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Effects of Topical Elk Velvet Antler on Cutaneous Wound Healing in Streptozotocin-Induced Diabetic Rats

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ABSTRACT

Objective: Wound repair is a finely orchestrated process involving cellular, molecular, physiologic, and biochemical interactions that restore the integrity of damaged tissue. Cyclic replacement of deer antlers requires rapid regenerative growth, in many ways analogous to that encountered during tissue repair. Molecular mechanisms regulating these processes are not fully understood, but it is increasingly apparent that growth factors are important mediators. Previous studies have shown that elk velvet antler (EVA) contains various growth factors and that a water-soluble extract stimulates dermal fibroblast growth *in vitro*.

Design: The efficacy of EVA water-soluble extract on wound healing in streptozotocin-induced diabetic rats was evaluated using a full-thickness cutaneous wound model. Animals were randomly selected to receive topical application of either control or EVA gel. Daily photographs of the wounds served to measure the rate of wound closure. Wound-edge biopsies obtained on postoperative days 2 and 10 allowed histologic evaluation and measurement of transforming growth factor-beta 1 (TGF-β_1) concentrations by enzyme-linked immunoabsorbent assay.

Results: Wounds treated with the EVA topical gel were significantly smaller by postoperative day 6. TGF-β_1 protein expression was not different in EVA-treated wounds compared to control wounds.

Conclusions: This study indicates that topical treatment with an EVA water-soluble extract accelerates repair of cutaneous wounds in diabetic rats. Further studies are warranted to reveal the mechanisms involved in EVA enhancement of wound closure and to determine if this compound is an economical pharmacologic agent in the treatment of normal and compromised wounds.

INTRODUCTION

Wound repair is a complex process involving inflammation, cellular proliferation and tissue remodeling and is regulated by a cascade of inflammatory mediators, including cytokines and growth factors. (Singer and Clark, 1999) Although numerous growth factors are involved, transforming growth factor-beta (TGF-β) arguably exerts the greatest fibrogenic effect during repair (Singer and Clark, 1999). It favors extracellular matrix accumulation through chemoattractant, proliferative, and synthetic influences on parenchymal cells, in particular, the fibroblast.

Complex spatial and temporal interactions between cells, the matrix, and mediators have yet to be wholly elucidated (Singer and Clark, 1999). This may explain the mitigated success of individual growth factors in accelerating repair of skin wounds. Furthermore, because normal wound repair proceeds at a rapid rate it is often difficult to establish an improvement in the speed or quality of repair confidently.

Conversely, wound healing in patients with underlying metabolic disorders is fraught with complications. For ex-

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ample, in both experimental and clinical diabetes, compromises in cellular migration, vascular proliferation, and extracellular matrix remodeling make a negative impact on tensile strength. The underlying cause of pathologic wound healing has yet to be established, but deficient growth-factor activity within the wound environment may play an important role (Bitar and Labbad, 1996; Moulin et al., 1998). Indeed, studies performed on animal models of impaired healing suggest that the addition of exogenous growth factors, in particular TGF-β, (Chesnoy et al., 2003) substantially promotes healing and achieves rates of repair comparable to those of unimpaired control animals (Pierce and Mustoe, 1995).

Extrapolating from United States data, approximately 1 million Canadians suffer from burns or chronic wounds annually. (Brigham and McLoughlin, 1996). Presently, only one recombinant growth factor–based product [becaplermin; platelet derived growth factor (PDGF)], the cost of which is prohibitive, is approved for use in Canada in the treatment of diabetic foot ulcers (Wieman et al., 1998). Consequently, the development of an affordable and efficacious growth factor-based therapy would represent a major advancement in the treatment of compromised wounds.

Elk velvet antler (EVA) has been used in Traditional Chinese Medicine for more than 2000 years as a preventative agent to treat a wide range of ailments. Of particular interest, EVA is alleged to enhance wound healing (Davidson, 2000). Although recent studies have shown that water-soluble extracts of EVA contain growth promoting factors that stimulate dermal fibroblast growth *in vitro* (Sunwoo et al., 1997), no published scientific data report on the *in vivo* wound healing effects of EVA. Because other naturally-occurring substances that exhibit growth factor activity have proven efficacious in enhancing wound repair in animal models of diabetes, (Moulin et al., 1998; Sidhu et al., 1999), we hypothesized that topical application of EVA to skin wounds in diabetic rats would accelerate repair, possibly through an increase in TGF-β_1 expression.

MATERIALS AND METHODS

Diabetes mellitus model

Ten (10) individually caged Sprague-Dawley rats (9 weeks old, 150–200g) were fasted for 24 hours before receiving a single tail vein injection of streptozotocin (STZ, 55 mg/kg) in citrate buffer (pH 4.5). Buprenorphine (0.03 mg/kg) was injected i.m. 30 minutes prior to STZ for prophylatic analgesia. Hyperglycemia was confirmed at the time of wounding and at the end of the study. Food consumption and body weight were also monitored throughout the study, which was carried out in accordance to guidelines established by the Canadian council on animal care (Junod et al., 1969).

Excisional wounding procedure

Twenty-one (21) days after induction of diabetes, rats were anesthetized with isoflurane. After clipping the animals' hair and cleaning the skin of their backs, two separate areas were outlined, one on either side of the midline, using a 10-mm diameter circular template. Full thickness wounds were made with a scalpel and iridectomy scissors.

Topical gel was applied immediately following surgery and then once daily to all wounds, which were left unbandaged and healed by second intention.

Topical EVA administration

Animals were randomly selected to receive either 500 μl/wound/day of control gel (Muko® Lubricating Jelly, Ingram and Bell, Don Mills, Ontario, Canada) or EVA gel (water-soluble extract of EVA mixed with Muko® Jelly). To obtain the water-soluble extract, 1.5 g of EVA were homogenized in 15 mL of deionized water (4°C). Homogenate was centrifuged at 40,000 $\times$ g for 30 minutes at 4°C, then a supernatant was filter-sterilized. Daily aliquots were stored at -80°C and thawed immediately prior to use. Protein content of the supernatant was approximately 7.0 mg/mL as determined by the Lowry protein method (Lowry et al., 1951). A volume of extract containing 400 μg of protein was mixed with 500 μL of gel prior to application.

Gross examination

All wounds were examined daily. The amounts and quality of granulation tissue, contraction, and epithelialization were assessed subjectively. One randomly selected wound per animal was photographed daily alongside a template until a biopsy of that wound was obtained at the end of the study (10 days postwounding). Photos were taken with a Nikon CoolPix 990 digital camera (Nikon Canada, Mississauga, Ontario, Canada) on a macro setting at maximum magnification and at a fixed distance of 25 cm. Wound areas were measured according to the two-dimensional scale displayed beside each wound, then calculated as percentage of the wound area on day 1, and log percentage of the day 1 wound areas. Mean values of treatment and control groups were plotted against time.

Biopsy samples

One wound per animal was biopsied at 48 hours to assess the acute phase of repair, while the second wound was photographed throughout then biopsied at the end of the study. Thirty (30) minutes prior to biopsy procedures, animals were given buprenorphine (0.03 mg/kg, i.m.) then induced and maintained in general anesthesia with isoflurane. Five (5)–mm full-thickness punch biopsies were taken from the wound edge to include the original epithelial border, new epithelium and granulation tissue. One half of the sample was prepared for histologic examination while the other was prepared for measurement of growth factor content.

Histology

Six (6)-μm sections of formalin-fixed, paraffin-embedded tissues were stained with either hematoxylin-eosin (HE) or Masson's trichrome (MT). Sections were scored on a scale of 0–3 (none, mild, moderate, and marked) for the following parameters: acute and chronic inflammation; angiogenesis; fibrosis and collagen organization; epithelial coverage; and epithelial hyperplasia (Theoret et al., 2001). A board-certified veterinary pathologist performed all evaluations in a blind fashion. Mean values were compared by a two-tailed unpaired *t*-test.

Growth factor analyses

Samples were weighed and placed in 0.5 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), then homogenized and centrifuged at 30,000 $\times$ g for 30 minutes to remove cellular material from the supernatant which were filter-sterilized and frozen at -80°C, in individual 400 μL aliquots.

Enzyme-linked immunosorbent assay

TGF-β_1 concentrations were measured using a commercial human cytokine enzyme-linked immunosorbent assay (ELISA) kit (R & D Systems, Inc., Minneapolis, MN), sensitive to 7 pg/mL and TGF-β_1 specific. All samples were acid-activated prior to measurement, while DMEM containing 10% FBS was used as a blank. Mean values were compared by a two-tailed unpaired *t*-test.

Statistical analysis

The rate of wound healing was determined by linear regression of the log percentage of day 1 wound area over time plot. Statistical analysis was performed on the resultant slope (k) values using a two-tailed *t*-test. Daily changes in wound closure were compared using ANOVA. Statistical analysis was conducted using Statview SE+ (Abacus Concepts Inc., Cary, North Carolina).

RESULTS

Blood glucose levels and changes in body weight

Analyses of blood glucose and body weight showed that all animals responded in a similar and positive manner to the STZ induction of diabetes.

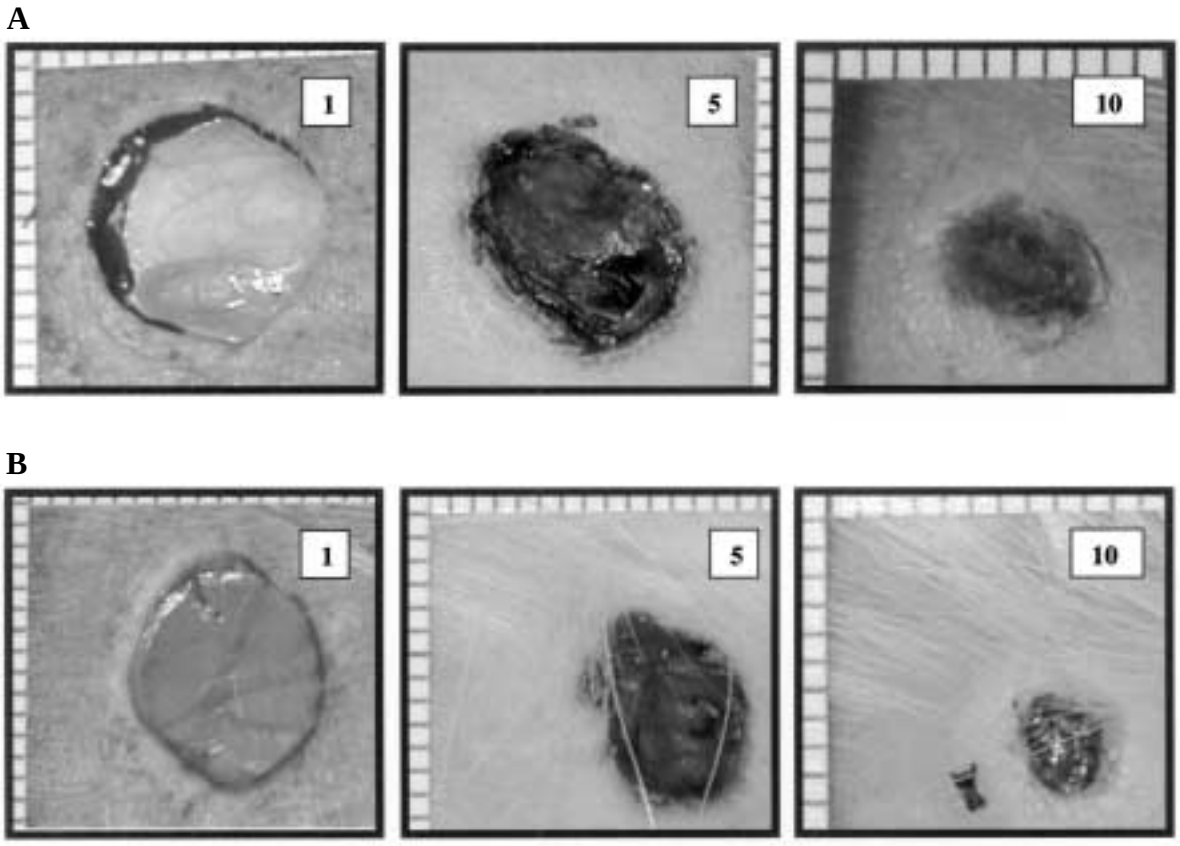

FIG. 1. Photos taken from a control (**1A**) and an EVA (**1B**) topically treated wound. Numbers inset identify the postwounding day at which the photo was taken. Template marks are at mm intervals.

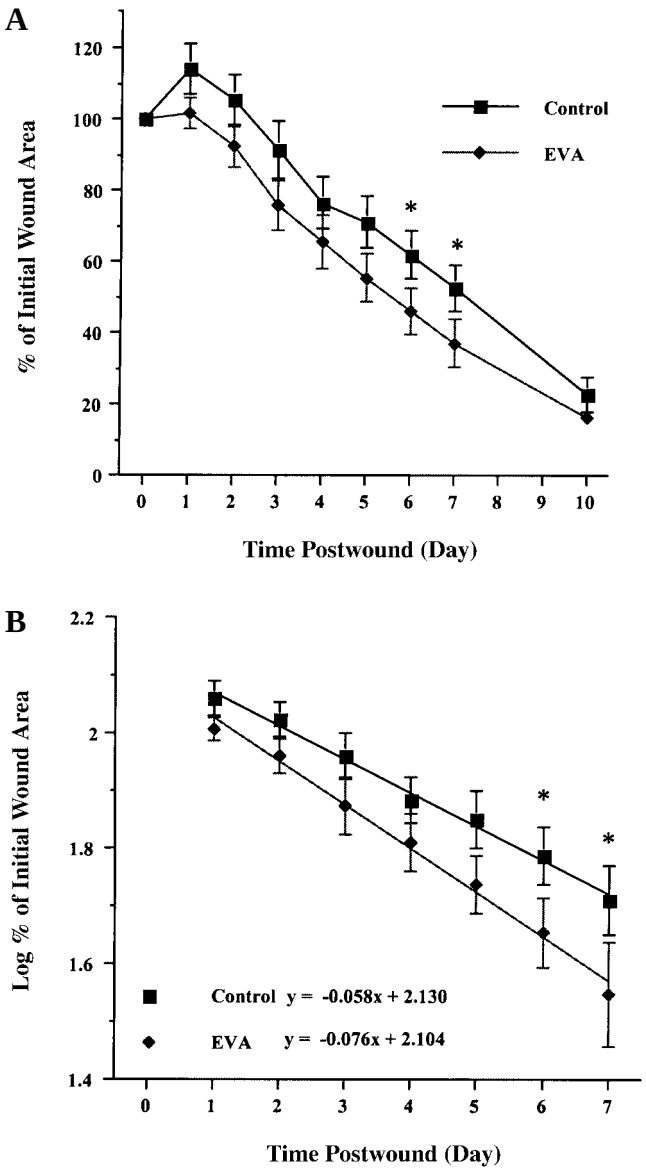

FIG. 2. **A.** Percentage of wound area versus time since surgery for both treatment groups. **B.** The log percentage of original wound area versus time since surgery for each treatment group from day 1 to day 7. The legend shows linear regression values for each treatment group with the closure coefficient (k) determined as the slope of the line. Data in both graphs are expressed as the mean ± standard error (n = 5) for each group. *Indicates $p < 0.05$ compared to control. EVA, elk velvet antler.

Gross findings

Twenty-four (24) hours after surgery wounds had attained maximal retraction and by 2 days, an epithelial border was apparent in all wounds (Fig. 1A and B). EVA-treated wounds were significantly smaller by days 6 and 7 ($p < 0.05$), however wound areas during the previous days, although consistently smaller in the EVA group, were not statistically different from those in the control group (Fig. 2A).

Coefficients of wound closure (k) were -0.058 ± 0.007 and -0.076 ± 0.010 for control and EVA-treated groups respectively. These coefficients of healing were similar to those reported in other studies (Cross et al., 1996) and showed a trend toward increased rate of wound closure in EVA-treated wounds.

Histologic findings

There was a trend toward decreased inflammation in EVA-treated wounds 2 days postoperatively, while angiogenesis was significantly inhibited by topical administration of EVA gel (Table 1).

On the tenth day postwounding, histologic evaluation was compromised by a thin and fragile neoepithelium. Although grossly it appeared that topical administration of EVA-accelerated epithelialization, we were unable to confirm this histologically.

Growth-factor levels

TGF-β_1 concentrations did not differ between control animals and those treated topically with EVA gel at either 2 or 10 days postsurgery.

DISCUSSION

To the best of our knowledge, this is the first controlled study investigating the effects of topically administered EVA on impaired wound healing *in vivo*. Results indicate that daily topical application of water-soluble EVA extract may accelerate second-intention wound healing in patients with diabetes.

We focused on compromised wound repair because it represents an enormous burden on the health care system both in terms of cost and labor and because deficient wound healing is more sensitive to pharmacologic manipulation. We chose an experimental model over a clinical trial because inherent variables present in the chronic wound environment and the myriad of underlying physiologic imbalances suf-

TABLE 1. HISTOLOGIC EVALUATION OF WOUNDS SAMPLES OBTAINED 2 DAYS POSTOPERATIVELY

Histologic variable	*Control*	*Topical EVA*
Acute inflammation (platelets, fibrin, PMN)	1.2 ± 0.6	0.8 ± 0.4
Chronic inflammation (macrophages, lymphocytes, plasma cells)	2.0 ± 0.4	1.2 ± 0.4
Angiogenesis (capillaries)	3.0 ± 0.0	1.6 ± 0.2*
Fibrosis and collagen organization (MT)	1.6 ± 0.2	2 ± 0.0

EVA, elk velvet antler; PMN, polymorphonuclear (cell); MT, Masson's trichrome (stain).

*Indicates $p < 0.05$ compared to control.

fered by affected patients limit the ability to find comparable populations of subjects with chronic wounds.

The diabetic model is particularly attractive for studying the effects of growth factors because deficient fibroplasia is thought to result from impaired cellular migration as well as diminished angiogenesis (Fahey et al., 1991). It is hypothesized that diabetes interferes with various phases of repair by decreasing growth-factor levels in the wound environment (Bitar and Labbad, 1996) Indeed, wound healing studies performed on STZ-induced diabetic rats have underlined the positive role of growth factors, such as bFGF and TGF-β. Furthermore, a greater impact is achieved through combinations of growth factors over single formulations, given that each mediator exerts a principal effect on a particular phase of repair, and that a number of phases may be deficient in impaired healing situations. (Moulin et al., 1998; Sidhu et al., 1999)

In our quest to develop an effective, yet affordable, growth factor–based therapy to promote impaired wound healing, we have investigated a naturally occurring product reported to stimulate dermal fibroblast growth *in vitro* (Sunwoo et al., 1997).

Although daily topical application of EVA to wounds of diabetic rats grossly accelerated repair, we were unable to confirm this histologically. We did, however, note a negative effect of treatment on capillary content of the wound bed 2 days after surgery. This was unexpected, as we had hypothesized that growth factors present in EVA would reverse the inhibitory effect of STZ on angiogenesis. Moreover, it appears to contradict the enhanced rate of repair noted macroscopically, although in some instances repair can proceed normally in the presence of deficient angiogenesis (Berger et al., 2000; Lange-Asschenfeldt et al., 2001; Nanney et al., 2001).

Since growth factor-like activity has been reported in velvet antler harvested from both elk (Sunwoo et al., 1997) and deer (Ko et al., 1986) and is credited for the rapid regenerative growth seen during the early phase of antler cycle (Gross, 1995), we speculated that concentrations of fibrogenic growth factors would be elevated in wounds treated topically with EVA. Following tissue injury, TGFβ_1 exerts chemoattractant and proliferative effects on fibroblasts (Singer and Clark, 1999) and is believed to enhance wound contraction by stimulating fibroblast attachment and by increasing the deposition of collagen matrix (Frank et al., 1996; Theoret et al., 2001). Results from our study suggest that acceleration of wound closure cannot be attributed to TGF-β_1 alone because its expression did not differ between control and EVA treated animals.

In diabetic mice, nerve growth factor (NGF) increases the rate of cutaneous wound healing (Matsuda et al., 1998) while epidermal growth factor (EGF) enhances oral wound healing (Nagy et al., 2001). Likewise, PDGF (beclapermin) accelerates dermal repair in humans with diabetes. (Cohen and Eaglestein, 2001). It is therefore probable that growth factors other than TGF-β_1 are present in EVA extract and are responsible for the observed acceleration in wound closure. Interestingly, EGF- and NGF-like activities have been reported in deer antler studied *in vitro* (Huo et al., 1997; Ko et al., 1986). Alternatively, EVA may positively influence repair by a mechanism unrelated to growth-factor content, for instance, by contributing components of the extracellular matrix, such as collagen, glycosaminoglycans, and proteoglycans (Sunwoo et al., 1998; Zhao et al., 1992).

CONCLUSIONS

In conclusion, our study has shown that topical treatment with water-soluble extracts of EVA positively influences the rate of second-intention repair in STZ induced diabetic rats. Further studies are necessary to determine active ingredients and to establish an optimum treatment regimen.

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