A Combination of Aloe Vera, Curcumin, Vitamin C, and Taurine Increases Canine Fibroblast Migration and Decreases Tritiated Water Diffusion across Canine Keratinocytes In Vitro

Timothy R. Fray, Adrian L. Watson, Julie M. Croft, Claire D. Baker, Julie Bailey, Nicola Sirel, Amanda Tobias, and Peter J. Markwell

WALTHAM Centre for Pet Nutrition, Melton Mowbray, Leicestershire, UK

EXPANDED ABSTRACT

KEY WORDS: • canine • fibroblast • keratinocyte • aloe vera • curcumin • migration • allergy • skin • atopy

Diet has a major role in the maintenance of healthy skin, and is a significant factor in the etiology and therapy of certain skin diseases (1). Canine skin diseases are common and some dietary components were shown to improve skin condition and help control disease. These include fatty acids (2), zinc (3), and certain proteins (4). This strategy was incorporated into a therapeutic diet3 intended to aid the management of canine allergic skin disease, including atopy, flea allergy, and dietary sensitivity (5).

Other antiinflammatory and antibacterial components to aid the recovery of skin were included in this diet by the addition of curcumin, aloe vera, vitamin C, and taurine. Curcumin exhibits antitumor, antioxidant, antiviral, immune modulating, and antiallergenic activity (6,7). Aloe vera contains a large number of active components that have recognized antioxidant, anti-inflammatory, and antimicrobial properties (8,9). Vitamin C has both antioxidant and antiinflammatory activities, and can significantly lower blood histamine in healthy adult humans (10) whereas taurine has known antioxidant properties (11,12).

In this study, the potential for a novel combination of aloe vera, curcumin, vitamin C, and taurine to effect the migration of canine fibroblasts and the permeability of keratinocytes to tritiated water was assessed in vitro.

METHODS

Vitamin C, aloe vera, curcumin, and taurine were combined (1:2.33:16.67:16.67 w:w:w:w) and dissolved in dimethyl sulphoxide (DMSO)4 before testing to create a final nutrient combination concentration of 0.125 g/L. The concentration of vitamin C used in vitro was based on the plasma concentration of vitamin C known to be achieved in vivo through dietary supplementation. In creating the in vitro model it was assumed that the absorption of aloe vera, curcumin, and taurine was the same as that of vitamin C. All materials for tissue culture were obtained from Invitrogen™ Life Technologies, Paisley, UK, unless otherwise stated.

Fibroblast migration assay

A two-dimensional assay of fibroblast migration was used, as described by Fray and Wood (13). Canine dermal fibroblasts were obtained from small tissue samples obtained from healthy dogs according to Lloyd (14), and passaged in fibroblast medium [Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FBS (fetal bovine serum), 1% penicillin/streptomycin, 1% nonessential amino acids, and 1% glucose (0.0684 mol/L)]. All cells were maintained at 37°C, in a 5% CO2:95% air-humidified atmosphere.

For the assay, tissue culture dishes (with lid and vent, sterile, 35 mm × 10 mm, 2 × 2 mm grid, Nalgene Nunc International, Hereford, UK) were set up with a line drawn on the underside approximately down the center to represent the "wound" line. Dishes were seeded with canine dermal fibroblasts between passages 3 and 10 (~4 × 105 cells per dish in 2 mL fibroblast medium), and allowed to reach confluency over a 24-h period. Cells were removed from the wound line over half the plate using a cell scraper (23 cm, Nalgene Nunc International, Hereford, UK), as confirmed by phase contrast microscopy. The remaining cells were washed and fresh fibroblast medium (2 mL) added. Finally, 20 μL of DMSO was added to control plates, 20 μL of the nutrient combination dissolved in DMSO (ingredient concentrations 0.125 mg/mL) added to test plates, and the cells incubated for 48 h. The cells were washed with phosphate-buffered saline (1 mL), fixed in 70% methanol (21.8 mol/L) (1 mL for 4 min), and washed again before staining (1 h in 1 mL Giemsa stain (Sigma Aldrich, Gillingham, UK), diluted 1:5 v:v in sterile water). Cells were washed and stored at 4°C before counting. Migration over the wound line was assessed by phase contrast microscopy. The mean

1 Presented as part of the WALTHAM International Science Symposium: Nature, Nurture, and the Case for Nutrition held in Bangkok, Thailand, October 28–31, 2003. This symposium and the publication of the symposium proceedings were sponsored by the WALTHAM Centre for Pet Nutrition, a division of Mars, Inc., and Quinton R. Rogers, University of California, Davis.
2 To whom correspondence should be addressed. E-mail: tim.fray@eu.effem.com.
3 WALTHAM® Veterinary Diet™ Canine Skin Support™.
4 Abbreviations used: CPM, counts per minute; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; FBS, fetal bovine serum.
migration was calculated from counting six fields on each slide. Five replicates were used per assay.

**Keratinocyte permeability assay**

The permeability of a sheet of keratinocytes to tritiated water was assessed using a new technique reported by Hidalgo et al. (15), which incorporated a new side by side diffusion cell for studying transport across epithelial cell monolayers. The method reported here measures the transepidermal diffusion of labeled water through a keratinocyte “barrier”.

The cells were constructed by culturing canine keratinocytes in Snapwell plates (Corning Costar, from Appleton Woods, Birmingham, UK) containing Green’s medium (DMEM/Ham’s F-12 mix) containing 3.4% NaHCO₃ solution (BDH, Derby, UK) (74 g/L in water, final concentration = 0.029 mol/L), 1% glutamine (0.0684 mol/L), 1% penicillin/streptomycin, 0.1% amphotericin, 10% FBS, 0.4% adenine solution (Sigma-Aldrich) (5 g/L solution in water, final concentration = 1.08 × 10⁻⁴ mol/L), 0.5% insulin solution (Sigma-Aldrich) (10g/L in 0.01 mol/L HCL, final concentration = 8.61 × 10⁻⁶ mol/L), 0.016% hydrocortisone solution (Sigma-Aldrich) (2.5 g/L in water), 0.02% epidermal growth factor (Sigma-Aldrich), and 0.1% Cholera toxin (Sigma-Aldrich). The plates were set up containing 2.6 ml Green’s Medium (made up as above) in the outer well and 400 µL Green’s medium in the inner well. The inner well was seeded with 1 × 10⁵ canine keratinocytes and incubated for 72 h. On d 4 the medium was removed from both inner and outer wells. The outewell received 900 µL Green’s medium containing the combined nutrient ingredients (0.125 mg/mL) (test) or DMSO (control). The plates were cultured again and on d 11 were ready for the diffusion assay.

For the diffusion assay, the inner well of each Snapwell was removed and placed in individual diffusion chambers (15). DMEM (6 mL) was added to each side. Each chamber was placed into the diffusion apparatus, which equilibrates the chambers to 37°C and enables gas (5%CO₂:95% air) to be continuously pumped through, thus ensuring movement of the medium. Tritiated water (³H) (100 µL) was added to the left-hand side of each chamber. Diffusion of the labeled water was monitored over the subsequent 90 min by taking a 30-µL sample from the right-hand chamber every 3 min; each time the volume was replaced by the addition of DMEM. The amount of radiolabel in each sample was quantified by scintillation counting.

**Statistical analysis**

Results are presented as mean ± SD. Differences compared to control were assessed using Statgraphics Plus (Manugistics, Rockville, MD) by Student-Newman-Keuls test; P < 0.05 was considered significant.

**RESULTS**

The migration of canine fibroblasts treated with the nutrient combination of Aloe vera, curcumin, taurine, and vitamin C was significantly greater (P < 0.05) than control cells (Fig. 1).

The diffusion of tritiated water across the canine keratinocyte cells was significantly less (P < 0.05) for those cells treated with the nutrient combination of Aloe vera, curcumin, taurine, and vitamin C, compared with control cells (Fig. 2).

**DISCUSSION**

Migration of fibroblasts is vital for the rapid and effective repair of damaged or diseased tissue. Fibroblasts are the major source of extracellular connective tissue matrix, and the recruitment, accumulation, and stimulation of these cells are thought to play important roles in both normal healing and the development of fibrosis. By populating the damaged area quickly, the barrier properties of the skin can be repaired quickly, thus protecting the body from pathogens. Enhanced fibroblast migration, as occurred in the cells cultured with the nutrient combination, might be beneficial to the host animal.

The migration of fibroblasts observed in the current study is likely to be a consequence of a combination of absolute cellular migration, proliferation, and cell death. It is argued, therefore, that this technique closely reflects the situation in vivo. Although a three-dimensional migration assay may appear to be a better measure of the healing response in vivo, it is more difficult to quantify the repair of the denuded zones accurately. The two-dimensional assay used in the current study is a reasonable alternative.

Integrity of the epidermal barrier is an essential natural defense of the body, protecting it against entry by pathogens and allergens (16). Although the epidermis is composed of several different cell types, keratinocytes form the outer layer, and it is these cells that eventually flake from the skin surface.

**FIGURE 1** Migration of canine fibroblasts incubated with (■) or without (□) the nutrient combination. Values represent mean cell number ± SD moved into the denuded zone on the tissue culture dishes. n = 5 for each treatment. Letters denote significant difference (P < 0.05; Student-Newman-Keuls test).

**FIGURE 2** Diffusion of tritiated water across canine keratinocytes incubated with (■) or without (□) the nutrient combination. Values represent the amount of radiolabeled water that diffused across the cell construct, in counts per minute (CPM/min) calculated as the mean ± SD. n = 3 for each treatment. Letters denote significant difference (P < 0.05; Student-Newman-Keuls test).
The keratinocyte membrane fabricated in vitro and used in the current study, therefore, provides an approximation of the first line of defense in vivo. If this barrier were enhanced, this may manifest as an improvement in skin health and appearance, as well as improved protection against skin disease and allergy. The results showed that the keratinocyte membrane permeability to tritiated water decreased by culturing the sheet with the ingredient combination. The decrease in permeability could be a consequence of increased cellular migration. However, permeability is more likely to be affected by proliferation causing a subsequent increase in sheet density or thickness. Proliferation was not measured in this study. Although there are a considerable number of structural and physiological factors involved that contribute to the complete barrier function of the skin, the ingredient combination might help to reinforce the barrier properties of the skin in vivo.

LITERATURE CITED