



Effects of Red Deer Antlers on Cutaneous Wound Healing in Full-thickness Rat Models

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ABSTRACT : The process of wound repair involves an ordered sequence of events such as overlapping biochemical and cellular events that, in the best of circumstances, result in the restoration of both the structural and functional integrity of the damaged tissue. An important event during wound healing is the contraction of newly formed connective tissues by fibroblasts. The polypeptide growth factors, like transforming growth factor- β (TGF- β), insulin-like growth factor I (IGF- I) and epidermal growth factor (EGF), play very important mediator roles in the process of wound contraction. Deer antlers, as models of mammalian regeneration, are cranial appendages that develop after birth as extensions of a permanent protuberance (pedicle) on the frontal bone. Antlers contain various growth factors which stimulate dermal fibroblast growth. They are involved in digestion and respiration and are necessary for normal wound healing and skin health. In order to investigate and evaluate the effects of red deer antlers on skin wound site, the speed of full-thickness skin wound healing and the expression of IGF-I, TGF- β , and EGF in skin wounds, three groups of skin full-thickness rat models with a high concentration of antler ointment, a low concentration of antler ointment and without antler ointment were compared. At post-injury days 0, 2, 4, 8, 16, 20, 32, 40 and 60, the skin wound area was measured, the expressions of IGF-I, TGF- β , and EGF mRNA were detected by reverse transcriptase polymerase chain reaction (RT-PCR) and collagen formation by sirius red dye and the localization of IGF-I, TGF- β , and EGF peptides were inspected by histological immunohistochemical techniques. Wound healing was significantly more rapid in antler treated skins. In addition, the wound treated with a high concentration antler ointment, a low concentration antler ointment, and the control closed completely at post-injury day 40, day 44 and day 60, respectively. Via RT-PCR, the expressions of IGF-I (day 8 and day 16), TGF- β (day 8, day 16 and day 20) and EGF (day 4, day 8, day 16, and day 32) were obviously up-regulated in high concentration antler-treated skins compared to control skins. Similar results could be seen in the histological detection of collagen dye and immunohistochemical methods using the corresponding polyclone antibodies of IGF-I, TGF- β , and EGF. These results illustrate that antlers stimulate and accelerate the repair of cutaneous wounds. (**Key Words :** Deer Antler, Full-thickness, Wound Healing, IGF-I, TGF- β , EGF)

INTRODUCTION

The skin provides the body with its first line of defense against both infection and invasion. Injury, illness, or surgery results in the loss of integrity of large portions of the skin and leads to major disturbances of this barrier function (Gallant et al., 2004). The ability of the skin to repair itself following minor injury is remarkable, but when the injury is severe, medical intervention is required, both to

speed the recovery of the skin itself and to protect the body from infection and fluid loss. Skin wound healing is the process of repair that follows injury to the skin and other soft tissues. Cutaneous wound healing is a complex process involving four major stages: homeostasis, inflammation, proliferation, and maturation/remodeling (Gallant et al., 2004). The cell types and the order in which they appear in the wound have been established. Inflammatory cells first migrate into the plasma clot, followed by fibroblasts, which elaborate collagen and other matrix components and eventually contract the newly formed connective tissue to bring together the edges of the wound (Montesano and Orci, 1988). This process is mediated in part, by the appearance of biologically active peptides or cytokines at the site of

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injury (Benn et al., 1996). Many polypeptide growth factors derived from platelets and inflammatory cells are important mediators of tissue repair processes and their functions have been elucidated.

Transforming growth factor- β (TGF- β) is a hormonally active polypeptide that controls cell growth and differentiation (Ignatz and Massague, 1986). It is present in a wide variety of tissues and is especially abundant in platelets, from which it is released at sites of injury. Experimental work with animal models has also demonstrated that TGF- β , in concert with other growth factors, can promote wound healing (Montesano and Orci, 1988). TGF- β is also a potent chemotactic agent for monocytes and fibroblasts, as it induces the synthesis of extracellular matrix proteins by fibroblasts. TGF- β has been found to directly accelerate the rate of healing of linear incisional wounds in rats (Pierce et al., 1989). Epidermal growth factor (EGF), a polypeptide of 53 amino acid residues, stimulates a variety of biological phenomena including proliferation of the skin and proliferation and differentiation of the epidermis. It stimulates keratinocyte division and epidermal regeneration, and the residual epithelial cells in wound sites proliferate in an integrated manner to regenerate intact epidermis. An early event after treatment of cultured epithelial cells with EGF is the remodeling of the actin cytoskeleton, which can lead to retraction of cells from the substrate surface and rounding (Haase et al., 2003). The effect of EGF on wound healing has been acknowledged, such as its improvement of the partial-thickness of pigs (Brown et al., 1986), acceleration of open-wound healing in rats (Okumura et al., 1990), and enhancement of the process of wound repair in rabbit ears (Franklin and Lynch, 1979).

Another growth factor involved in wound healing is the insulin-like growth factor I (IGF-I). The IGF system is essential for normal embryonic and postnatal growth and plays an important role in the function of a healthy immune system, lymphopoiesis, myogenesis, and bone growth, among other physiological functions. IGF-I is a peptide hormone with a structural homology to proinsulin. It is produced by dermal fibroblasts and macrophages (Rappolee et al., 1988) in skin and is thought to signal to basal epidermal keratinocytes that express the IGF-I receptor (Haase et al., 2003). IGF-I can induce the chemotaxis of inflammatory cells and the proliferation of some cells (Wang and Jiang, 2000). It was reported that IGF-I is a major mediator in all stages of wound healing, including inflammation, and its absence dramatically impairs wound healing across multiple parameters (Kurtz et al., 1999). It is further hypothesized to play a major role in tissue repair as a peptide present in wounds by stimulating extracellular matrix deposition and fibroblast growth (Suh et al., 1992).

Deer Velvet Antler is proving to be one of the most beneficial products in natural medicine. Almost 2000 years ago, oriental medicine practitioners regarded it as essential for health maintenance, as it boosted the immune system, enhanced athletic performance and strength, and promoted anti-inflammatory properties. Its effects on liver function protection, toxicology, stress, and osteoporosis and its role in growth, arthritis, and wound healing were highly regarded. Deer velvet antlers were prescribed in combination with a number of herbs, according to the treatment required. It is a unique mammalian organ that has an annual cycle of regeneration and grows very rapidly from the tip at up to 1 cm/day in red deer within a 90- to 120-day period. Therefore, locally produced growth factors are thought to be the mediators supporting the rapid growth. Growth factors and growth-factor receptors have already been proven to be a factor in the rapid growth and differentiation of antler tissue. These growth factors include insulin-like growth factor (IGF) (Elliott et al., 1992), epidermal growth factor (EGF) and its receptor (EGFR) (Barling et al., 2005), bone morphogenetic protein 4 (Feng et al., 1995), neurotrophin-3 (NT-3) (Garcia et al., 1997), transforming growth factor- β 1 (TGF- β 1), transforming growth factor- β 2 (TGF- β 2) and transforming growth factor- α (TGF α) (Francis and Suttie, 1998), and Nerve Growth Factor (NGF). It has been suggested that the high concentrations of hormone-like substances in deer velvet, or even the cartilaginous concentration of the antler itself, are responsible for the rapid tissue repair after injury.

Understanding how the skin repairs damaged tissue and the factors that influence the wound healing process helps the surgeon ensure an acceptable outcome from surgery. Although several animal models were studied for wound healing through the treatment of growth factors, our goal in this study is to prove the multi-function of antlers in skin repair. We examine the possibility that the application of antlers accelerate wound healing and stimulate the biochemical targets for TGF- β , IGF-I, and EGF.

MATERIALS AND METHODS

Animals and full-thickness skin wound preparation

A total of 48 Sprague-Dawley rats aged six to seven weeks were purchased from Danhan Biolink Inc. (Korea) to be used in this experiment. They were weighed between 180 and 200 g and were arranged into three groups: the Control group (n = 16), Low concentration group (n = 16), and High concentration group (n = 16). Before the initiation of the studies, the rats were acclimated after arrival for a period of one week for the purpose of habituation before experiments. They were housed in per cage under artificial illumination and given unrestricted food and water. The

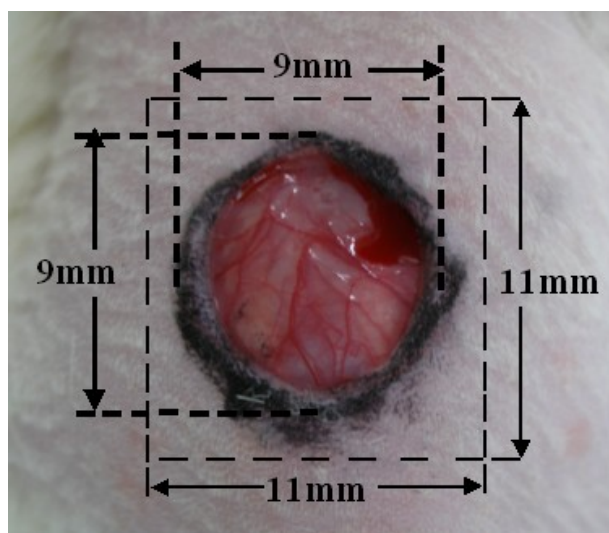


Figure 1. The full-thickness excisional wounds in rats. After anesthetization and shaving the dorsal hair, four portions of full-thickness dorsal skin wound (9 mm diameter) were excised from each mouse. From post-injury day 2, every wound was treated with 0.1 g antler ointment of low concentration, high concentration, and control. On the indicated days after wounding, the major (a, mm) and minor (b, mm) axes of the wounds were measured. Two rats were sacrificed in every group on post-injury days 0, 2, 4, 8, 16, 20, 32, and 40, and full-thickness skin wound (9 mm) containing the 2mm normal margin (2 mm) samples were excised.

ambient temperature was 22-23°C. The surgical damage procedure was carried out under general anesthesia with ether. Immediately after anesthesia, four round sections of full-thickness skin incisions (around 9 mm in diameter) were made using a puncher on the clipped dorsal skin of the animals and the skin margins were approximated with three 0.2 cm. The wounds were not covered with dressing and the day of the surgical damage procedure was taken as day 0. From day 2, every wound was treated with 0.1 g antler ointment of low concentration, high concentration and control Vaseline and covered with bandage. The wounds were retreated with the ointment at days 4, 8, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, and 60, and the curative effect was observed and expressed as the remaining wound area. We calculated the wound area from the product of cross-diameters of the wound site (Toyokawa et al., 2003). The post-excision wound size was calculated using the formula: wound remaining area (mm²) = (wound major a × wound minor b) × π/4 (Akiko et al., 2005). We sacrificed two rats in every group on days 2, 4, 8, 16, 20, 32, and 40, respectively. Wound skin including the 2 mm normal margin (Figure 1) was incised and immediately put in liquid nitrogen. All the skin samples were transferred to -70°C for further experiments.

Preparation of ointment

Fresh red deer (*Cervus elaphus* L. 1758) antlers were collected from an anesthetized four-year old red deer stag from a local deer farm during the late spring (Jinshan, Korea). Two different concentrations of antler extract in ointment were used in this study, one of high concentration and the other of low concentration. Fresh antler tissues (tip) at 90 g (used for high concentration ointment) and 45 g (used for low concentration ointment) were homogenized in a Waring blender. The homogenized antler tissues were defatted by extraction with acetone and a 2:1 chloroform-methanol mixture (Ha et al., 2005). The fat-free antler was suspended in 200 ml of 0.02 M NaCl-HCl buffer (pH 6.0), and incubated in 40°C for eight h and 4°C overnight. The suspension supernatant was filtered and concentrated by dialyzing in polyethylene 8000 (PEG 8000) (Sigma, USA) to 20 ml, followed by adding 100 ml of 80% ethanol for final precipitation. The sample was mixed, allowed to stand for six h, and then centrifuged for 20 min at 10,000 g. The precipitate was dissolved in 5 ml water and mixed in 25 g white Vaseline (Duksan, Korea). The control ointment was prepared without any antler extract. Potein content of antler in each process of extraction was measured using Bradford (Bio-Rad, USA) method at 595 nm absorption.

SDS-PAGE and western blot

And IGF-I, TGF-β1, and EGF were considered as the criterion to assess the efficacy of the antler extract by western blot. The extract from last process was performed the 12% SDS-PAGE (Mini Protean II; Bio-Rad). Proteins were electro-transferred to a nitrocellulose membrane pre-soaked with transfer buffer (Millipore, USA) at 90 mA for 2.5 h after SDS-PAGE. The membrane was blocked overnight at 4°C with an ECL blocking agent containing 5% skimmed milk (Amersham, USA) in 1×TTBS (20 mM Tris PH 7.5, 100 mM NaCl, and 0.5% Tween 20). Immunodetection was performed by incubation with a 1:500 dilution of primary antibody, specific polyclonal IgG (Santa Cruz Biotechnology, USA) for 2 h at room temperature. The primary antibody used was TGF-β rabbit polyclonal IgG (Santa Cruz Biotechnology, CA), IGF-I goat polyclonal IgG (Santa Cruz Biotechnology, CA), and EGF rabbit polyclonal IgG (Santa Cruz Biotechnology, CA). Followed by incubation for 1 h at room temperature with ECL anti-rabbit IgG hoseradish-peroxidase-linked whole second antibody (Amersham, USA) at 1:3,000 dilution in a blocking solution. Blot were detected by chemiluminescence using an ECL Advance Western-blotting detection kit (Amersham, USA).

Total RNA extraction

The total RNA extraction was performed using a modification of the Trizol reagent method described by the

Table 1. Protein content of antler extract in each process

Extract process	90 g antler (n = 3)		45 g antler (n = 3)	
	Total protein (g)	Protein content (%)	Total protein (g)	Protein content (%)
Defatted	14.00	15.56	8.80	19.56
NaAc-HAc	14.61	16.23	10.16	22.58
80% Ethanol	43.80	48.66	23.60	52.44

Table 2. Primers and expected sizes of PCR products with each primer pair

Primer	Sequence (5'→3')	Size (bp)
Actin sense	CCCTAAGGCCAACCG TGA AAA GAT G	220
Actin antisense	GTCCCGGCCAGCCAGGTCCAG	
TGF- β sense	CTTCAGCTCCACAGAGAAGAAGTGC	298
TGF- β antisense	CACGATCATGTTGGACAAGTCTCC	
IGF-I sense	AAATCAGCAGTCTTGGAAACC	395
IGF-I antisense	CTTCTGGGTCTGGGCATGT	
EGF sense	TATGTCTGCCGGTGCTCAGAA	415
EGF antisense	AGCGTGGCGCAGTTCACCA	

manufacturer's instructions (Invitrogen, USA). The integrity of the RNA was electrophoretically verified by ethidium bromide staining in 1.2% agarose gel under denaturing conditions, and the quality of the RNA was estimated by optical density (OD) absorption ratio $OD_{260}/OD_{280} > 1.9$ using an Agilent 2100 bio-analyzer.

Reverse-transcription

One microgram total RNA from the sample preparation was reverse transcribed by using a Maxime RT premix kit (iNtRON, Korea). Two μg of total RNA were heated at 75°C for five min, immediately after, it was cooled on ice for over two min. Template RNA and sterilized DEPC dd H_2O were added into the Maxime RT premix kit tubes containing Oligo dT primers for a total final volume of 20 μl . Reactions were carried out at 45°C for 60 min in a thermocycler system (Bio-Rad, USA), followed by a five min step at 95°C to denature the enzyme, and then cooled at 4°C. The reactant was diluted by adding 50 μl sterilized dd H_2O into a tube containing the complementary DNA (cDNA) obtained at RT reactant and then stored at -20°C.

RT-PCR

RT-PCR was carried out by using i-Taq DNA polymerase (Biosesang, Korea). The cDNA (1 μl) was amplified in a 20- μl reaction mixture including: 1 μl 10 mM dNTP, 1 μl 10 mM forward, 1 μl 10 mM reverse primers (Genotec, Ltd, Korea), 2 μl reaction buffer (containing 80 mM KCl, 4 mM MgCl_2), and 0.25 μl i-Taq (5 U/ μl). The reaction was performed by the thermocycler (Bio-Rad, USA). All the primer sequences specific for the genes of Actin- β (Garcia et al., 1997), IGF-I (Bamman et al., 2001), EGF (Lee and Joo, 1999), and TGF- β (Tsunoda et al., 2001) were examined and the RT-PCR condition for every gene is shown in Table 2.

Histology and immunohistochemistry

The skin specimens for immunohistochemistry were fixed in 10% paraformaldehyde and embedded in paraffin. Immunohistochemistry was carried out by using the Zymed lab-SA detection system (Invitrogen, USA). The sections were deparaffinized and placed in 100 mM PBS for 10 min before starting the staining procedure. The tissue sections were deparaffinized, rehydrated in graded alcohols, and submerged in the peroxidase quenching solution (1 volume H_2O_2 in 9 absolute pure methanol) to eliminate the endogenous peroxidase activity for 10 min, followed by being rinsed in PBS in three steps for two min. Sections were blocked in 10% non-immune goat serum for 10 min and incubated at 37°C for 1 h in 1:200 primary antibody. The primary antibody used was TGF- β rabbit polyclonal IgG (Santa Cruz Biotechnology, CA), IGF-I goat polyclonal IgG (Santa Cruz Biotechnology, CA), and EGF rabbit polyclonal IgG (Santa Cruz Biotechnology, CA). This was followed by washing in PBS and reacting with streptavidin-peroxidase conjugate for 10 min. The peroxidase activity was detected with the peroxidase substrates (DAB) system controlled under the microscope. After counterstaining with hematoxylin, the slides were dehydrated with grade series of alcohol, cleared in xylene, and mounted with a coverslip.

Collagen determination

The collagen content change in the skins of every sacrificed rat was examined by sirius red F3B staining (Chen et al., 2004). Paraffin sections were deparaffinized in graded alcohols and stained with 0.1% (w/v) sirius red F3B (Sigma, USA) for one h in a saturated aqueous solution of picric acid (Sigma, USA). After staining, the slides were rinsed for five min in two changes of acidified water (5 ml acetic acid in one liter of water), followed by dehydration in alcohol and then mounted in a resinous medium. The presence of collagen fibers was determined by light

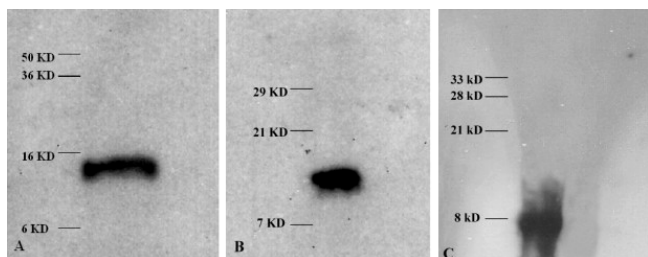


Figure 2. Western-blot of IGF-I, TGF-β1, and EGF. The extract for ointment showed the IGF-I blot band, 12 kDa (A), TGF-β1 blot band, 7-21 kDa (B), and EGF blot band, 8 kDa (C).

microscopy in histological preparations of the skin wound on different post-wound healing days.

RESULTS

Protein content of antler extract in each process and the

detection of growth factors by Western-blot analysis

Protein content of antler extract in each process during ointment preparation was measured. Table 1 showed that protein content of 90 g antler extract, for high concentration ointment. In defat process, NaAc-HAc extract process, and 80% ethanol precipitation process, the total protein was 14.00 g, 14.61 g, and 43.80 g and the protein content was 15.56%, 16.23%, and 48.66%, respectively; And the total protein in each process of 45 g antler extract, for low concentration ointment was 8.8 g, 10.16 g, and 23.60 g, and the protein content was 19.56%, 22.58%, and 52.44%, respectively. To assess the efficiency of ointment, IGF-I, TGF-β1, and EGF were considered as the efficient components and tested by western-blot. From Western-blot analysis revealed the presence of the bands that bound to the antibodies are likely to be IGF-I (Figure 2A), TGF-β1 (Figure 2B), and EGF (Figure 2C).

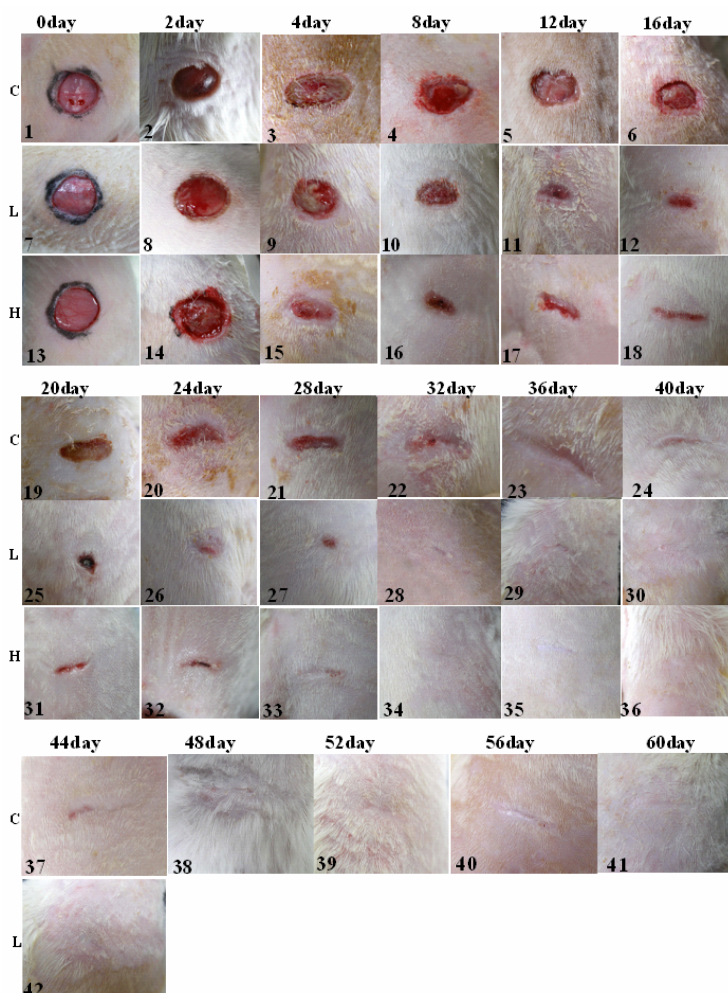


Figure 3. Effects of antler ointment on closure of full-thickness excisional wounds in rats. From post-injury day 2, each wound was treated with 0.1 g antler ointment of low concentration (L) (photos 7-12, 25-30, and 42), high concentration (H) (photos 13-18, and 31-36), or Vaseline (C) (photos 1-6, 19-24, and 37-41). The wounds were covered with bandage. Wounds were again treated at post-injury days 4, 8, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, and 60. The curative effect was observed and expressed as the remaining wound area. The post-excision wound size was calculated using the formula: wound remaining area (mm²) = (major a × minor b)(π/4) (Akiko et al., 2005).

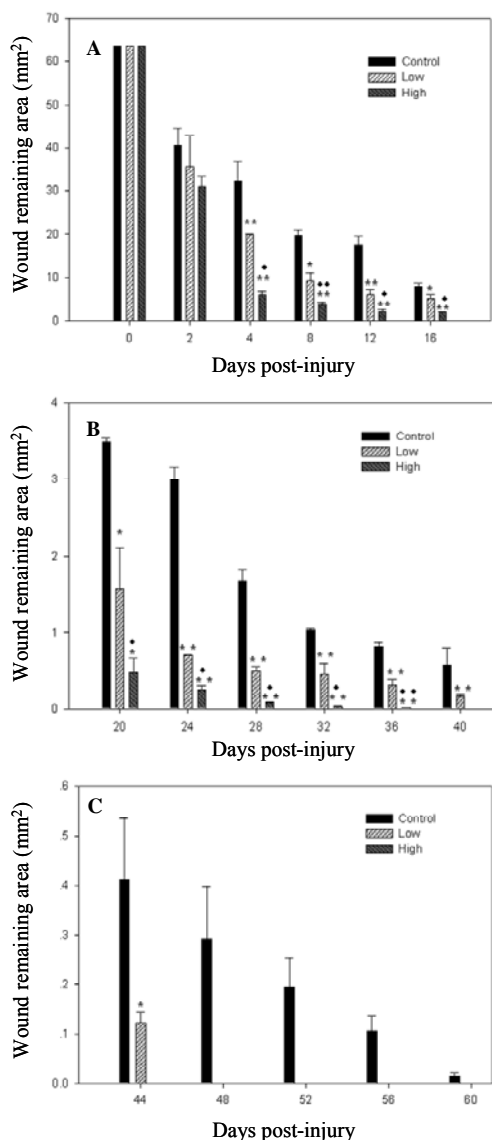


Figure 4. Effects of deer antler ointment on stimulation of wound closure on post-injury days in rats. Thickness excisions were treated with a topical application of antler ointment with low and high concentration and Vaseline treatment was taken as the control. At the indicated times post-injury, the remaining wound region was quantitated and the remaining wound areas were assessed for closure according to the formula: wound remaining area (mm^2) = $(a \times b) \times \pi / 4$ (Akiko et al., 2005). (A) The remaining wound area on post-injury days 0 to 16; (B) remaining wound area on post-injury days 20 to 40; (C) The remaining wound area on post-injury days 24 to 60. Data shown are combined results from three independent experiments, error bars represent the mean \pm SEM. $0.01 < * p < 0.05$, and $** p < 0.01$: compared with low concentration-treat and control, as well as high concentration-treat and control; $0.01 < \blacklozenge p < 0.05$, and $\blacklozenge p < 0.01$: compared with low concentration-treat and high concentration-treat.

Closure of excisional full-thickness skin wounds during healing process

Full-thickness dermal excisions were removed from the

backs of the test rats, and the topical application of low and high concentration deer antler extracts and the Vaseline as the control were applied to the wounds. As shown in Figure 3, wounds treated with the antler ointment are consistently smaller than those treated with the Vaseline ointment. The difference in the sizes of the remaining wound area is apparent at post-injury day 4: 32.18 mm^2 in the Vaseline control group, 19.83 mm^2 in the low concentration ointment group, and 6.08 mm^2 in the high concentration ointment group. At post-injury day 40, the wounds treated with high concentration antler ointment have completely closed, while the control wound remained slightly open. The remaining wound area was 0.156 mm^2 in the low concentration ointment group and 0.57 mm^2 in the control Vaseline group. Only at post-injury day 44 does the wound in the low concentration antler ointment group become completely closed, while the wound of the control group still had a remaining wound area of 0.016 mm^2 at post-injury day 60. The differences in the sizes of the remaining wound area can be seen in Figure 4. These data indicate that the effects of the antler extract ointment on wound closure are dose-dependent.

Expression profiles of IGF-I, EGF, and TGF- β in the skin during wound healing

In semi-quantitative RT-PCR using β -actin (Figure 5A) as an internal control, we utilized a set of highly specific primers for each gene and examined the expression change of the growth factors during wound healing. Expression of the growth factors such as IGF-I, EGF, and TGF- β strongly affected growth cycle (Kawano et al., 2004). We found that each gene studied was expressed at the same level in the healthy skins at post-injury day 0. TGF- β expressions were found to be concentration-dependent. In wounds treated with antler ointment, especially in those treated with high concentration ointment, the TGF- β levels were found to be elevated at day 8 after the wounding occurred, as opposed to the levels in the control group (Figure 5B). Expressions in the wounds treated with antler ointment were found to continuously increase and are significantly stronger than that in the wounds treated with the Vaseline ointment. Particularly at day 16, the levels of TGF- β reached its maximum and then declined after. However, compared with the control group, expression levels in wounds treated with the antler ointment were still higher. This suggests an induction of the endogenous rat TGF- β after application of the antler ointment treatment. Similar results of IGF-I expressions were found (Figure 5C). The expressions in the wounds treated with antler ointments, both in the low and high concentration treatment groups, appear to increase at post-injury day 4. It was previously shown to be expressed stronger in the antler ointment-treated wounds at post-injury day 8 and day 16. High levels of IGF-I mRNA were

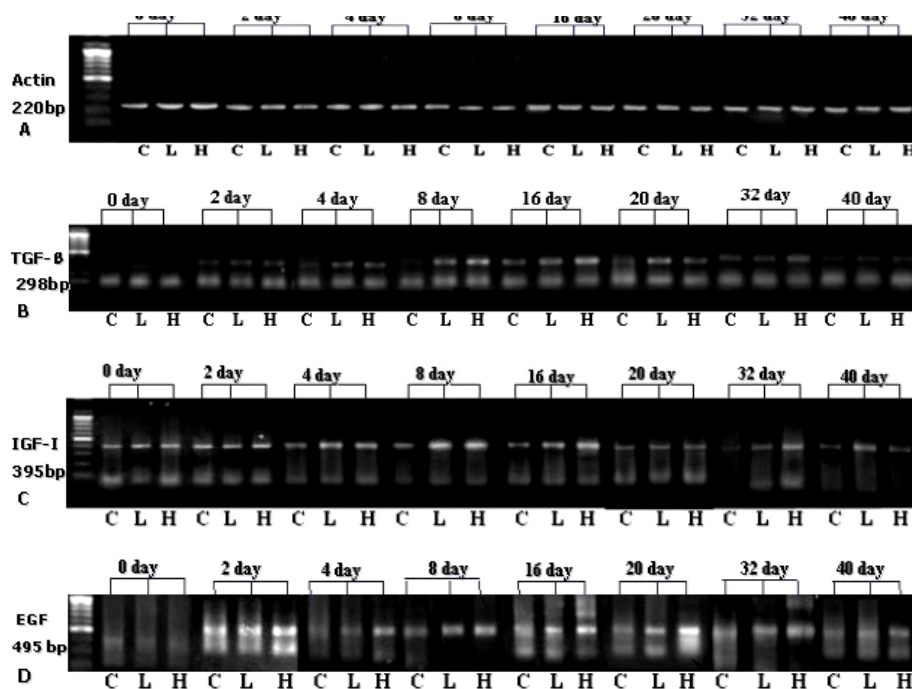


Figure 5. Reverse transcription-polymerase chain reaction (RT-PCR) amplification of TGF- β , IGF-I, and EGF mRNA at indicated days post-injury. RT-PCR was performed on RNA isolated from wound samples. To determine relative changes of TGF- β (B), IGF-I (C), and EGF (D) in mRNA levels during development, RT-PCR was performed using specific primers and fractionated on a 1.0% agarose gel. The size of the predicted amplified product for TGF- β (298 bp), IGF-I (395 bp), and EGF (415 bp) is indicated on the right. A 220 bp β -actin fragment was amplified from the same RT reaction to serve as an endogenous internal control (A). C = Control Vaseline ointment group; L = Low concentration of antler ointment group; H = High concentration of antler ointment group.

apparently maintained at post-injury day 32 compared to the levels in the control skins. In addition to this, we also found that the levels of mRNAs encoding EGF were all significantly upregulated at post-injury days 8, 16, and 20 in the wounds treated with the antler ointment (Figure 5D). The strongest expression in antler ointment-treated wounds was at post-injury day 16 as compared to the control wounds where EGF was expressed at low levels.

Collagen determination

Sirius red staining was performed in order to evaluate the collagen formation in the wounds. In bright-field microscopy, the collagen is red on a pale yellow background. There is no distinctly different collagen staining between the control wound and the wound treated with antler ointment until post-injury day 4. However, from post-injury day 8, lesser collagen staining in control slides compared to the amount of red-stained collagen were observed in the antler ointment treatment slides, especially in high concentration antler ointment-treated skins (Figure 6). The fibers appeared as wavy structures of variable width and intermediate length. Collagen staining in high concentration antler ointment treatment slides is significantly stronger and dense beneath the epidermis in post-injury days 16, 32, and 40. These results demonstrate

that local application of deer antler extract accelerates collagen formation during the wound healing process.

Immunohistochemistry and Immunoreactivity of IGF-I, TGF- β , and EGF during wound healing

Soon after injury, the wounded skins were fixed in 10% paraformaldehyde and embedded in paraffin. The sections to which the antibody immunologically saturated by TGF- β antigen was applied showed a difference in the staining between control and antler ointment-treated skins (Figure 7). At post-injury day 0, no normal skin epidermis could be seen in the slides. During the healing process, acute inflammatory infiltrates showing positivity to TGF- β in the wound side and positive infiltrates cells that have the tendency to move toward the epidermis (Figure 7-6, black arrow) in wounds treated with high concentration antler ointments could be observed. Eight days post-injury, the thin layer epidermis formed in antler ointment-treated skins and TGF- β positive staining intensity increases accumulated, occupying most of the skin dermis surface (Figure 7-8, 7-9, black arrow). The inflammatory cells containing TGF- β were also seen throughout the wound healing process. The positive intensity shows that the concentration-dependence and intensity is always stronger in high concentration antler ointment-treated skins than that

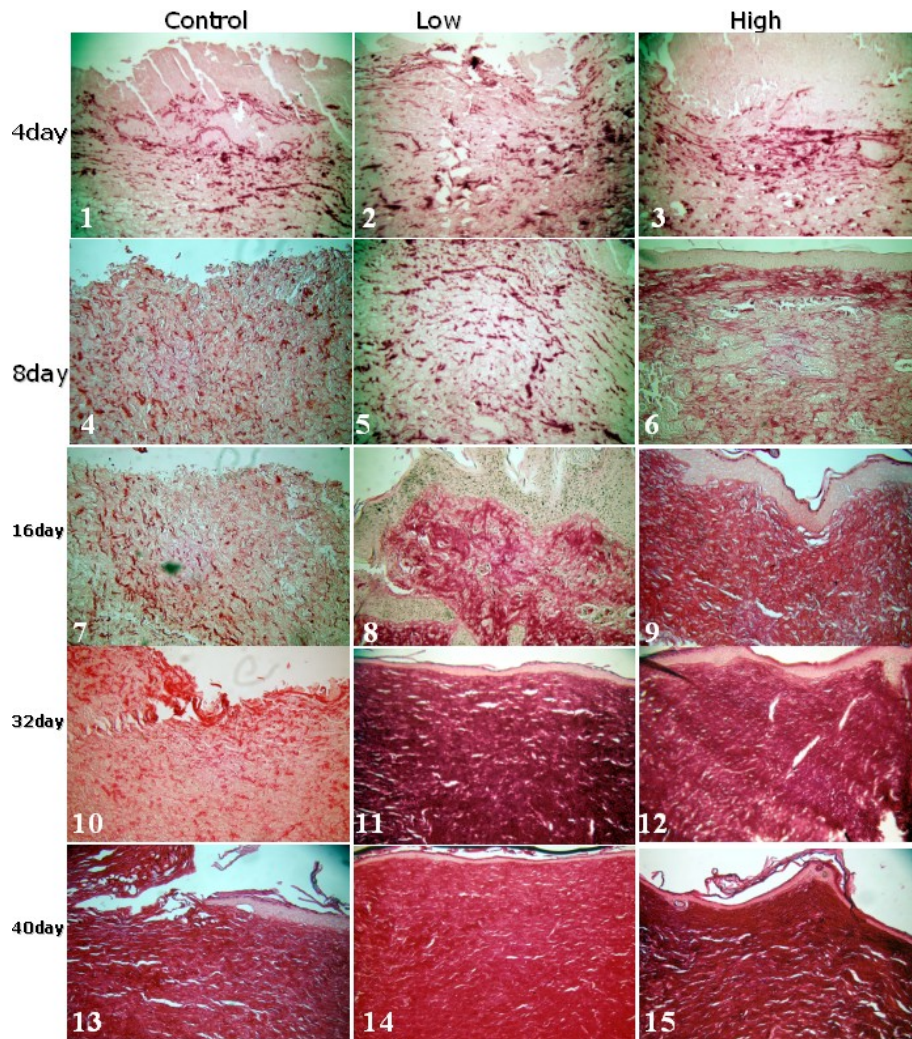


Figure 6. Collagen orientation in full-thickness wound samples from Vaseline (1, 4, 7, 10, and 13), low concentration (2, 5, 8, 11, and 14), and high (3, 5, 9, 12, and 15) concentration of antler ointment groups at indicated days post-injury. Sections were stained with sirius red in picric acid (Original magnification $\times 100$).

in those of low concentration antler ointment-treated skins. At post-injury day 40, when the wound is almost closed, infiltrating inflammatory cells and fibroblasts in the wound show high positivity to TGF- β in antler ointment-treated skins than that in control skins (Figure 7-20, 7-21, black arrow). No positive staining to TGF- β in the wounds treated with Vaseline was seen at the early post-injury days (Figure 7-7). Positive cells were only found on post-injury day 20 (Figure 7-13, black arrow). At post-injury day 40, the wounds were not completely closed and the remnant of the wound without TGF- β positive staining could be seen.

Detection of IGF-I in wound skins by immunohistochemistry and immunoreactivity for IGF-I was found in early antler ointment-treated wound skins at post-injury day 4 (Figure 8-5, 8-6 black arrow), and the positive intensity increased successively after post-injury day 4. However, for the control group, the positive stained cells were first found only on post-injury day 16 (Figure 8-10, black arrow),

specifically, a fainter staining at the sides of the wound. On the same day, new epidermis with almost complete positive staining could be found in wounds from high antler concentration ointment treatment groups (Figure 8-12, black arrow).

From DAB-stained sections, control samples and antler ointment treatment samples have the same skin histology at post-injury day 0 (Figure 9-1, 9-2, 9-3). However, at post-injury day 4, positive immunoreactive cells can be seen in the high concentration antler ointment-treated wound skins (Figure 9-6, black arrow) and the intensity increases at day 8 (Figure 9-9, black arrow). Meanwhile, in low antler concentration ointment-treated skins, few positive cells appear at the edges of the newly grown skin at post-injury day 16 (Figure 9-11, black arrow). However, EGF immunoreactive cells are retarded and can only be seen in the control skins at post-injury day 20 (Figure 9-13, black arrow). Although more positive cells have been detected in

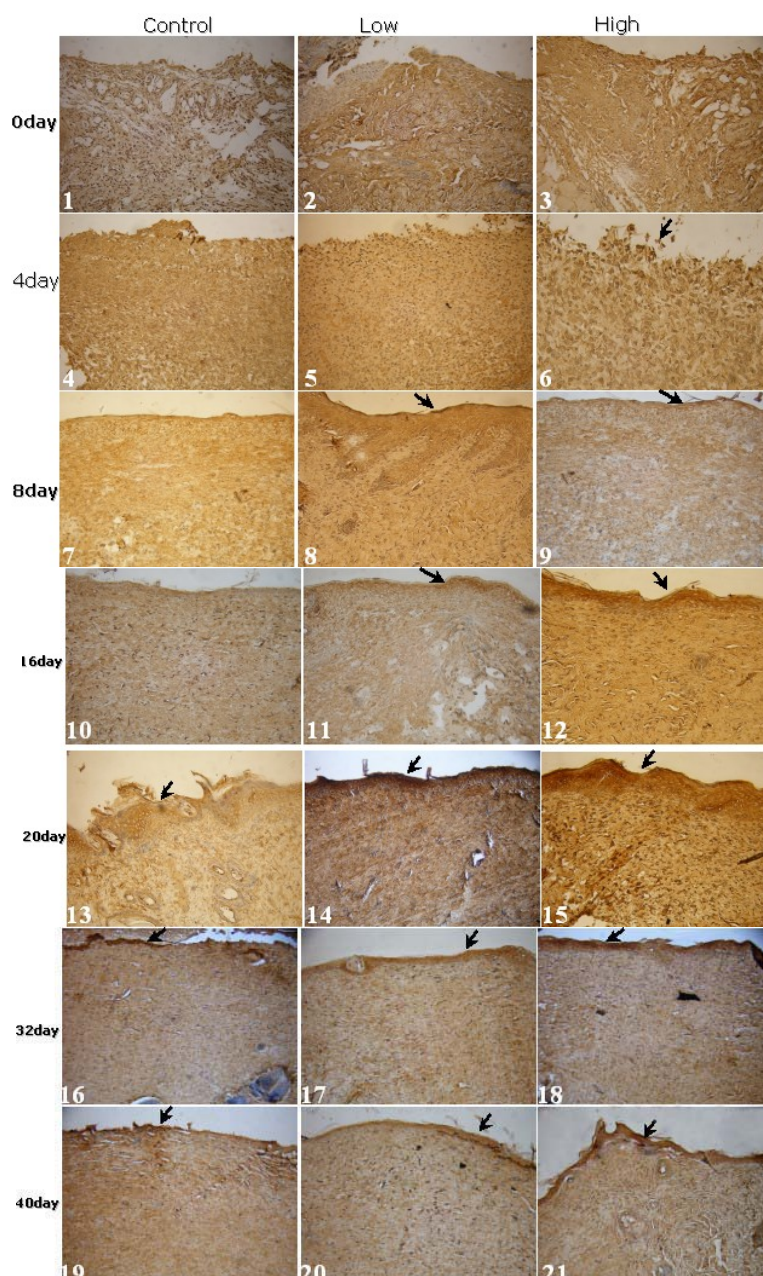


Figure 7. Immunohistochemical localization of TGF- β in full-thickness wound samples from Vaseline (1, 4, 7, 10, 13, 16, and 19), low concentration (2, 5, 8, 11, 14, 17, and 20), and high (3, 6, 9, 12, 15, 18, and 21) concentration of antler ointment groups at wound healing stages. Sections of wounds harvested at indicated days after wounding were immunostained for TGF- β using the DAB technique detection (brown is positive staining). Positive reaction products in high concentration treatment samples show high positivity accumulates on the epidermis of healing wound skin. The epidermis of cutaneous wounds from the control stained much less intensely for TGF- β . The presence of immunoreactive TGF- β was primarily noted at post-injury day 4 in wound skin from high concentration antler ointment treatment sample (6, black arrow), at post-injury day 8 in wound skin from low concentration antler ointment treatment sample (8, black arrow), and at post-injury day 20 in the control group sample (13, black arrow), respectively. Arrows indicate the wound site as determined microscopically from the wound (Original magnification $\times 100$).

the control skins from day 20, a significant difference in the intensity of positive cells in the control group and in those in the antler ointment treated skins is obvious. Control skin samples show a thicker epidermis when compared with the antler ointment-treated skins at post-injury days 32 and 40. There was an increased intensity in the low concentration

antler ointment-treated skins at the basal layer as well as in the high concentration antler ointment-treated skins, where the complete continuous epidermis are formed (Figure 9-18, 20, 21), suggesting epidermal proliferation and an acceleration in epidermal differentiation after application of the antler ointment.

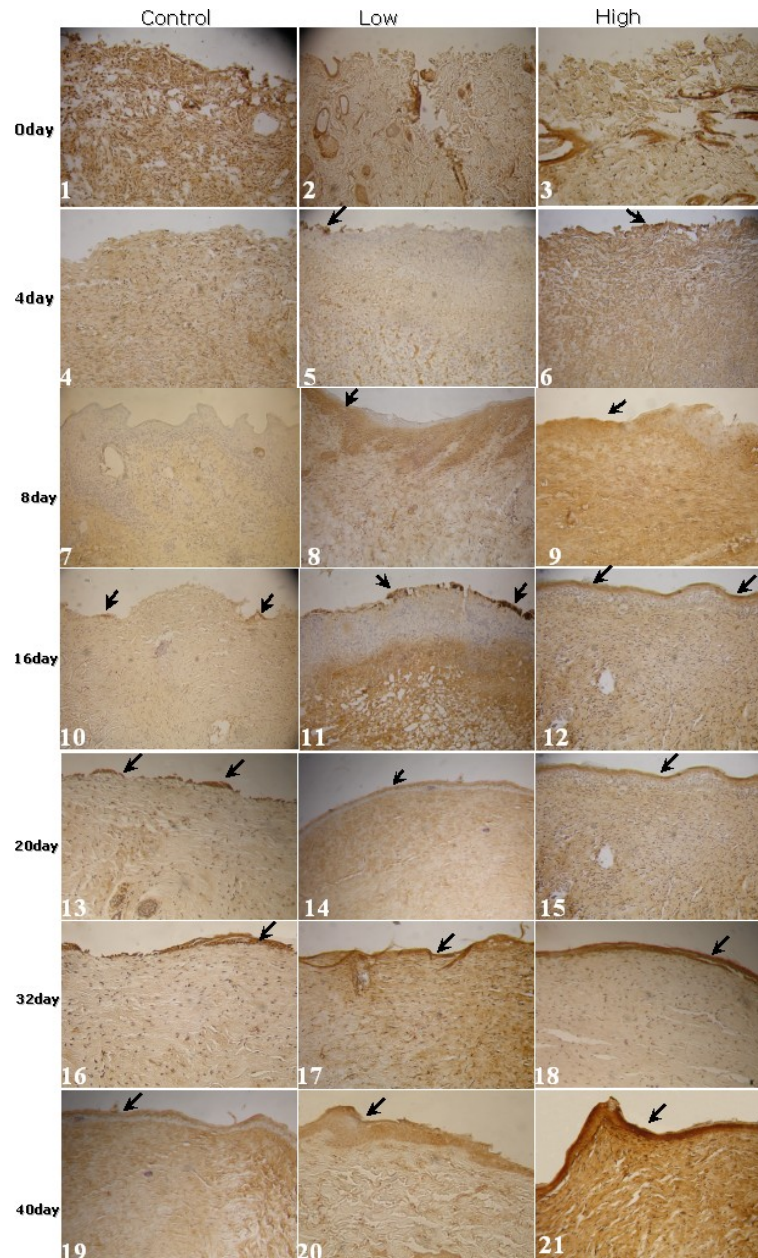


Figure 8. Immunohistochemical localization of IGF-I in full-thickness wound samples from Vaseline (1, 4, 7, 10, 13, 16, and 19), low concentration (2, 5, 8, 11, 14, 17, and 20), and high (3, 6, 9, 12, 15, 18, and 21) concentration of antler ointment groups at wound healing stages. Sections of wounds harvested at indicated days after wounding were immunostained for IGF-I using the DAB technique detection (brown is positive staining). Positive reaction products in high concentration treatment samples show high positivity accumulates on the epidermis of healing wound skin. The epidermis of cutaneous wounds from the control stained much less intensely for IGF-I. The presence of immunoreactive IGF-I was primarily noted at post-injury day 4 in wound skin from high concentration antler ointment treatment sample (6, black arrow), at post-injury day 4 in wound skin from low concentration antler ointment treatment sample (5, black arrow), and at post-injury day 16 in the control group sample (10, black arrow), respectively. Arrows indicate the wound site as determined microscopically from the wound (Original magnification $\times 100$).

DISCUSSION

Deer velvet antler has been found to contain a variety of complex elements in growth progress (Jeon et al., 2006; Jeon et al., 2007), including amino acids (almost half of its substance), enzymes, minerals, vitamins, fats, male and

female hormones, leuteinizing hormone, a very potent growth-promoting hormone known as IFG-1 (insulin-like growth factor), Epidermal Growth Factor (EGF), and transforming growth factor- β 1 (TGF- β 1). These growth hormones and factors have growth promoting actions on the skin. In this study, we explored and demonstrated the

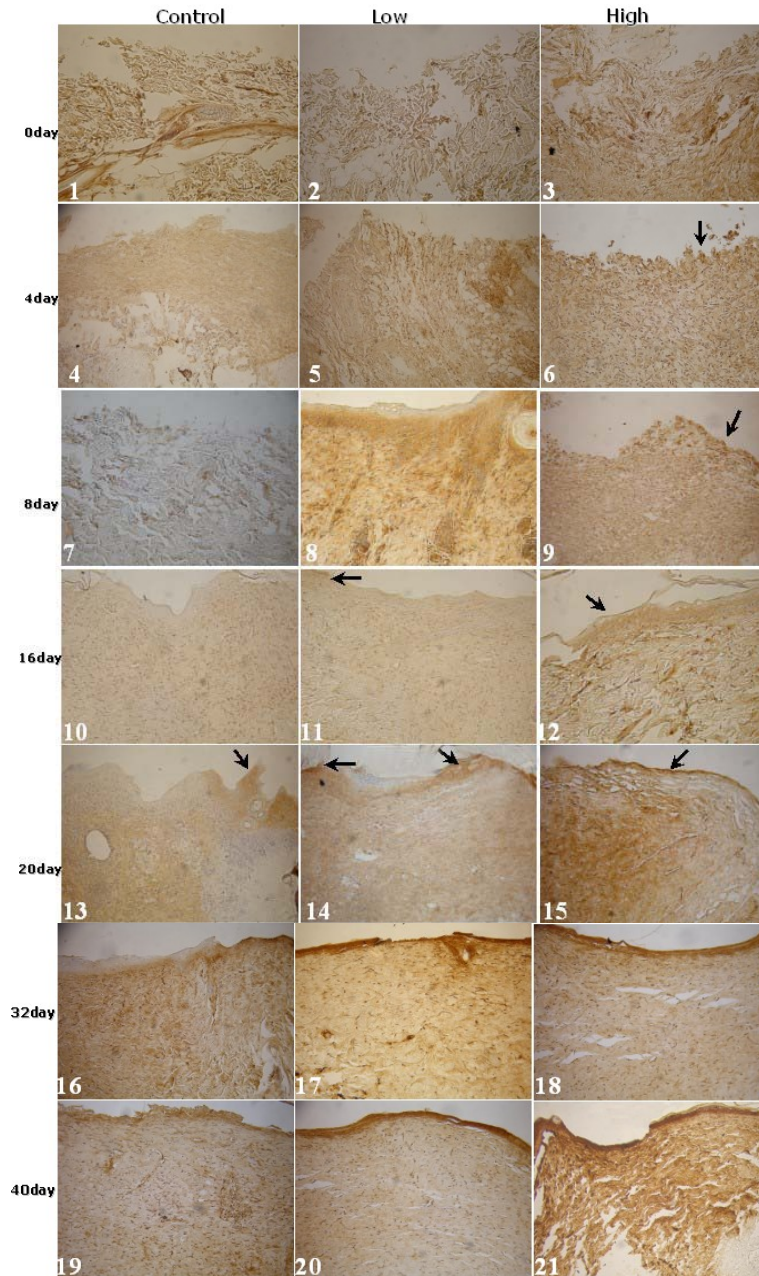


Figure 9. Immunohistochemical localization of EGF in full-thickness wound samples from Vaseline (1, 4, 7, 10, 13, 16, and 19), low concentration (2, 5, 8, 11, 14, 17, and 20), and high (3, 6, 9, 12, 15, 18, and 21) concentration of antler ointment groups at wound healing stages. Sections of wounds harvested at indicated days after wounding were immunostained for EGF using the DAB technique detection (brown is positive staining). Positive reaction products in high concentration treatment samples show high positivity accumulates on the epidermis of healing wound skin. The epidermis of cutaneous wounds from the control stained much less intensely for EGF. The presence of immunoreactive EGF was primarily noted at post-injury day 8 in wound skin from high concentration antler ointment treatment sample (9, black arrow), at post-injury day 16 in wound skin from low concentration antler ointment treatment sample (11, black arrow), and at post-injury day 20 in the control group sample (13, black arrow), respectively. Arrows indicate the wound site as determined microscopically from the wound (Original magnification $\times 100$).

hypothesis that deer antlers may promote wound repair. The epithelium of the wound skin showed smooth regeneration and complete resurfacing within 40 days after abrasion in antler ointment-treated skins.

In the wound healing process, the inflammatory phase is

characterized by hemostasis and inflammation. Collagen exposed during wound formation activates the clotting cascade (both the intrinsic and extrinsic pathways), thereby initiating the inflammatory phase. Moreover, glycosaminoglycans (GAGs), the important component stimulating chondrocytes to make new collagen and

proteoglycans, are proofed present in the antler (Newbrey and Banks, 1983). And the content of GAGs was also already detected in stages of antler development (Lee et al., 2007). The fibroblast is the connective tissue cell responsible for collagen deposition that is needed to repair the tissue injury (Diegelmann and Evans, 2004). Following acute inflammation from about four days after injury, intense positive reaction to TGF- β was seen in inflammatory cells in the antler ointment treatment group, suggesting that TGF- β may play a role in the inflammatory response (Hayashi et al., 1989). TGF- β exerts chemoattractant and proliferative effects on fibroblasts (Singer and Clark, 1999). Wound contraction was enhanced by stimulating fibroblast attachment and by increasing the deposition of collagen matrix (Theoret et al., 2001). It has been demonstrated that TGF- β is a hormonally active polypeptide that controls cell growth and differentiation and has strong chemoattractivity to fibroblasts and monocytes (Sporn et al., 1983). Therefore, TGF- β expressed in the inflammatory phase may be a mediator that draws fibroblasts and macrophages, the cellular components of wound healing, into the inflammatory focus.

The main mechanism of healing during primary intention is connective tissue matrix deposition, where collagen, proteoglycans, and attachment proteins are deposited to form a new extracellular matrix. Collagen, a key wound resource, is a biologically active and very dynamic wound healing material specific to skin tissue. Collagen's performance as one of the body's primary wound healing resources has been well known and documented for more than seventy years. It creates the proper environment for the skin tissues' cellular activity to take place. This cellular activity is necessary for the skin tissues' natural healing process to occur. It has been demonstrated that TGF- β increases the expression of the major extracellular matrix proteins, fibronectin, and collagen (Ignatz and Massague, 1986). There is the evidence that TGF isolated from bovine accelerated the accumulation of total protein, collagen, and DNA in treated chambers (Sporn et al., 1983). TGF- β has been shown to be chemotactic for inflammatory cells and for fibroblasts, and has the ability to induce fibroblasts to produce increased amounts of collagen and fibronectin (Montesano and Orci, 1988). TGF- β further enhances fibroblast and smooth muscle cell chemotaxis and modulates collagen and collagenase expression (Diegelmann and Evans, 2004).

TGF- β in concert with EGF, accelerates the accumulation of protein, collagen, and DNA in a wound chamber model. EGF was demonstrated to be able to mediate an increase in collagen content in wound repair (Hiramatsu et al., 1982). However, EGF itself is not a chemoattractant for fibroblasts or inflammatory cells. It is possible that EGF mediates its effect via a mixture of both mitogenesis and recruitment. Moreover, the study also revealed that EGF alone did not improve wound healing

(Okumura et al., 1990). It is possible that EGF may exert its wound-healing properties via a mitogenic effect on granulation tissue, or it may involve recruitment or interactions among other factors and multiple cell types (Buckley et al., 1985). In addition, as our results report here, eight days after injury, there was a significant increase in collagen content in antler-treated skins. Collagen accumulation appeared to be the result of an equivalent rate of synthesis by increase in the expression of EGF and TGF- β .

Some differences have been noted in the efficiency of low concentration and high concentration treatment in promoting wound healing in this study. One of the explanations may be the dosage form administered to the wound site. This hypothesis was authenticated by the observation of significant wound healing when EGF was released continuously (Buckley et al., 1985). The cellular interactions of the antler ointment suggest that the wound healing properties of these growth factors in the antler might be enhanced by constant exposure of the tissue to the growth factors.

Functions of epidermal keratinocytes are thought to be regulated by a multitude of extracellular influences, such as contact to extracellular matrix molecules and soluble factors that act in an autocrine or paracrine fashion. IGF-I, which is known to have multiple points of action such as mediation of the growth-promoting action of growth hormone (GH), also has normal wound healing as a major physiological mediator. IGF-I has been shown to act in several phases of wound healing (Suh et al., 1992) which exerts a similar function as EGF in epidermal keratinocytes; it regulates keratinocyte shape by stimulating plasma membrane protrusion and spreading. This action of IGF-I results in the acceleration of wound epithelialization *in vitro* (Haase et al., 2003). Studies made with rats demonstrated that IGF-I reverses the impairment of wound healing (Suh et al., 1992). IGF-I may directly affect all cells involved in musculoskeletal soft tissue repair. *In vitro* experimentation has shown that IGF-I directly regulates tenoblastic function by stimulating tenoblastic activity, migration, and proliferation (Kurtz et al., 1999). During the next stage of healing, fibroblasts are attracted into the wound, proliferate, and produce collagen. The expression of the IGF-I gene is increased in antler-treated skins at post-injury day 16, which demonstrates that the IGF-I released by fibroblasts can act in an autocrine mode, stimulating proliferation and collagen synthesis.

A variety of animal models, such as rat model, rabbit model, and pig model, have been carried out in the wound healing treatment with growth factors. Every model suggests that the addition of growth factors to wounds is beneficial. However, there is still one mechanism that needs further study, that is, the apparent uniformity of growth factors in deer antler and whether or not this collaborates or

individually affects the keratinocyte proliferation and migration in the wound healing process. Because wound healing is a complex process that can be influenced, it is unlikely that a single growth factor will be able to resolve all issues of repair or strengthen all vulnerabilities of chronic wounds (Robson et al., 1998). Although these growth factors are proven to be the key physiological mediator of wound healing, tissue oxygen, lactate, cytokines, other biologic agents, and other growth factors are also important in this process. Multiple synergistic actions are highly probable (Suh et al., 1992). Deer antler, which contains abundant factors, has promising targeting growth factor therapy for wound healing.

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