technique reported by Nastruzzi's [43]. Briefly, an aqueous gelatin solution was added dropwise into paraffin oil while the mixture was mechanically stirred at 1000 rpm to form a water–oil emulsion. Then the solution was rapidly cooled by immersing in ice–water medium. The formed gelatin microspheres were filtered, washed with acetone and dried at room temperature.

In order to prepare the EGF containing microspheres, EGF (50 or 750 μg in 1 ml phosphate buffer, pH = 7.4) and heparin (50 μl) were added into the aqueous gelatin solution at the beginning of microsphere preparation stage and the resultant microspheres were designated as GM-EGF.

2.3. Gelatin sponge preparation

2.3.1. Preparation of gelatin sponges

Aqueous gelatin solutions stirred at about 2000 rpm for 30 min at room temperature and glutaraldehyde solutions were added to form crosslinkings. Then the solutions were poured into molds, frozen in liquid nitrogen and freeze-dried for 24 h. The resultant sponges (thickness ca. 1 cm) were exposed to UV for 1 h prior to in vivo applications to achieve sterilization. These sponges were labeled as GS.

2.3.2. Preparation of gelatin sponges with EGF

Required amounts of EGF (50 or 750 μg EGF in 1 ml phosphate buffer, pH = 7.4) and heparin (50 μl) were added into the gelatin solutions (prepared as above) and poured into molds. The general procedure of sponge formation was followed. These sponges were labeled as GS-EGF.

2.3.3. Preparation of gelatin sponges with EGF loaded microspheres

Microspheres containing EGF were added into foaming solutions and the general procedure of sponge formation was followed. These sponges were labeled as GS-MS-EGF.

2.4. Preparation of bilayer wound dressing

In the present study, bilayer wound dressing was constructed in situ, at the wound site, by initially applying the sponges and then covering with commercially available, adhesive polyurethane, OpSite®. However, in order to see the adhesion of sponge onto polyurethane membranes; some bilayer dressings were prepared by pouring the foaming gelatin solution on polyurethane films prepared in our laboratory. But these dressings were not used in in vivo applications.

2.5. Characterization of microspheres and sponges

2.5.1. Morphological analysis

The morphology of microspheres and sponges were examined using a scanning electron microscope (SEM, Jeol Model 6400) after coating the samples with a thin layer of gold under vacuum.

2.5.2. Particle size analysis

The average sizes and size distribution curves of microspheres were obtained by a particle size analyzer (Mastersizer S Version, Malvern Instruments Ltd., UK) by using acetone as solvent.

2.6. In vivo studies

2.6.1. Animal model

Thirty, 3-month-old male rabbits, weighing about 2–3 kg, were used for in vivo experiments. The animals were fed with a standard diet and housed individually in the animal care facilities of the University.

2.6.2. Surgical procedure

The rabbits were anaesthetized by one intramuscular injection of a mixture of ketamine hydrochloride and rompun (3 ml). The hair of the dorsal area was removed with an electrical shaver and the skin was sterilized with baticone alcoholic solution. Four or five circular full-thickness skin wounds (diameter = 0.8 cm, area = 0.50 cm^2) were created using a sterile punch.

On each animal, nothing was applied to the first wound area and it was kept as control. The second wound was covered with gelatin sponge (1 cm^2). The third and fourth wounds were covered with free-EGF containing sponges and EGF-loaded microsphere containing sponges, respectively (1 cm^2) (Fig. 1). The total amount of EGF in each sponge was 1 μg. Then, the total wound area was completely covered with a commercially available adhesive polyurethane film (OpSite®).

Everyday, the rabbits were checked and the length and width of the lesions were measured. To evaluate the biocompatibility and efficiency of the systems, specimens encompassing the whole area were removed under general anesthesia on 7th, 14th and 21st days after the operation. Specimens were fixed in formaldehyde to be processed for histology. Five implantations were performed for each period of each sponge sample.

2.6.3. Influence of EGF dose

These experiments were carried out to study the influence of EGF dose on wound repair. For this set, the EGF load was 15 μg instead of earlier 1 μg/cm^2 of sponge. In this set an extra control wound (#5) was created. This control was not covered at all and left as is all through out the test. The other four lesions were processed as described previously.

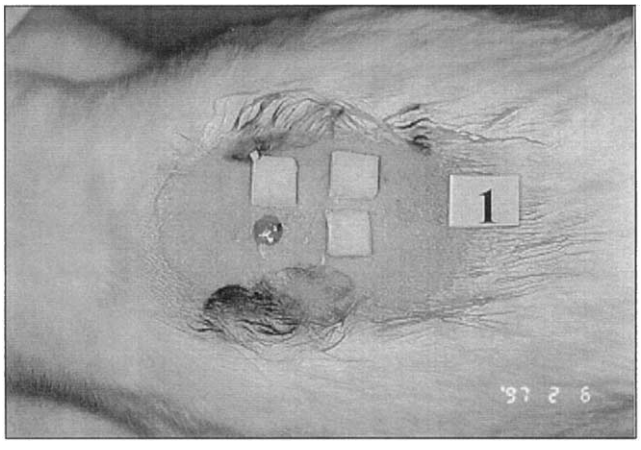

Fig. 1. Wounds covered with gelatin sponges.

2.6.4. Wound area measurements

Length and width of the wound regions were measured by using a microruler that was sterilized prior to every measurement. Assuming that the lesion is in the shape of an ellipse (due to skin anisotropy) the following equation for the area of an ellipse was used as suggested by Baker and Haig [44]:

$$\text{Wound area } (cm^2) = [\text{long axis (cm)} \times \text{short axis (cm)} \times \pi]/4.$$

2.7. Histological examination

Skin samples containing the whole wound area were removed in the first, second and third weeks of post-operation, and all specimens were immersed in 10% buffer formalin. They were dehydrated in a graded series of ethanol and embedded in paraffin. Five to seven micrometer thin sections were prepared and stained with Hematoxylin, Eosin and Masson's Trichrome. Photomicrographs were obtained by an Olympus BH-7 light microscope. For each wound of each rabbit at least 10 sections were taken and examined.

3. Results and discussion

3.1. Characterization of microspheres

3.1.1. Morphological analysis results

SEM micrographs of unused GM and GM-EGF samples are given in Fig. 2. The micrographs indicate that the microspheres did not aggregate. It is noticed that the samples were quite spherical with smooth surfaces (Fig. 2A) and loading of EGF into microspheres caused roughness on the surfaces (Fig. 2B).

3.1.2. Particle size analysis of microspheres

It is possible to obtain different-sized microspheres by changing the experimental conditions such as stirring speed, concentration of gelatin or by addition of surface active materials to the reaction medium during preparation [45]. Since the addition of surfactants might cause some adverse effects in vivo and decrease biocompatibility, in this study, no surfactants were used.

Microspheres prepared had mean diameters in the range 42.80–58.60 μm (Table 1). The size distributions were as follows: 10% of the particles were found to be under 16.50 and 21.17 μm, 50% of the particles were under 38.11 and 47.97 μm, 90% sample was below the 76.16 and 92.29 μm for GM and GM-EGF samples, respectively.

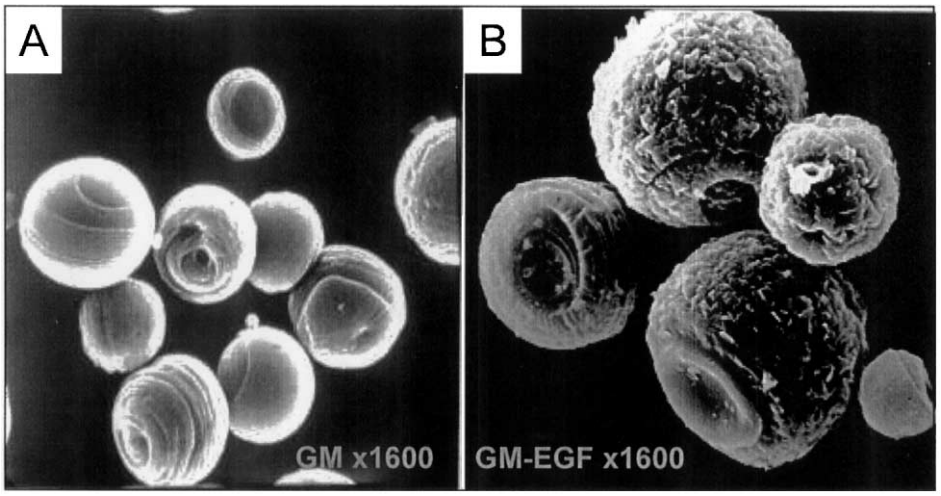

Fig. 2. SEM of gelatin microspheres: (A) GM (×1600), (B) GM-EGF (×1600).

Table 1
Particle size analysis results[a]

Microsphere type	D (v, 0.1) (μm)	D (v, 0.5) (μm)	D (v, 0.9) (μm)	VMD (μm)	SMD (μm)
GM	16.50	38.11	76.16	42.80	17.85
GM-EGF	21.17	47.97	92.29	58.60	21.63

[a] D (v, 0.5) is the size of particle at which 50% of the sample is smaller and 50% is larger than this size. This value also known as the mass median diameter (MMD). D (v, 0.1) is the size of particle for the which 10% of the sample is below this size. D (v, 0.9) gives a size of particle which 90% of the sample is below this size. VMD is the volume mean diameter. SMD is the surface area mean diameter. Also known as the Sauter mean.

3.2. Gelatin sponges and bilayer wound dressing

The SEM photographs of the gelatin sponges are shown in Fig. 3. The gelatin sponges have highly porous lattice structures with cell radii around 100 μm. Upon loading with microspheres, two or more microspheres appear to occupy a single cell in the sponge structure. The full structure of a bilayer wound dressing prepared in this study (not used in in vivo tests) is shown in Fig. 4A. This wound dressing is composed of a thin polyurethane film (PU) over which the gelatin sponge (GS) containing EGF-loaded microspheres was attached. The polyurethane film did not affect the porous structure of the gelatin sponges and the two layers adhered firmly to each other. At higher magnifications, the microspheres inside pores could be clearly seen (Fig. 4B and C).

3.3. In vivo studies

The prepared sponges were applied on the full thickness skin defects created on the dorsal regions of rabbits and controlled daily. The wound areas were measured and photographed. The observations are presented below.

3.3.1. Macroscopic observations

One week after the operation, skin defects were observed in all lesions and all the wounds were covered with hemorrhagic crutes. Two weeks after the operation, the skin defects and crutes were partially observed for the lesions 1, 2 and 3 in all animals. At lesions 4, where EGF loaded microsphere containing sponges were applied, neither skin defect nor crute were observed. Three weeks after the operation, all skin defects were covered with epidermis. Hemorrhagic crute disappeared from all the lesions and a thin layer of squam covering the wound regions was observed over epidermis.

It is known that, one of the most frequent complications of the wounds and wound dressing is the infection. It is very important that, in this study, no infection was observed for any of the animals throughout the experiments.

The area of the wounds originally produced by a circular punch was 0.50 cm^2. One, two and three weeks after the operations the wound areas were calculated from the measured values and the average values are given in Fig. 5. In the OpSite® covered control group (#1), the decreases in the wound areas were found to be 42, 60 and 66%, at the end of 1, 2 and 3 weeks post-operation, respectively. In the unloaded sponge (GS) applied lesions (#2), the decrease percentages were 48, 70, and 74% for the same periods. Free EGF containing gelatin sponge (GS-EGF) applied lesions (#3) gave 70, 74 and 88% healing. The extent of healing reached 88% for lesions #3 and #4 at the end of 3 weeks. However, the #4 (GS-MS-EGF applied) did not have any measurable wound area after 2 weeks post-operation even though the third week #4's still showed some measurable wounds

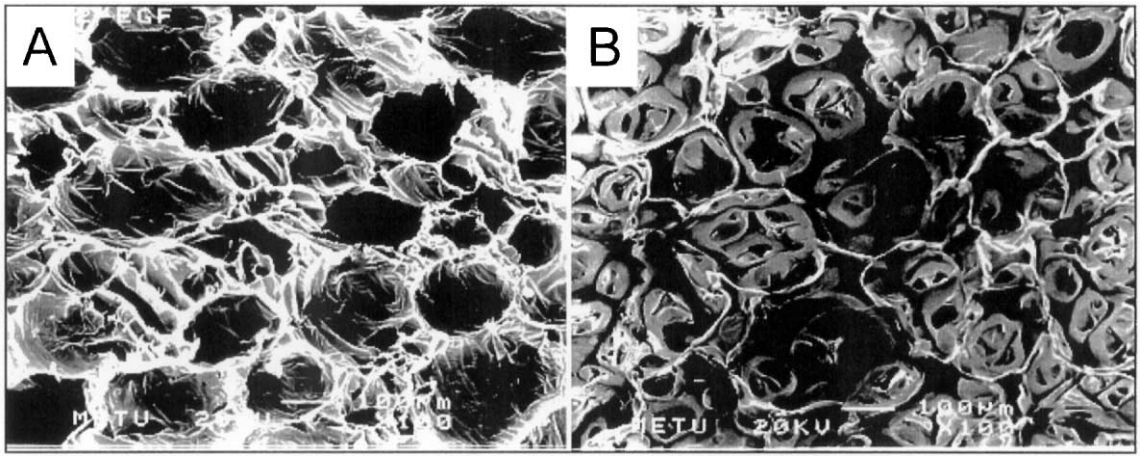

Fig. 3. SEM of gelatin sponges: (A) GS-EGF cross-section (× 100), (B) GS-MS-EGF surface (× 100).

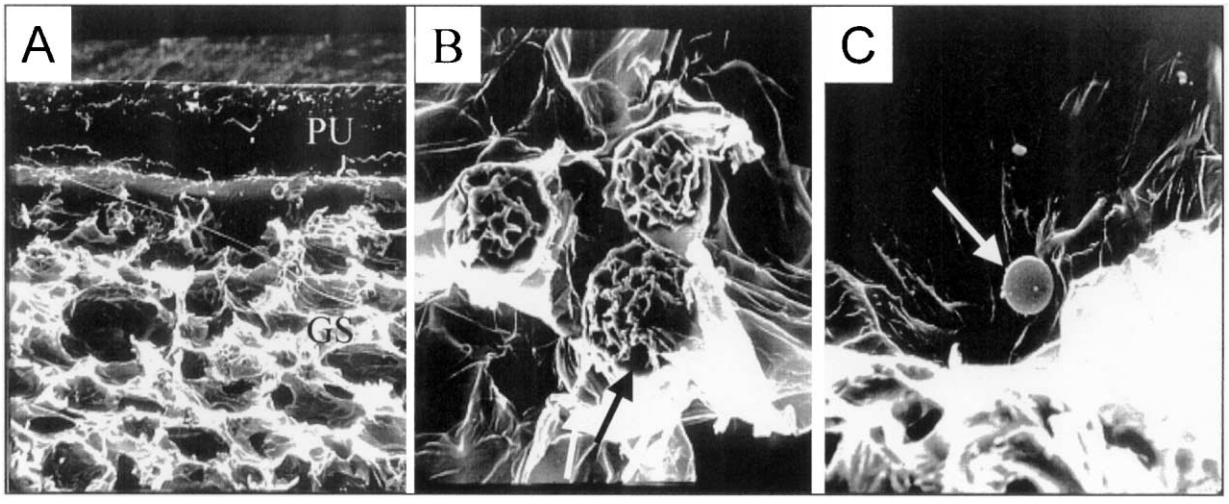

Fig. 4. SEM of bilayer wound dressing cross-sections at different magnifications: (A) × 90, (B) × 900, (C) × 1800 (PU; polyurethane, GS; gelatin sponge, arrow; GM).

for another group of rabbits. In summary, there was a progressive decrease in the wound areas with time. It was also observed that the decrease of areas of wounds on which GS-EGF and GS-MS-EGF applied was higher than those of the control groups.

3.3.2. Influence of EGF dose on healing

In order to observe the effect of EGF dose, sponges containing a higher dose of EGF ($15\,\mu g/cm^2$) were prepared and applied to wounds on the dorsal region of the rabbits. For each set five rabbits were used. Fig. 6A and B show the wound sites, 7 and 10 days after operations, respectively. Fig. 7 gives the wound areas of various lesions at different times of post-operation.

The appearances of the wound sites after 7 days of operation are shown in Fig. 6A. Higher-dose EGF containing sponges were applied to the lesions 3 and 4. In these experiments, the lesions 5 were also controls and these wound were not covered with any material (# 1 was covered with OpSite®). After 1 week, thick, depressed hemorrhagic crutes existed on these wounds with white–gray-colored centers. The sizes of the wounds were recorded as the originals and the wound areas were calculated as $0.50 \pm 0.12\,cm^2$. In lesions, 1, the wound areas were also depressed and covered with hemorrhagic crute with a dark center. Lesion 2 was similar to lesion 1 although the skin defects were not as deep as the lesion 1. For both sets, areas were calculated as $0.33 \pm 0.09\,cm^2$. For lesion 3, a decrease in the wound areas from 0.50 to $0.28 \pm 0.08\,cm^2$ was observed. There was a thin hemorrhagic crute with a thicker and dark-colored center. In the lesions 4, there were only a very thin hemorrhagic crute on the wounds, the periphery of the wounds were well vascularized and the skin defects had vanished. The wound areas decreased from 0.50 to $0.23 \pm 0.07\,cm^2$.

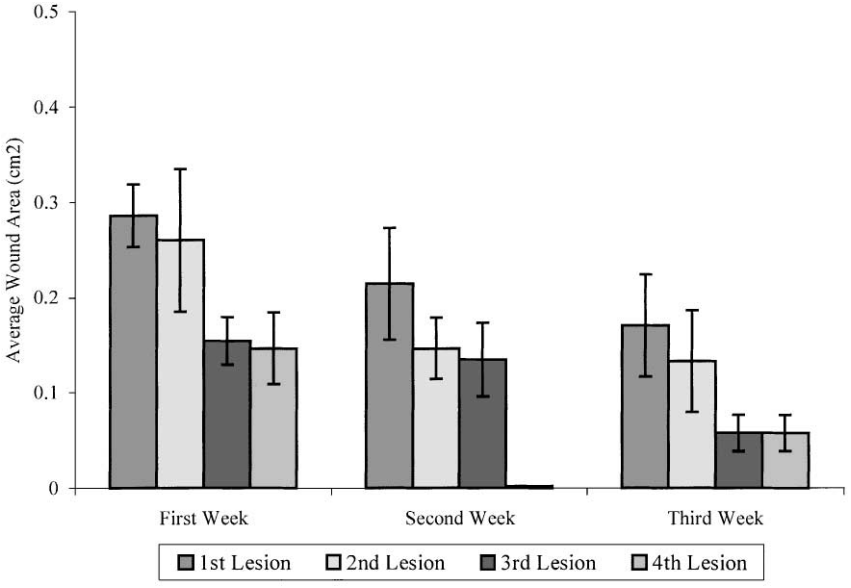

Fig. 5. Change in wound area of lesions treated with various dressings (EGF amount was 1 μg/cm^2): lesion 1: covered with OpSite, lesion 2: covered with GS, lesion 3: covered with GS-EGF, lesion 4: covered with GS-MS-EGF.

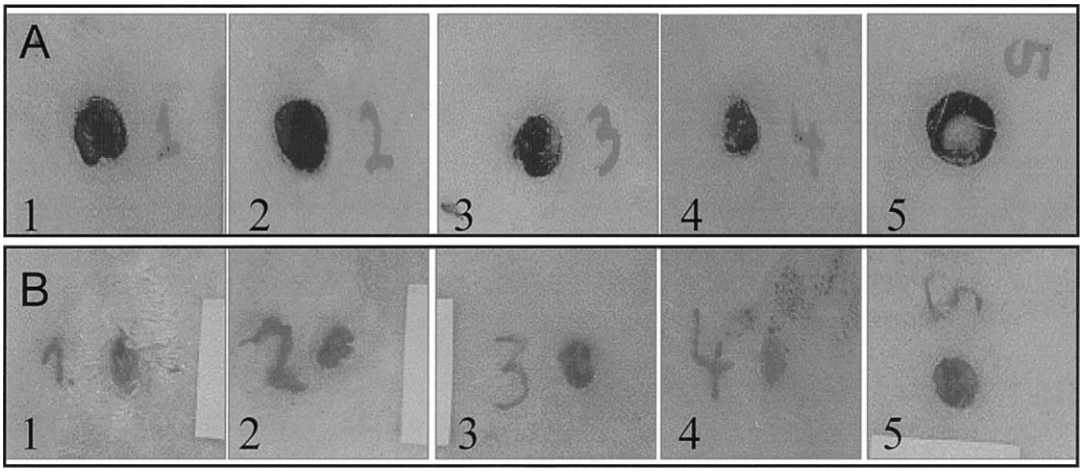

Fig. 6. Macroscopic observations of lesions after: (A) 7 days, (B) 10 days (EGF amount was 15 μg/cm^2): lesion 1: covered with OpSite, lesion 2: covered with GS, lesion 3: covered with GS-EGF, lesion 4: covered with GS-MS-EGF, lesion 5: untreated (control).

The appearance of the wound sites after ten days of post-operation are shown in Fig. 6B. For the control wound (lesion 5) a full thickness skin defect was observed. In this lesion, a deep (about 2 mm) skin defect, covered with a thick hemorrhagic crute was observed. Wound areas were reduced from 0.50 to $0.44 \pm 0.14\,cm^2$. In lesion 1, deep skin defects with a white–gray-colored fluid filling the wound areas were observed. The wound areas were $0.44 \pm 0.19\,cm^2$. Lesions 2 were covered with thick hemorrhagic crute and so were the lesions 3. The wound areas decreased to 0.20 ± 0.05 and $0.16 \pm 0.04\,cm^2$, respectively. Compared with the control group the decrease was remarkable. In the lesions 4, the wound was covered with a very thin hemorrhagic crute, the defect had disappeared and the wound area had closed. There was an increase of vascularization in the surrounding area.

3.3.3. *Histological findings*

Seven days after the operations, in all examined groups, the wound regions were observed to be covered by thick crutes and epidermal parts were missing under the crute. In some of the sections, the scab on the wound was detached from its place during sectioning. The

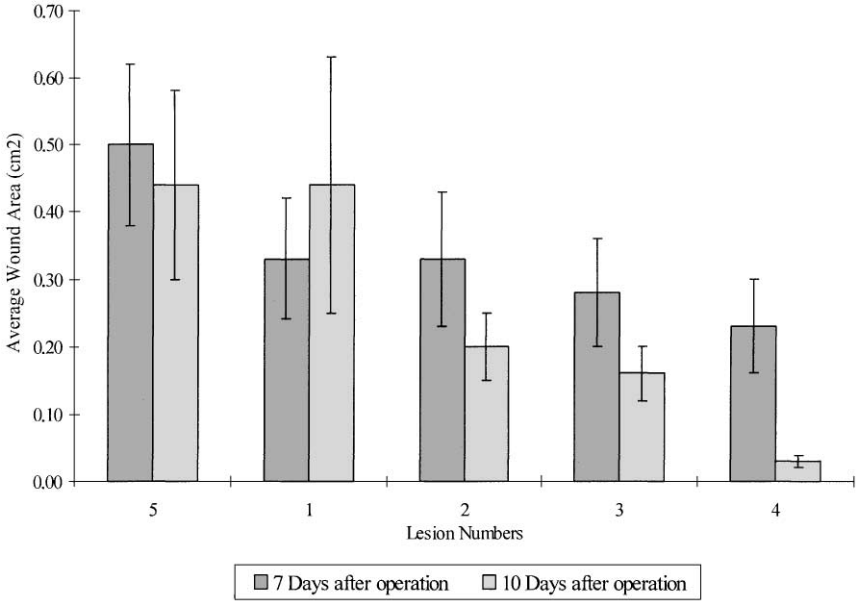

Fig. 7. Change in wound area of lesions treated with various dressings (EGF amount was 15 μg/cm^2): lesion 1: covered with OpSite, lesion 2: covered with GS, lesion 3: covered with GS-EGF, lesion 4: covered with GS-MS-EGF, lesion 5: untreated (control).

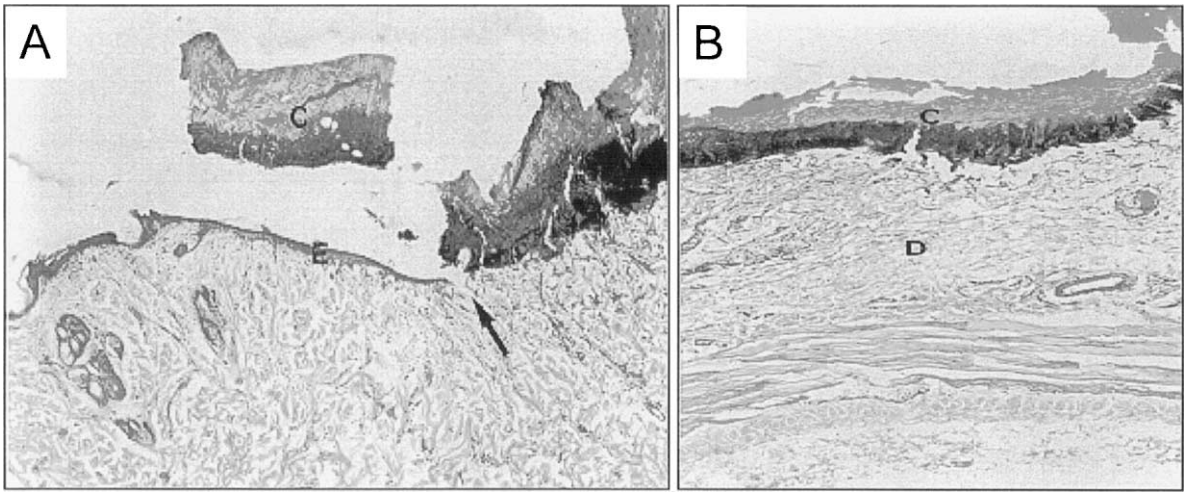

Fig. 8. Low-dose (EGF amount 1 μg/cm^2) in vivo histology results after 1 week. (A) Wound region of the control group (#1) seven days after the operation. There is a junction in between the normal skin and the wound area (arrow). The wound region is covered by a thick crute (C). Dermis; D. HE (×4). (B) Wound region of the GS-MS-EGF group (#4) 7 days after the operation. The wound region is covered by a thick crute (C). The granulation tissue is formed mainly by thin collagen fibers (arrow) parallel to the surface. The dermis has almost the same appearance as the healthy region of the skin. D; dermis. HE (×4).

structure of the dermis was not affected by the operation and it was observed to be the same as the normal dermis in all groups examined. No hair follicles were present in the wound regions and no mononuclear cells were observed (Fig. 8A and B).

Fourteen days after the operation, the crute had disappeared and wounds were covered by a continuous epidermis in all examined groups. The stratified squamous epithelium that formed the epidermis (12 layers) was much thicker than that of the normal skin (3–4 layers) and the wound regions were filled with the connective tissue of the dermis. The junctions of the normal dermis and the dermis in the wound regions were very prominent in all groups examined. Thick, coarse collagen fibers of the normal dermis continued horizontally with the newly formed thin collagen fibers. Still there were no hair follicles in the wound regions (Fig. 9A and B). In the control group (#1) the skin surface was not repaired and

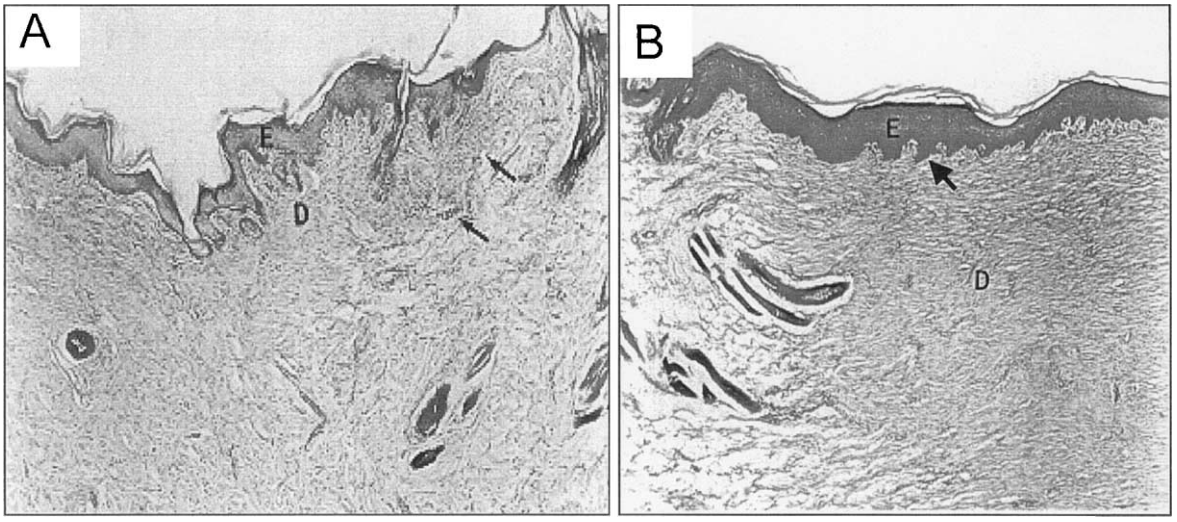

Fig. 9. Low-dose (EGF amount 1 μg/cm^2) in vivo histology results after 2 weeks. (A) Wound region of the control group (#1) 14 days after the operation. The wound is covered by a continuos epidermis (E). Development of the small dermal papillae (arrow head) are observed. The underlying dermis (D) is composed of thin collagen fibers parallel to the surface. The junction in between wound area and the normal dermis is prominent (arrows). HE (×4). (B) Wound region of the GS-MS-EGF group (#4) 14 days after the operation. A thick epidermis (E) with papillac (arrow head) lies on the newly formed dermis that has collagen fibers mainly parallel to the surface. HE (×4).

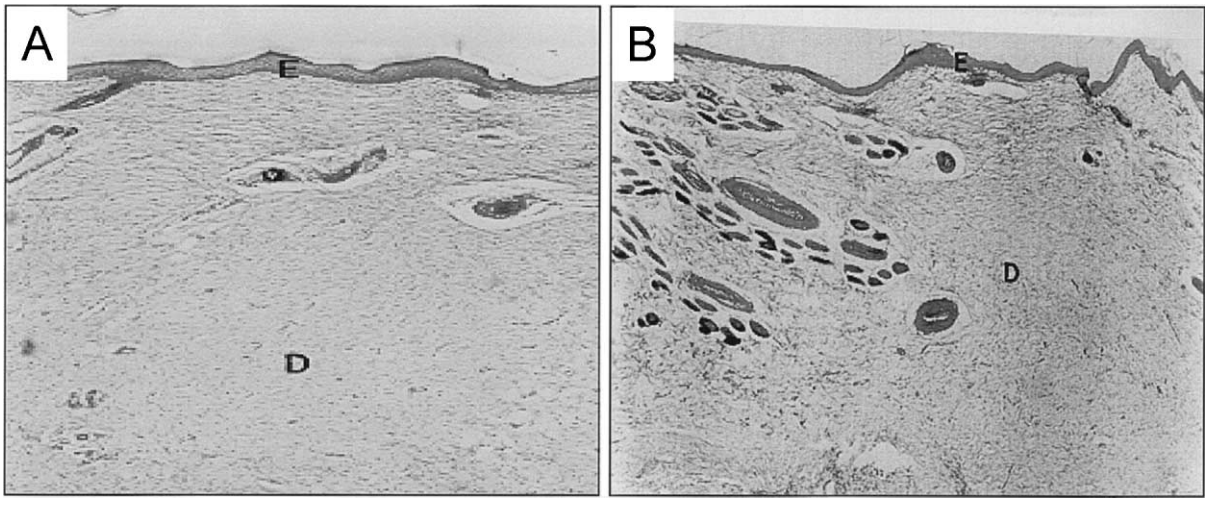

Fig. 10. Low-dose (EGF amount 1 μg/cm^2) in vivo histology results after 3 weeks. (A) Wound region of the control group (#1) 21 days after the operation. The epidermis (E) and dermis (D) appear to be almost the same as the normal skin except the superficial regions where the collagen fibers are parallel to the surface. The papillae had disappeared in epidermis. HE (×4). (B) Wound region of the GS-MS-EGF group (#4) 21 days after the operation. There is a thin epidermis (E) on the dermis (D) which resembles normal skin. The normal and newly formed dermis have almost the same structure. The gentle passage (arrows) of the newly formed dermis into the normal dermis is observed. HE (×4).

did not gain the normal appearance. There were still some invaginations in some regions (Fig. 9A). In the GS-MS-EGF applied wounds (#4) the dermis consisted of thin collagen fibers which were mostly arranged parallel to the skin surface. This was especially prominent in the superficial regions (Fig. 9B). In the GS (#2), GS-EGF (#3), and GS-MS-EGF (#4) applied wounds, development of small dermal papillae were observed which were not a feature of the normal rabbit skin.

Twenty-one days after the operation, the structure of the epidermis became almost the same as that of the normal in appearance and thickness. Small hair follicles appeared in all groups. No mononuclear cell infiltration and foreign body reactions were observed for any of the examined groups. In the control group (#1) (Fig. 10A) and GS (#2) group, the junctions of the normal and newly formed dermis were still prominent though not so distinct as the 14th day groups. In GS-EGF applied

group (#3), the junction was less well demarcated with some horizontal fibers extending between the two regions. In these groups, the horizontal organization of the fine collagen fibers in the superficial dermis was still observed. In the GS-EGF-MS group (#4), there was a marked difference (Fig. 10B). The structure of the dermis was rather loosened and the junction was much less prominent. The collagen fibers of the two regions continued and intermingled gently with each other.

Histological findings of the wounds, to which higher dose EGF containing sponges were applied, are summarized below (Figs. 11 and 12).

Seven days after the operation, the epithelium on the wound region was missing in the control group (#5) (Fig. 11A). Most of the crute covering the wound cavity was removed during sectioning. An incomplete granulation tissue formation was observed with enlarged blood vessels. In Fig. 11B, GS-MS-EGF applied wound (#4) is shown 7 days after operation. No epidermis formation under the crute was observed. The wound region was filled with a better-organized tissue.

In control group (#5), 10 days after the operation, the wound region was covered with a thin surface epithelium consisting of few layers (Fig. 12A). Thin collagen fibers, which were mostly arranged parallel to the skin surface, were observed. In GS-MS-EGF group (#4), 10 days after the operation, epithelization was complete and appeared to be thick at the periphery of the wound region. The wound had healed and was covered with a newly

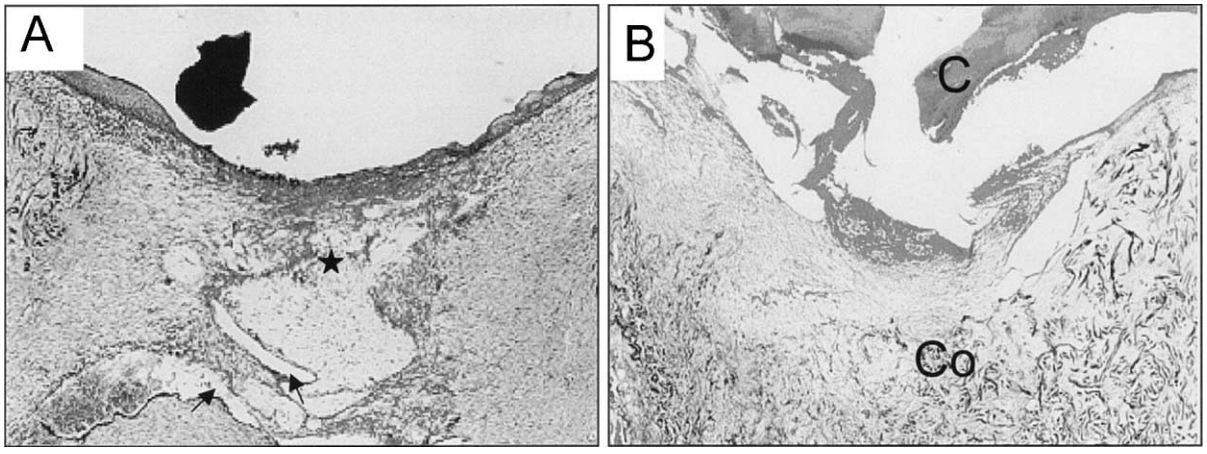

Fig. 11. High-dose (EGF amount 15 μg/cm^2) in vivo histology results after 1 week. (A) Wound region of the control group (#5) 7 days after the operation. An incomplete granulation tissue formation (*) with enlarged blood vessels (arrows) is observed. M Trichrome (×4). (B) Wound region of the GS-MS-EGF group (#4) 7 days after the operation. The wound region is filled with a better organized granulation tissue. Collagen fibers (Co) oriented mainly in their characteristic way of strucuture. C; crute, M Trichrome (×4).

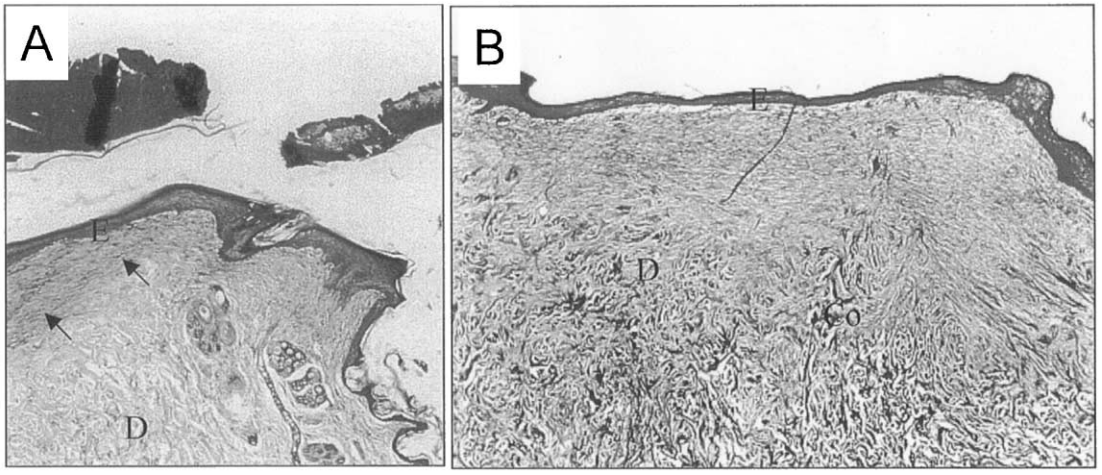

Fig. 12. High-dose (EGF amount 15 μg/cm^2) in vivo histology results after 10 days. (A) Wound region of the control group (#5) 10 days after the operation. The surface of the wound is covered with a thin epidermis (E). Dermis (D) consists of thin collagen fibers (arrows). HE (x 4). (B) Wound region of the GS-MS-EGF group (#4) 10 days after the operation. Epithelization is completed (E). Dermis (D) is infiltrated with collagen fibers (Co) most of them in a mesh structure. The wound area is completely healed. M Trichrome (×4).

formed dermis. The collagen fibers in the dermis layer were in a more organized form (Fig. 12B).

As a result, the prepared sponge systems were found to be biocompatible. Mononuclear cell infiltration or foreign body reactions were not observed for any of wounds. The time of repair of epidermis was almost the same in all groups. In the GS-MS-EGF applied lesions, the wound cavity was filled with a dermis that appeared to have a structure resembling the normal dermis, without any scar tissue formation. There was a soft passage between the dermis and the newly formed dermis. The high quality of the newly formed dermis in the wound cavity was attributed to the application of EGF.

In summary, EGF containing gelatin sponges have a substantial effect on decreasing the wound area and removal of histological signs of tissue damage in experimentally created rabbit skin lesions. This effect was more distinct in the samples that received higher doses.

4. Conclusion

The aim of this work was to prepare a bilayer wound dressing containing epidermal growth factor as a novel system in healing skin defects. For this purpose, various types of gelatin sponges and bilayer wound dressings were prepared and their compatibilities were tested with a series of preliminary in vivo experiments by applying them on full thickness skin defects created on rabbits.

Histological investigations showed that these EGF loaded, biodegradable materials are biocompatible. Mononuclear cell infiltrations or foreign body reactions were not observed. The other properties of these materials are as follows: the preparation process is relatively simple, there are no toxic substances among the constituents, they have a high body fluid absorption capacity, the release rate of macromolecules and growth factors from them are controllable, they are very soft and therefore cause no disturbances during the application. In vivo experiments showed that the rate of wound area decrease was much faster compared to the control groups and the quality of the newly formed dermis as judged by the histological examinations was almost as good as the normal skin. In case of very deep wounds or ulcers, the applied dose of EGF could be increased or frequent application of fresh EGF-loaded sponges could be made. It could also be more effective if a mixture of growth factors is used instead of only EGF. We believe that after the optimization of the composition and testing in vivo for other signs of wound healing (such as DNA, collagen and glucoseaminoglycans contents and wound tensile strength), these constructs would meet a demand for wound dressings in the medical field.

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