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Effect of Topically Applied Silver Sulfadiazine on Fibroblast Cell Proliferation and Biomechanical Properties of the Wound

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The effect of silver sulfadiazine (SSD) on the proliferation of human dermal fibroblast (HDF) was studied to determine the impact of the drug on the wound healing process and dermal mechanical strength. Human dermal fibroblasts were cultured to 80% confluency using DMEM with 10% FBS and viability of the cell was estimated using neutral red assay. In addition, the 2nd degree burn wound was prepared on the anterior part of rabbit ear skin and dressings containing SSD were applied for 96 h. Presence of inflammatory cells and degree of re-epithelialization were investigated in the wound. After 15 day of the induction of burn wounds, the treated area was excised and dermal mechanical strength was quantitatively measured with a constant speed tensiometer. SSD was found to be highly cyto-toxic in cultured HDF cells. The topical application of SSD (2%) could control the infection as evidenced by the lack of accumulation of inflammatory cells in histological evaluation. Therefore, these observations suggested that the impairment of dermal regeneration and decreased mechanical strength of dermal tissue was resulted from the cyto-toxic effect of SSD on dermal cells. Since the decreased mechanical strength may lead to reduction in resilience, toughness and maximum extension of the tissue, the identification of optimum dose for SSD that limits infection while minimizes the cyto-toxic effect may be clinically relevant.

Key words: Silver-sulfadiazine, Fibroblast, Dermis, Wound healing, Burn

INTRODUCTION

Although the use of topical antimicrobial agents, such as silver sulfadiazine (SSD) is essential in the establishment of the bacterial balance in contaminated wounds, the use has been associated with delayed healing of wounds in which the process of skin cell proliferation and collagen deposition play a primary role (Cooper *et al.*, 1991; McCauley *et al.*, 1994). SSD has been used clinically as a standard treatment for burns over the past three decades since Fox first synthesized from silver nitrate and sodium sulfadiazine for an increased potency and negligible adverse effects including minimal pain on application (Fox *et al.*, 1983; Gear *et al.*, 1997; Wright *et al.*, 1998). SSD was reported to be particularly effective as a topical antibacterial agent in the control of Pseudomonas infection in burns (Fuller *et al.*, 1994). However, disturbing evidence has appeared that commonly used antimicrobials may be harmful to the cells involved in wound healing (Mccauley *et al.*, 1992). Currently, no strategy has been devised for SSD to minimize the harmful effect in wound healing while controlling the microbial infection.

Wound healing occurs chiefly by the processes of epithelialization and wound contraction, which is defined as closure of open wound by inward movement of the surrounding integument (Leitch *et al.*, 1993). The process of re-epithelialization has been indicated to be generally keratinocyte dependent, while the process of contraction is fibroblast dependent. Thus, impaired proliferation of these two cell types could lead to the delayed and complicated wound healing (McCauley *et al.*, 1992).

We previously reported the cyto-toxic effect of SSD (1-100 μg/mL) on the viability of cultured keratinocytes, which may be lead to a delayed re-epithelialization process in 2nd degree burn mouse model (Cho Lee, 2002). In general, the process of wound healing follows three phases: the inflammatory response, the migratory response, and the proliferation response. Collagen gives the dermis its structural integrity and is formed within the ribosome of fibroblast

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cells in dermis (Piscatelli *et al.*, 1994; Reddy *et al.*, 1999). Thus, the cyto-toxic effect of SSD on the fibroblast is likely to impede the proper maturation of the collagen matrix, which provides dermal strength. However, it is not clear whether similar effect may be obtained with the fibroblast, which may be associated with the mechanical strength of dermal tissue. Thus, in this study we further investigated the effect of SSD on the human dermal fibroblast (HDF) cell growth and its effect on the wound healing process. In addition, the mechanical properties of the wound was evaluated in 2nd degree burn rabbit ear model since the mechanical properties of a wound could be characterized by the physical attributes of a healing wound of the collagen fibers such as fiber diameter and alignment and cross-linking as well as on other matrix components. (McCauley *et al.*, 1994).

MATERIALS AND METHODS

Materials

Silver sulfadiazine was donated from Dong Wha Pharmaceuticals (Seoul, Korea). Human dermal fibroblast was provided by the department of Dermatology, Seoul National University. All other chemicals were used as received. Duoderm® occlusive dressing (Convatec Co., NJ, USA) was used as a control dressing.

Collagen sponge preparation

Collagen sponges, the primary component in the test dressing, were prepared from collagen extracted from the pig skin. Briefly, 5 mL of 1% collagen solution was placed in 35-mm petri-dish and freeze-dried. Resulting sponge was cross-linked with hexamethylene diisocyanate (1%) for 10 min. Chondroitin-6-sulfate (CS) solution was supplemented at time of molding (Cho Lee *et al.*, 1999).

Effect of SSD on fibroblast proliferation and cyto-compatability of sponge

HDF cells were cultured in 35-mm petri-dish to a 80% confluency in 2 mL DMEM containing 10% of fetal bovine serum. Samples of collagen sponge disk (0.2 cm^2) were prepared by soaking in different concentration of SSD solutions. The resulting sponge was freeze-dried. Gamma irradiated collagen sponge (3 cm diameter) was placed into six well plastic dishes and fibroblast cells (0.8×10^5 cells) were seeded on 8 cm^2 collagen sponge in DMEM media.

Human fibroblasts (10,000 cells/cm^2) seeded on the sponge were incubated in 5% CO_2 and 95 % humidity for one week. When it was necessay to determine the number of cultured cell, the standard MTT assay was used.

Evaluation of wound healing

New Zealand White rabbit (average body weight, 2.0±0.2 kg) was used as the animal model in this study. One circular wound (1 cm diameter) was made on the anterior surface of both sides of the ears. In one side, the tested dressings were applied for 96 h and the wound was remained open thereafter. The extent of wound exudates, the sign of infection and the process of re-epithelialization and wound closure rate were evaluated at predetermined time intervals till the complete wound closure. Upon the complete wound closure, histological evaluation was carried out with hematoxylin-eosin (H-E) staining. Since the wound healing process is dependent on the degree of wound exudates absorption and occlusion, after each tested dressing was applied on the wound, a non-adherent dressing, Duoderm® was applied as a control dressing.

Determination of mechanical properties of wound

After 15 day of the induction of wound, the treated area was excised (15×25 mm^2) and the thickness of each excised skin specimen was determined. The excised skin specimens were analyzed within 2 h of excision to minimize the effect of autolysis. The skin strips were mounted on a Texture analyzer (Stable micro Systems, UK) and secured with modified grips. Using a 500 N load cell, each clamped skin specimen was pulled to rupture at a cross-head speed of 0.2 mm/sec. The load and the elongation of the strip were monitored by the tensiometer, and stress-strain curve profile was plotted for each strip. The mechanical properties such as maximum tension (breaking strength, F_{max}/unit area), skin toughness, resilience, % elongation and elastic modulus constant were determined from the load versus strain curves obtained from a constant speed tensiometer interfaced to PC.

Data analysis

When it was necessary to compare means of test parameters between the control and test formulation, students *t*-test was carried out. $P<0.05$ was accepted as denoting statistical significance. Data are expressed as mean ± standard deviation.

RESULTS

Effect of SSD on the viability of cultured human dermal fibroblast

The effect of collagen sponge containing SSD on the viability of cultured human fibroblast cells was studied. Observation under phase-contrast microscopy (Fig. 1) indicated that apparent morphology HDF was different in the presence (100 μg/mL) and the absence of SSD. Sponge containing SSD showed high cyto-toxicity. This observa-

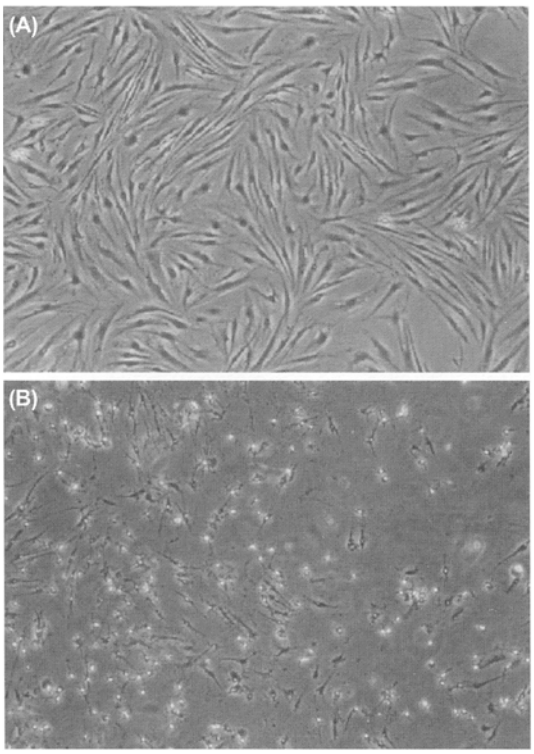

Fig. 1. Phase-contrast microscopy (magnification 100X): Effect of Silver sulfadiazine on the viability of cultured HDF cells. (A) Morphology of HDF cultured in 35 mm petri-dish to a 80% confluency. Bi-axial cell configuration and well stained nucleus. (B) Morphology of HDF after exposure to SSD (100 μg/mL) for 24 h: Loss of elongated configuration and significant cellular destruction reflecting cell death.

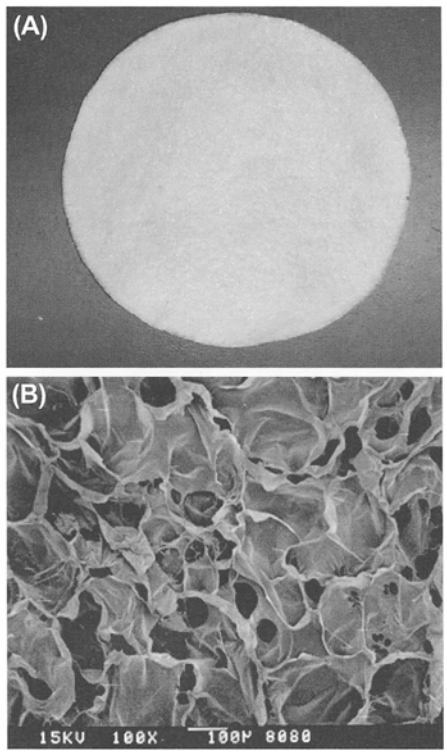

Fig. 2. General appearance (A) and SEM micrography (B) of collagen sponge dressing.

tion indicated that the addition of SSD lead to a significant cell damage in HDF.

Preparation of collagen sponge dressing

Fig. 2 shows the general appearance (A) and the scanning electron micrograph (SEM, B) of collagen sponge. The sponge was fabricated into a disk shape of 35 mm in diameter and 2 mm in thickness. Pore size of the sponge was estimated to be 50-100 μm. Interconnect channels of the sponge were readily apparent in SEM (Fig. 2B). Water absorption capacity of the sponge was estimated to be 500-700% of the dry weight. Mechanical strength was augmented by chemical cross-linking and resulting sponges showed an acceptable cyto-compatibility and mechanical strength (data not shown).

Histological evaluation

In open wound with no dressing, the histological examination indicated a significant accumulation of inflammatory cells as denoted an arrow and clotting of red blood cells (Fig. 3A), probably by the impaired regeneration of stratum basal layer and dermis layer. In addition, epidermis and dermis has been grossly destroyed. Fig. 3B shows the SSD (2%) treated wound after 96 hr of the application of the test dressing. Lack of accumulation of inflammatory cells was readily apparent. However, due to some cytotoxic effect of SSD of cells, the regeneration of epidermis layer was not observed. And decreased density of fibroblasts and interwoven collagen bundles were observed.

Fig. 3C depicts the epidermis, and dermis of normal rabbit ear skin. In epidermis, the outer most cells of the normal stratum corneum are anucleate, and thus are technically dead, and stained eosinophilic. The basal cells form a single layer, are columnar, and lie with their long axes perpendicular to the dividing line between the epidermis and the dermis. In dermis, the extra cellular matrix composed of collageneous and elastic fibers are embedded into ground substances. All three components are formed by fibroblasts (Murphy, 1997). The collagen fibers, as denoted an arrow, are present as a fine woven network.

Evaluation of mechanical strength of SSD of treated wounds

Fig. 4 shows the load versus extension curves for a normal skin and SSD-treated skin. Biomechanical parameters derived from the stress-strain curves are summarized in Table I. Fig. 4A shows the stress-strain profile of ear strips (1×2 cm^2) obtained from normal rabbit skin. Maximal point on stress axis (US: ultimate strength), maximal point on strain axis (total deformation at rupture and area under

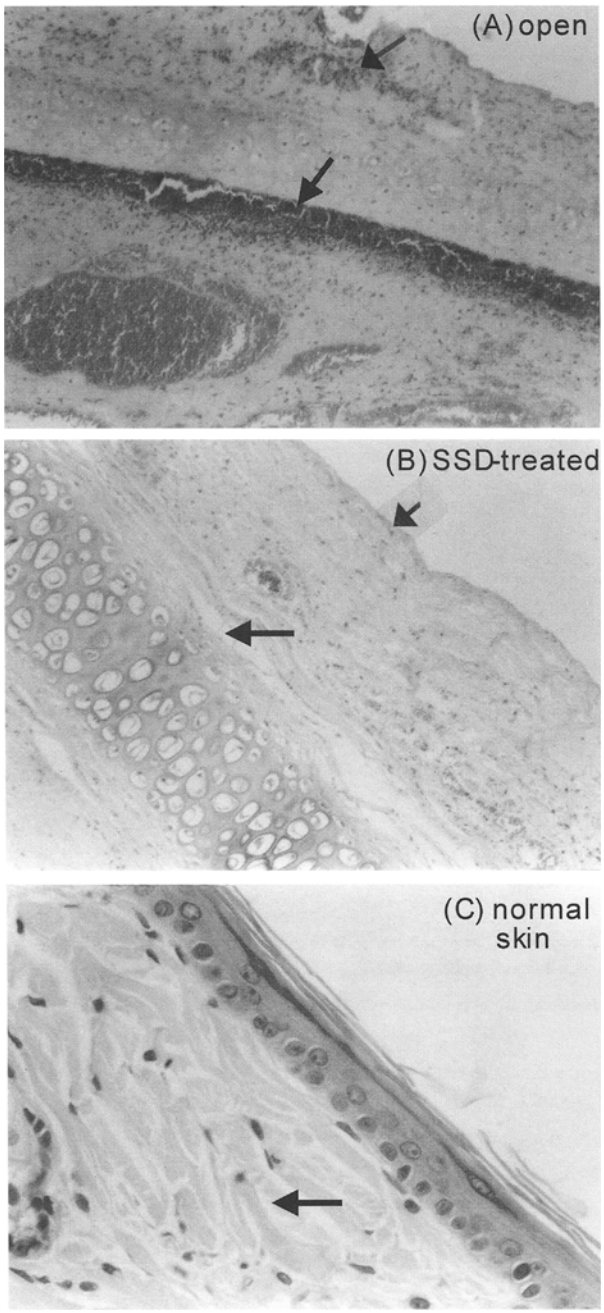

Fig. 3. Histology of H-E stained 2nd degree burn wound after 96 h application of tested dressings. A: Open wound: a lot of inflammatory cells as denoted an arrow have been observed. B: collagen + SSD (2%): Due to some inhibitory effect of SSD on epidermal cell growth, regeneration of epidermal cells has not been progressed yet. C: Normal, unwounded skin: Histological examination indicates well stained epidermis layer and high density of fibroblasts and weave like collagen bundles as denoted an arrow in dermis layer. Original magnification×100.

the curve (A: resilience; A+B: toughness) and modulus of elasticity (related to tangent α) are derived from this curve (Davis *et al.*, 2000). The area under the stress-strain curve represents the product (stress×elongation), which is the energy or work necessary to break the skin specimen, constituting a measure of the toughness or brittleness. Strong (as opposed to brittle) skin specimen has high tensile strengths. Tough (as opposed to brittle) skin specimen has large area under its stress-strain curve and requires large amounts of energy to break under stress (Beaubien *et al.*, 1994). Resilience (A) is the property of material enabling to endure loads without inducing a tension exceeding the elastic limit. The toughness (A+B) is the energy absorbed, which is the maximum extension and average force, up to the point of rupture. These quantitative parameters are likely to be correlated with the degree of cell proliferation of fibroblasts and the alignment, density of collagen fiber networks in dermis. Fig. 4B shows the typical stress-strain profiles of ear strips (1×2 cm^2) obtained from rabbits with wound treated with silver sulfadiazine (2%) for 96 hrs. A significant loss in biomechanical properties was observed in SSD treated wound. That is, SSD significantly decreased breaking strength and % elongation as compared with normal skin. The decrease of maximum extension in SSD treated wound suggests that skin become stiff and brittle.

DISCUSSION

In this study, rabbit model was used to study the effect of wound healing according to dressing containing with or without SSD. Since the study is in comparative in nature, wound in the anterior surface of the rabbit ear seems suitable for comparison of the progressive epithelial growth and dermal regeneration. The biomechanical aspect of wound closure is a complex process, and, thus, conclusion drawn from a single mechanical parameter (e.g., tensile strength) may be misleading. In this study, several quantitative properties were derived from the load versus strain curves. Based on our study, mechanical parameters such as tensile strength could be affected by the thickness and the distribution of the skin matrix. The effect of excised wound site on rabbit ear upon mechanical strength should be carefully considered and controlled. The strength of the wound relates to the size, number, and architectural arrangement of collagen fibrils (Berthod *et al.*, 2001; Brown *et al.*, 1998). In comparative wound healing study *in vivo*, controlling wound exudates absorption capacity of the dressing, aeration and oxygen tension are the important factors to be controlled. After preparing burn wound, tested dressing was applied on the wound and occlusive dressing was placed to control the wound healing environment.

Successful wound closure has been shown to be related to the amount of bacteria in the wound. The closure of a contaminated wound is largely determined by their ability to decrease the number of bacteria within the tissue of the wound. Contaminated wounds must be monitored. When

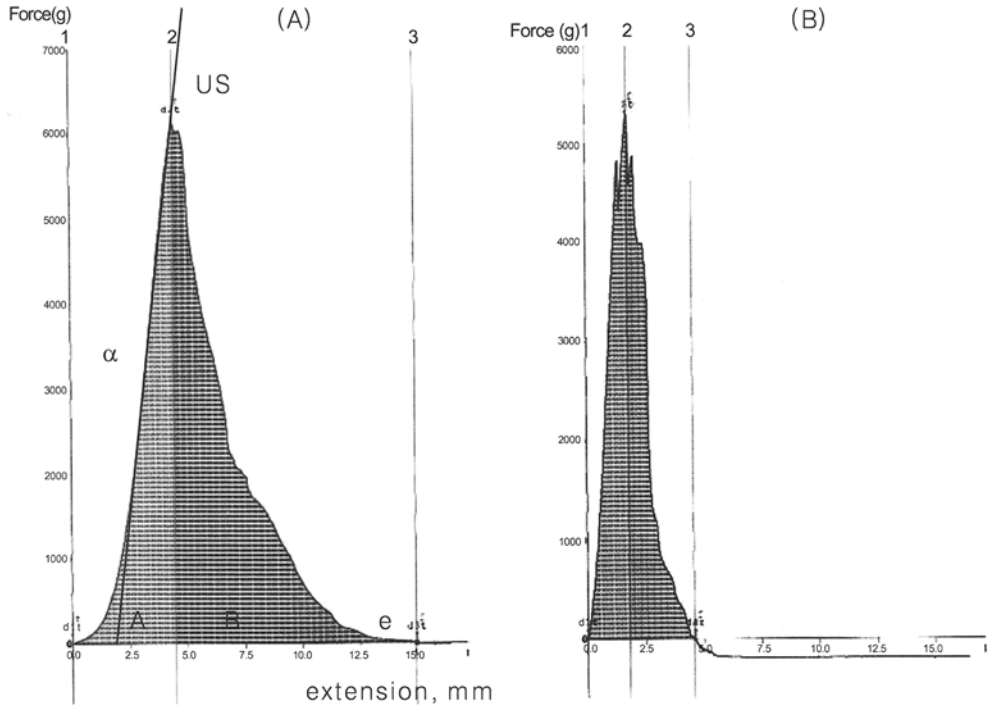

Fig. 4. The load versus extension curve. Panel A: for a normal skin excised from anterior part of rabbit ear. Skin thickness: 1.35 mm; m aximum force: 6100 g, maximum extension: 14.929 mm; Area A (Resilience): 8713.9 g · mm; Area A+B (Toughness) : 24468 g · mm. Panel B: for 2% SSD treated wound excised from anterior part of rabbit ear. Skin thickness: 1.2 mm; maximum force: 5239 g, maximum extension: 4.632 mm; Area A (Resilience): 4816.9 g · mm; Area A+B (Toughness): 9653.9 g · mm.

Table I. Comparison of Biomechanical properties of excised wound from 15 day-post second degree burn rabbit ear model. Data are expressed as mean±S.D. of quadraplicate measurements.

Factors	Normal skin	SSD-treated skin	*p*-value
Ultimate strength (Fmax, g mm^{-2})	256.4±23.1	144.9±22.9	0.004
Resilence (mJ)	40.8±12.2	16.2± 2.8	0.027
Toughness (mJ)	113.4± 7.6	38.9± 3.4	0.0001
Maximum tensile strain (% elongation)	38.1± 9.0	16.6± 5.5	0.024
Elasticity constant, Modulus (g/mm)	809 ±91.2	1132.3±85.5	0.011

the bacteria are at levels of 10^5 or fewer organisms per gram of tissue, then less toxic substances more favorable to specific wound healing processes should be used (Wilgus *et al.*, 2003).

Due to a loss of elastic property, SSD treated wound showed a significant increase in elastic modulus (α: slope). Increase in elastic modulus in SSD treated group suggests that SSD inhibits the regeneration of the fibroblasts and collagen deposition in the dermis result in fragile and stiff membrane. Since fibroblasts are the major components of the dermis, impaired wound healing due to decreased fibroblast cell replication may disturb the new dermal matrix regeneration and proper alignment of collagen fiber networks in dermis result in decreased mechanical strength of the wound.

Many clinicians have believed that inflamed wounds exhibit impaired wound closure (Smoot *et al.*, 1991). Yet, in the absence of inflammation, wound closure is retarded. However, the role of extraneously supplied collagen structural bases and antibacterial agents, SSD, on the prevention of hyper-scar formation needs to be further studied. The alignment and density of collagen fiber networks in dermis after SSD treatment are being investigated by employing Trans emission Electron Microscopy.

In conclusion, antibiotics, SSD in collagen sponge showed cyto-toxicity on the HDF cell growth and caused a significant impairment in wound healing process and a decrease in wound tear strength. Since the decreased mechanical strength may lead to reduction in resilience, toughness and maximum extension the tissue, the identification of optimum dose for SSD that limits infection while minimizes the cyto-toxic effect may be clinically relevant.

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