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Bulbine Natalensis and *Bulbine Frutescens* promote cutaneous wound healing

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ABSTRACT

Ethnopharmacological relevance: The gel from the leaves of *Bulbine natalensis* (BN) and *Bulbine frutescens* (BF) is commonly used as a traditional medicine in South Africa for the treatment of skin wounds and burns. Treatment with both leaf gel extracts has previously been demonstrated to increase tensile strength and protein and DNA content in pig dermal wounds. This study examined the effect of the leaf gel extracts *in vivo* on histology of wound healing in pigs to elucidate the mechanism of increased tensile strength.

Materials and methods: Mirror imaged wounds on the dorsum of 12 post weaning female pigs were treated with either BN or BF, biopsied at days 2, 4, 7, 10 and 16 post-wounding and fixed. Sections of wound tissue were then stained with haematoxylin and eosin and Mallory's stain to analyse the general morphology and collagen arrangement; and smooth muscle actin, vascular endothelial growth factor (VEGF) and transforming growth factor β (TGFβ) receptors were immunolocalised.

Results: Histological analysis of the wound tissue in the study indicated earlier wound contraction and collagen deposition in both treatment groups with re-organisation of the collagen (indicating collagen maturation) evident as early as at day 10.

Conclusion: The results of this study suggests that the leaf extracts increase tensile strength by increasing fibroplasia, differentiation of fibroblasts into myofibroblasts, and increased collagen deposition and maturation. This study further validates the use of the Bulbine leaf gels for the treatment of skin wounds.

1. Introduction

There is a trend towards natural and traditional medicine as a source of affordable, readily available treatments of wounds (Makunga et al., 2008). In South Africa, there are a number of plants used for traditional medicine, but many of these have not been scientifically validated. An example is *Bulbine natalensis* (BN) and *Bulbine frutescens* (BF) of the *Asphodelaceae* family (Van Wyk and Gericke, 2000) used commonly in traditional and indigenous medicine (Hutchings et al., 1996) for the treatment of wounds, burns, rashes, itches, ringworm, cracked lips and herpes. The clear leaf gel is obtained from fleshy leaves of the plants which is similar in appearance and consistency to the gel of *Aloe vera*.

Wound healing is a complex process. Among different systems which are active in early wound healing, fibroblasts invade the wound space where they proliferate and differentiate into myofibroblasts, which contain α-smooth muscle actin (αSMA) and a lesser amount of vimentin in their cytoplasm. These myofibroblasts are functionally different from fibroblasts in that they are essential to neodermal formation and wound contraction (Moulin et al., 2000; Bates and Pritchard-Jones, 2003). Myofibroblasts increase the rate of contraction by using an actin–myosin complex. Intracellular actin microfilaments terminate in adhesion complexes at the cell surface which connect them with the surrounding extracellular matrix (ECM) resulting in matrix reorganisation and wound contraction (Mirastschijski et al., 2004).

The processes of wound healing are controlled by a milieu of cytokines and chemokines. Transforming growth factor (TGF)-βs are mitogenic for fibroblasts. Furthermore, TGF-β has been shown to stimulate differentiation of fibroblasts into myofibroblasts at high concentrations (Slavin, 1996). The differentiated phenotype is associated with increased ECM production. The role of TGF-β in keratinocyte proliferation and wound re-epithelialisation is controversial (Margadant and Sonnenberg, 2010). On one hand TGF-β has been shown to inhibit keratinocyte proliferation while on the other hand, it induces the migratory phenotype in keratinocytes (Margadant and Sonnenberg, 2010). It is thought that TGF-βs stimulate re-epithelialisation and granulation tissue formation. TGF-β1 plays a central role in cutaneous scar formation and is a potential target for anti-scarring agents. Rapid induction of TGF-β1 and TGF-β2 occurs early in wound healing while TGF-β3 expression is seen in later stages of wound healing. The associated receptors,

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TGFβ-R1 and TGFβ-R2 are widely distributed and found on essentially all cell types (Gold et al., 1997 and Schmid et al., 1998).

VEGF, a well-established pro-angiogenic factor, is critical and widely expressed after cutaneous injury (Brown et al., 1992, Frank et al., 1996). It is produced in large quantities by epidermal keratinocytes during the healing of a wound (Frank et al., 1996, Tonnesen et al., 2000) especially in the basal layer (Kapoor et al., 2006). Myofibroblasts have also been shown to express VEGF mRNA (Bates and Pritchard-Jones, 2003). VEGF is critical for angiogenesis during the proliferative phase of granulation tissue formation from day 4 to day 7 (Nissen et al., 1998; Tonnesen et al., 2000) and peaking on day 7 following injury (Kapoor et al., 2006). The associated receptors, VEGF-R1 and VEGF-R2, have been detected in the blood vessels found in granulation tissue (Lauer et al., 2000).

We previously reported increased tensile strength in pig dermal wounds treated with BN and BF (Pather et al., 2011). Biochemical analysis of these wounds demonstrated increased protein and DNA in treated wounds compared to untreated wounds. We postulated that this may be due to increased cell proliferation and/or collagen synthesis in treated wounds. To elucidate the mechanism by which the leaf gel extracts of BN and BF increased tensile strength, this study examined the effect of the leaf gels *in vivo* on the histology of wound healing in pigs.

2. Materials and methods

BN and BF plant materials (voucher specimens, J95814 and J95815, respectively, have been deposited in the C.E. Moss Herbarium of the School of Animal, Plant and Environmental Sciences of the University of the Witwatersrand) for this study was provided by the Walter Sisulu Botanical Gardens in Johannesburg, South Africa following confirmation of the taxonomic identity of the plants by the principal horticulturalist. Fresh gel from leaves of BN and BF (average pH of the leaf gels were 4 and 5, respectively) was extracted under sterile conditions as described by Pather et al. (2011). Briefly, the mature leaves were washed, wiped in 70% alcohol and the fresh gel was collected into a sterile tube. This method of using the fresh gel was chosen because it is the fresh gel from the leaves that is used by the indigenous people of South Africa for treating skin ailments and wounds.

2.1. Experimental animals and wound creation

Twelve post-weaning pigs (*Susscrofa domestica*) were used in this study (University of the Witwatersrand, Johannesburg, Animal Ethics Clearance #2006 2804) due to the similarities between pig and human skin (Vardaxis et al., 1997; Sullivan et al., 2001). Wound contraction varies depending on the site of the wound (Sullivan et al., 2001) and therefore mirror-imaged control wounds were made to provide control tissue at the anatomical location of the 'experimental' wound (Sullivan et al., 1995; Gonçalves et al., 2007).

Following anaesthetising, five mirror-imaged, 4 mm full-thickness **excisional** wounds were made on the dorsum of each pig. The wounds were treated with 1 ml of the corresponding leaf extract ($n=6$ animals each for BN and BF) applied topically twice a day and photographed. All wounds were covered with Opsite Flexigrid® and a veterinary sock to keep the wound site clean as described previously (Pather et al., 2011). The wounds together with a uniform perimeter of surrounding tissue were harvested on days 2, 4, 7, 10 and 16 following wounding. The excisional wound tissue for histological analysis was fixed in 10% buffered formalin and prepared for histology and immunohistochemistry.

Table 1
Mean maximal distance between wound edges (mm) and per cent closed from day 0.

Day	*Untreated*		*BN-treated*		*BF-treated*	
	Mean distance	**% Closed**	**Mean distance**	**% Closed**	**Mean distance**	**% Closed**
2	3.71 ± 0.38	7.00	3.18 ± 0.40	20.50	3.44 ± 0.41	14.00
4	3.14 ± 0.33	21.50	2.69 ± 0.24	32.75	2.58 ± 0.36	35.50
7	1.68 ± 0.24	58.00	1.39 ± 0.32	62.25	1.43 ± 0.45	64.25
10	1.18 ± 0.35	70.50	0.79 ± 0.22	80.25	0.82 ± 0.19	79.50
16	Wound contraction complete					

2.2. Histological analyses of the excisional wounds

The general morphology and regeneration of the wound tissue was examined by staining sections with haemotoxylin and eosin (Bancroft and Gamble, 2002). Collagen deposition in the wound tissue was studied by staining sections with Mallory's trichrome using three stains: 1% acid fuschin, 5% phosphotungstic acid water and the Mallory's mixture (Bancroft and Gamble, 2002).

2.3. Wound contraction

The time for the excisional wounds to 'close' was calculated from the digital photographs and the wound tracings using ImageJ® (software developed by US National Institute of Health). This was repeated three times for each wound tracing with the repeatability of the measurements tested for accuracy and repeatability using the Lin's concordance correlation co-efficient test (Lin, 1989). The change in wound size over a period of 16 days was then calculated as the percentage of the original wound area that was created.

2.4. Immunolocalisation of smooth muscle actin, VEGF, TGFβ-R1 and Anti- TGFβ-R2

For VEGF and TGFβ-receptor localisation, antigen retrieval was carried out by microwaving the sections in citrate buffer (pH 6) and cooling to room temperature.

Endogenous peroxidase activity was blocked with hydrogen peroxide and non-specific binding was blocked with normal rabbit serum for SMA and VEGF and with goat serum for TGFβ-receptors. Sections were incubated with either monoclonal anti-smooth muscle actin (SMA) antibody (Santa Cruz Biotechnology) for 60 min, primary mouse monoclonal VEGF antibody (sc-7269, Santa Cruz Biotechnology) overnight or TGFβ-R1 and TGFβ-R2 antibodies (sc-398 and sc-220, respectively; Santa Cruz Biotechnology) for 30 min. After washing, the sections were incubated in a secondary antibody for 30 min-mouse immunoglobulins (Dako, Denmark), rabbit anti-mouse antibody (Dako, Denmark) and goat anti-rabbit antibody (Dako, Denmark), respectively. The sections were then washed and then incubated with a strepavidin horseradish peroxidase complex (Dako, Denmark) for 30 min. The sections were then washed and stained with diaminobenzidine (DAB) (Dako, Denmark) and counterstained with Mayer's haematoxylin.

Negative controls for all the immunohistochemistry were prepared using adjacent sections to those on which the relevant immunolocalisation was carried out. These controls were treated identically, except that the primary and secondary antibodies were respectively replaced with the buffer (PBS for αSMA and VEGF, and TBS for TGFβ-R1 and TGFβ-R2). Formalin fixed paraffin embedded mouse heart (Animal ethics clearance # 2007 232 A), human breast carcinoma tissue (Human ethics clearance # M05052) and mouse