An immunohistological study of anhydrous topical ascorbic acid compositions on *ex vivo* human skin

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Summary

Background Ascorbic acid has numerous essential and beneficial functions in normal and photoaged skin. Ionisation of ascorbic acid in aqueous topical formulations leads to oxidative degradation. Ascorbic acid in an anhydrous vehicle would inherently have greater stability.

Objective The objective of this study was to observe the effects of two anhydrous formulations containing microfine particles of ascorbic acid on neocollagenesis and cytokeratin production in *ex vivo* human skin.

Methods Vitamin C preparations were applied topically onto the surface of freshly excised human abdominal skin. Following an exposure time of 48 h with appropriate controls, skin discs were cut into sections, placed on slides and assessed using immunohistochemical (antibodies: collagen type I, III, cytokeratin) staining. Analysis was performed using microscopy and descriptive rating.

Results Both formulations resulted in increased production of collagen types I and III and cytokeratin.

Conclusion The application of anhydrous formulations containing microfine particles of ascorbic acid to *ex vivo* human skin in this study resulted in neocollagenesis and increased production of cytokeratin. This approach appears to enable biological effects of ascorbic acid in the skin using a vehicle which would provide it greater stability than an aqueous vehicle.

Keywords: ascorbic acid, collagen, topical therapeutic

Ascorbic acid has been reported to have numerous essential and beneficial functions in normal and photoaged skin including up-regulation of neocollagenesis by dermal fibroblasts,[1–3] being a cofactor for various hydroxylating enzymes,[4] down-regulation of matrix metalloproteinase enzymes,[3] protection of the skin against the effects of ultraviolet B and ultraviolet A radiation,[5,6] inhibition of melanogenesis,[7–9] stimulation of ceramide synthesis,[10,11] stimulation of cytokeratin synthesis,[12] and angiostasis.[13]

Vitamin C is defined as "ascorbic acid" in dictionaries and cosmetic and scientific reference books.[14–17] Ascorbic acid is sparingly soluble in alcohols and insoluble in other substances except water. However, as ascorbic acid is readily oxidized in aqueous formulations, such formulations are relatively unstable.[18–20] Lowering the pH of aqueous formulations to increase the protonation of ascorbic acid would be expected to help but not solve this problem, as ionization of ascorbic acid would still be in a state of flux. An anhydrous vehicle containing ascorbic acid would be expected to be inherently more stable than an aqueous vehicle, as the oxidization of ascorbic acid is triggered by its ionization in aqueous solutions.[20]

Many skin care products that make label claims to contain "vitamin C" do not contain ascorbic acid but

derivatives of ascorbic acid such as magnesium ascorbyl phosphate, sodium ascorbyl phosphate, ascorbyl palmitate, or ascorbyl glucoside. However, the commonly used ascorbic acid derivative, ascorbyl palmitate, is readily hydrolyzed in aqueous solutions and is reported to be unstable.[18,21] Effectiveness for magnesium ascorbyl phosphate depends upon its conversion *in vivo* to ascorbic acid.[22] Magnesium ascorbyl phosphate and sodium ascorbyl phosphate applied topically have been reported not to increase levels of ascorbic acid in the skin.[23] Ten times the concentration of sodium ascorbyl phosphate compared to ascorbic acid was required to induce equivalent neocollagenesis in cultured dermal fibroblasts.[24] Both magnesium ascorbyl phosphate and sodium ascorbyl phosphate have been reported to have negligible free radical scavenging activity.[21] After topical application, sodium ascorbyl phosphate was reported to be less effective than ascorbic acid in reducing oxidative stress in the skin.[25] At the time of writing there are no references accessible through the *Medline* database on the use or effects of ascorbyl glucoside.

Microfine particles of L-ascorbic acid predominantly less than 20 microns in size have been reported to penetrate into the dermis from an anhydrous vehicle.[26]

The objective of this study was to observe the effects of two anhydrous formulations containing microfine particles of ascorbic acid less than 5 microns in size on three known effects of ascorbic acid, neocollagenesis of collagen types I and III, and cytokeratin production, in *ex vivo* human skin. Microfine particles of ascorbic acid were produced by ball milling. Formulation A contained 23% microfine ascorbic acid particles in an oil/wax vehicle. Formulation B contained 20% microfine ascorbic acid particles in a silicone/oil/wax vehicle (Fig. 1).
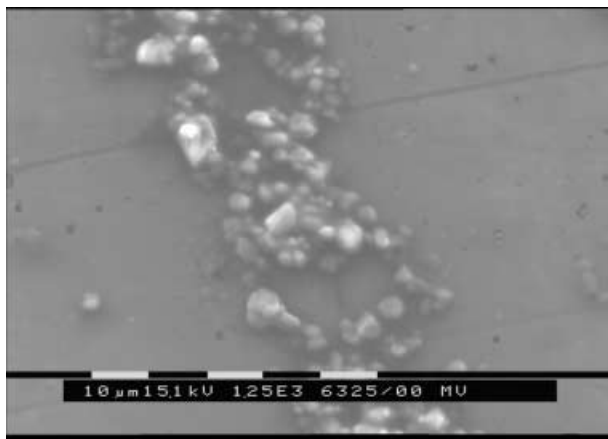
Figure 1 Ascorbic acid microfine particles in silicone/oil/wax vehicle formulation B, SEM × 1250.

Materials and methods

In vitro percutaneous absorption testing procedures using modifications of the Organization for Economic Co-operation and Development (OECD) test guideline 428 (2004): skin absorption: *in vitro* method and other relevant publications were followed.[27,28] Methodologies for *in vitro* percutaneous absorption, immunohistochemistry and histopathology have been published in a number of papers.[29–31]

The *in vitro* percutaneous absorption assay

An extensive range of procedures for determining the percutaneous absorption of test compounds through human skin have been developed.[27,28,32,33] Although skin viability is not a prerequisite for penetration studies and biotransformation may be of minor importance to the majority of chemicals, the use of freshly excised human skin gives the potential to simultaneously explore the cellular histology of skin following topical exposures.[27]

Procedure for preparation of skin discs in Franz cells

Percutaneous absorption studies were performed using a Franz cell diffusion system (Crown Glass Co., New Jersey) consisting of nine vertical glass diffusion cells (2.5 cm diameter) with flat ground glass O-ring joints. Each diffusion cell was mounted on a PermeGear (http://www.permegear.com) stainless steel vertical cell stirrer with water bath recirculator.[30]

For each experiment, a freshly excised full thickness human abdominal skin was obtained from a patient undergoing surgical resection (Eastern Suburbs Private Hospital, Randwick), collected in a saline solution of Hanks Balanced Salt Solution (HBSS, Invitrogen) and immediately transported to Chemical Safety and Applied Toxicology (CSAT) laboratories (University of New South Wales Human Research Ethics Committee Approval HREC 04004: Assessment of *in vitro* percutaneous absorption of topical preparations using skin biopsies). Skin was assessed by visual examination for physical damage and suitable preparations were washed thoroughly with sterile color-free Dulbecco's Modified Eagle Medium/Hams F12, Invitrogen (DMEM/F12) to remove surface debris. The subcutaneous fat layer was removed by gross dissection. Circles of full-thickness skin, 2.5 cm in diameter (4.9 cm^2 in surface area), were cut using a sharpened stainless steel cork borer and surgical scissors.

Skin circles were placed epidermis-side up on each of the diffusion cells between two clamped ground glass joints, and allowed to equilibrate and hydrate for approximately

30 min. Prior to the placement of skin circles, receptor chambers of the diffusion cells were filled (mean volume 15.0 mL) with color-free DMEM/F12 + 1% (v/v) of antibiotic (200 mM L-glutamine, 10 units penicillin, 10 mg streptomycin per mL: Sigma, USA) and stirred constantly using a mini magnetic stirrer. The temperature of the jacketed diffusion cells was regulated by water thermostat maintained at 34 °C ± 1 °C.

Test compounds

Vitamin C preparations were supplied by Ultraceuticals Pty. Ltd. Formulation A contained microfine particles of ascorbic acid in an anhydrous oil/wax vehicle. Formulation B contained microfine particles of ascorbic acid in an anhydrous silicone/oil/wax vehicle.

Exposure and doses

Aliquots (100 mg) of each vitamin C preparation were applied topically (in duplicate) to skin discs using a cotton bud and rubbed into the skin for a period of 1 min. The skin was then occluded with paraffin and a watch glass placed over each of the ground glass joints. A 48-h exposure period was used. Skin discs were subjected to the same experimental conditions (i.e., 48-h exposure) without the test substance and skin discs with the silicone/oil/wax vehicle without ascorbic acid served as controls and were used as reference standards for the immunohistochemical assessment.

Dissection of skin disc

At the completion of the exposure period, the surface of each treated and control skin disc was swabbed using a cotton bud and removed from the Franz cell. Excess untreated skin from the circumference was removed and the exposed areas cut into halves. One-half of the exposed skin was snap frozen in liquid nitrogen and the offer stored in buffered formalin [4% formaldehyde in phosphate buffer saline (PBS)] for 48 h.

Procedure for microscopic slide preparation

The detailed procedure has been published previously.[31] Following the percutaneous absorption experiment, formalin-fixed skin sections were processed into paraffin blocks (stored at room temperature) using a Leica RM 2165 (Leica Microsystems Nussloch, Nu Bloch, Germany) automated microtome (Leica) and frozen sections were processed into cryoblocks (stored at −70 °C) using a Reichert Jung Cryocut 1800 (Leica) at the School of Medical Sciences, University of New South Wales. Tissue sections were cut from each block and placed on coated slides for immunohistochemical methods. Paraffin-coated slides were labeled and heated in an oven at 70 °C for 5 min. Slides were washed in the following solvents for a minimum of 2 min each: (i) xylene (three changes), (ii) 100% ethanol (two changes), (iii) 95% ethanol (one change), (iv) 80% ethanol (one change), (v) water (one change), and (vi) 1× PBS (two changes) and decanted. Frozen-coated slides were thawed at room temperature, fixed for 1 min in acetone/methanol, and air dried.

All slides were blocked for nonspecific binding sites for antibodies by the addition of 100 μL of 2% (w/v) bovine serum albumin (BSA; Trace) in HBSS, to each slide for 15 min. The slides were then decanted and 100 μL of the desired primary antibody was added. These included: (a) antihuman collagen type I antibody (Chemical Credential, ICN, MP Biomedicals, Aurora, Ohio, USA); (b) antihuman collagen type III antibody (DakoCytomation, Dako Denmark, Glostrup, Denmark); and cytokeratin clone LP34 (Dako-Cytomation).

All antibodies were diluted according to manufacturers' specifications in HBSS containing 1% (w/v) BSA, added to each slide, and left at room temperature for 30 min. The antibody was decanted and slides were washed four times with PBS containing 0.05% (v/v) Triton X-100. The secondary antibody, a biotinylated Multi-Link (DakoCytomation), was diluted (1 : 100) and applied in the same manner as the primary antibodies. Following a 30-min exposure, slides were washed four times in PBS with 0.05% Triton X-100 and then placed in TBS buffer 1 for 5 min.

Detection

Slides were decanted and biotin on the bound secondary antibody was detected using a single application of streptavidin-biotinylated alkaline phosphatase (Dako-Cytomation) diluted (1 : 100) in TBS buffer 1. Slides were washed four times in TBS buffer 1, incubated in TBS buffer 2 for 5 min, and decanted.

Visualization

Naphthol AS/MX phosphate/fast red substrate (100 μL) containing 5 mM (1.20 g/L of distilled water) levamisole (Sigma) was added to each section and left at room temperature for 20–40 min. The color development was assessed periodically by microscopic examination (Leitz Laborlux 12 Pol). Once optimal color development was achieved (usually 20 min, with no color development in the controls), slides were washed as follows: twice in TBS buffer 2; once by tap water; twice in TBS buffer 2;

and once by tap water. Slides were counter-stained with filtered Mayer's hematoxylin for approximately 2 min. Slides were then washed as follows: tap water two washes (to remove excess stain); dilute ammonium solution for 30 s and tap water two washes, and then mounted with cover slips in glycerol-gelatin (Sigma).

Slides were visually assessed for expression of collagens types I and III and cytokeratin compared to controls by the three authors independently, with the second author examining the slides coded as to the treatment applied.
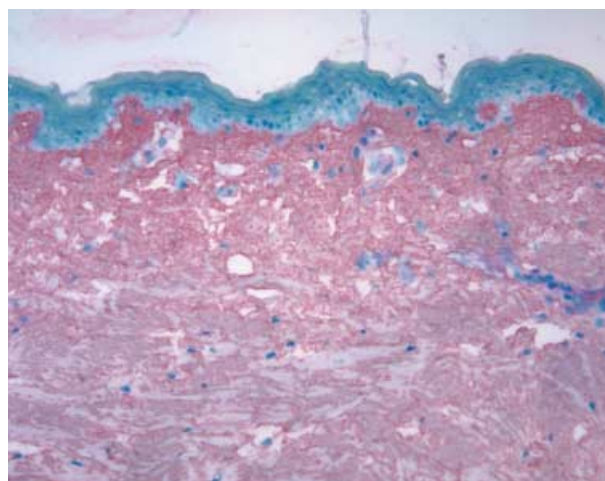
Results

Both formulations A and B resulted in increased expression of collagen type I compared to control (Figs 2–4).
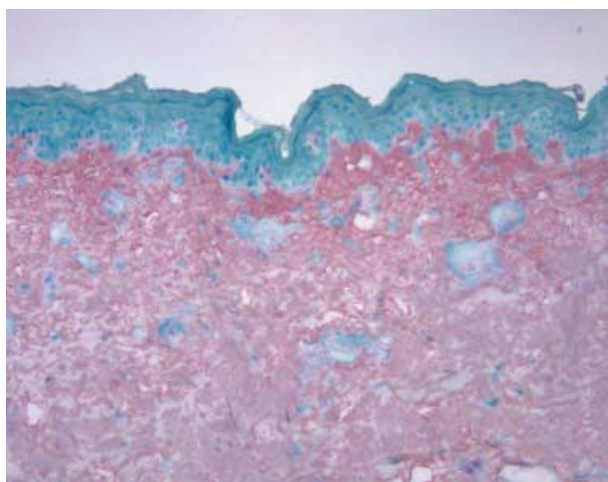
Figure 2 Control after 48 h, collagen I stain × 200.
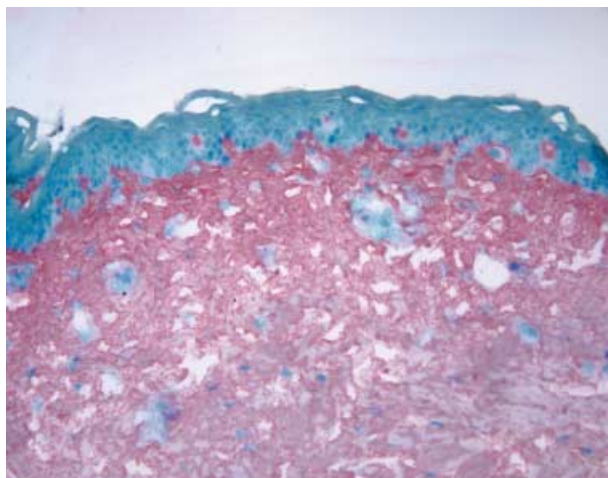
Figure 3 Anhydrous ascorbic acid formulation A after 48 h, collagen I stain × 200.

Figure 4 Anhydrous ascorbic acid formulation B after 48 h, collagen I stain × 200.

Both formulations also resulted in increased expression of collagen type III compared to control (Figs 5–7). Expression of both types of collagen appeared to be higher with formulation A, the oil/wax vehicle, compared with formulation B, the silicone/oil/wax vehicle. Cytokeratin expression was increased in the epidermis of treated skin compared to control by both formulations (Figs 8–10).

Discussion

This is the first report to describe effects of ascorbic acid in the skin resulting from its topical application as microfine particles in anhydrous formulations. Both formulations tested in this study resulted in the increased expression of collagen types I and III and cytokeratin in *ex vivo* human skin.

Expression of both types of collagen appeared to be higher with formulation B, the oil/wax vehicle, compared with formulation A, the silicone/oil/wax vehicle. The oil/wax vehicle contained 23% ascorbic acid compared to 20% ascorbic acid in the silicone/oil/wax formulation. It is also possible the oil/wax vehicle delivered the ascorbic acid more effectively into the skin but reasons for this would be unclear.

Clinically, increased dermal collagen would result in improvement in some signs of photoaging such as smoothing of texture, increased skin elasticity, and reduced skin wrinkling. Increased cytokeratin would result in improved skin barrier function and perhaps increased skin moisturization.
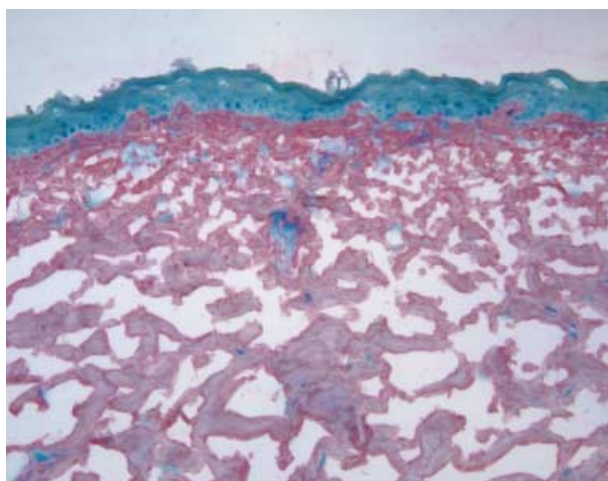
The method of percutaneous absorption of ascorbic acid particles is a matter for speculation. L-ascorbic acid in an anhydrous vehicle as microfine particles predominantly
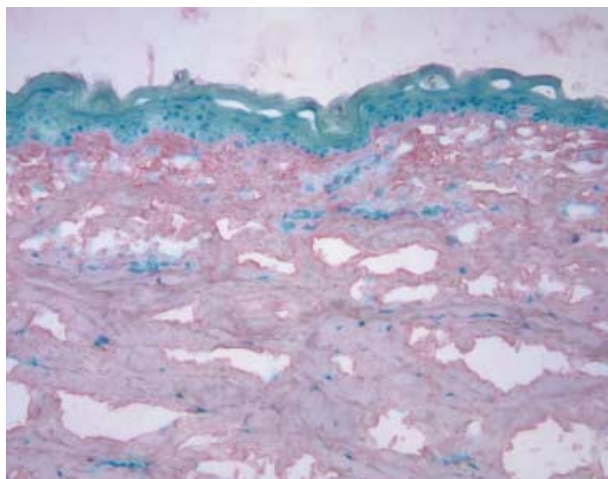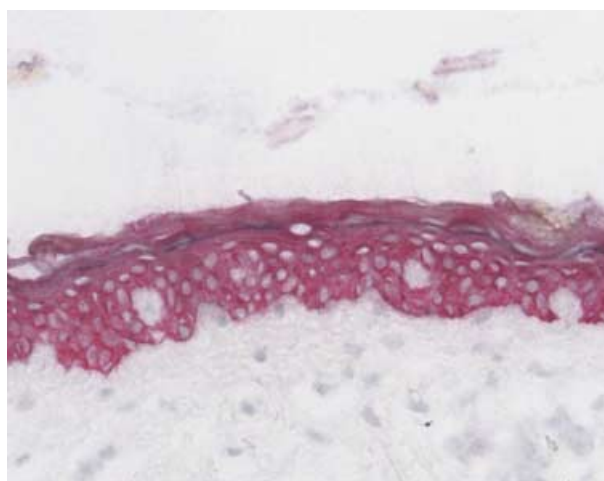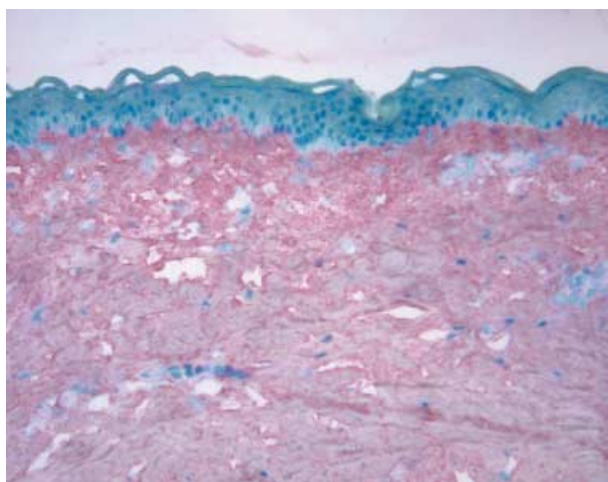
Figure 5 Control after 48 h, collagen III stain × 200.
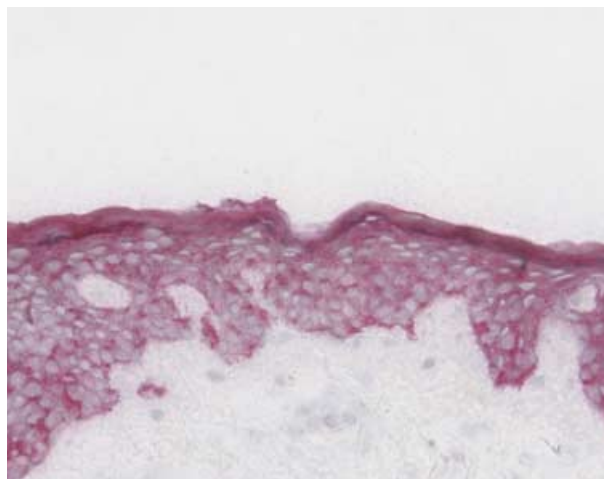
Figure 8 Control after 48 h, cytokeratin stain ×200.
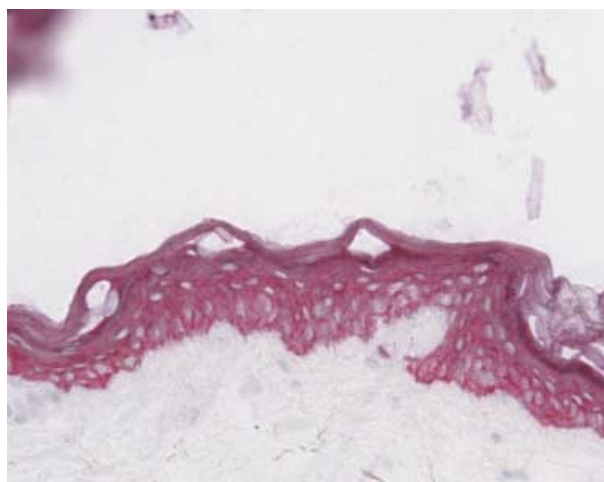

Figure 6 Anhydrous ascorbic acid formulation A after 48 h, collagen III stain × 200.

Figure 9 Anhydrous ascorbic acid formulation A after 48 h, cytokeratin stain × 200.

Figure 7 Anhydrous ascorbic acid formulation B after 48 h, collagen III stain × 200.

Figure 10 Anhydrous ascorbic acid formulation B after 48 h, cytokeratin stain × 200.

less than 20 microns in size has been reported to penetrate into the dermis.[26] Polystyrene microspheres of 5 μm have been reported to show a high concentration in follicular ducts without penetrating the stratum corneum after topical application.[34] Polystyrene microspheres less than 3 μm have been reported to penetrate into skin appendages and into superficial layers of the SC.[35]

The size of the ascorbic acid particles in the formulations tested in this study was less than 5 μm, with a range of approximately 1 μm to 5 μm. An anhydrous formulation applied topically would increase skin hydration and absorption of hydrophilic ingredients. Ascorbic acid particles on the surface of the skin would be expected to dissolve into water released transdermally under the topical anhydrous vehicle and proceed into aqueous channels in the lipid bilayer of the SC. Ascorbic acid: which had penetrated as particles less than 3 μm into the superficial SC through skin appendages, would be expected to dissolve into the aqueous channels in the lipid bilayer and travel to the dermis.

The delivery of ascorbic acid using microfine particles in an anhydrous topical formulation appears to offer many advantages over currently available therapeutic preparations. The main advantages are the reduced potential for oxidation of ascorbic acid in an anhydrous base and the higher biological activity that ascorbic acid has over its various therapeutically used derivatives.

Acknowledgements

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