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Research paper

Anti-inflammatory activity of *Punica granatum* L. (Pomegranate) rind extracts applied topically to *ex vivo* skin

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ABSTRACT

Coadministered pomegranate rind extract (PRE) and zinc (II) produces a potent virucidal activity against *Herpes simplex* virus (HSV); however, HSV infections are also associated with localised inflammation and pain. Here, the objective was to determine the anti-inflammatory activity and relative depth penetration of PRE, total pomegranate tannins (TPT) and zinc (II) in skin, *ex vivo*. PRE, TPT and $ZnSO_4$ were dosed onto freshly excised *ex vivo* porcine skin mounted in Franz diffusion cells and analysed for COX-2, as a marker for modulation of the arachidonic acid inflammation pathway, by Western blotting and immunohistochemistry. Tape stripping was carried out to construct relative depth profiles. Topical application of PRE to *ex vivo* skin downregulated expression of COX-2, which was significant after just 6 h, and maintained for up to 24 h. This was achieved with intact stratum corneum, proving that punicalagin penetrated skin, further supported by the depth profiling data. When PRE and $ZnSO_4$ were applied together, statistically equal downregulation of COX-2 was observed when compared to the application of PRE alone; no effect followed the application of $ZnSO_4$ alone. TPT downregulated COX-2 less than PRE, indicating that tannins alone may not be entirely responsible for the anti-inflammatory activity of PRE. Punicalagin was found throughout the skin, in particular the lower regions, indicating appendageal delivery as a significant route to the viable epidermis. Topical application of TPT and PRE had significant anti-inflammatory effects in *ex vivo* skin, confirming that PRE penetrates the skin and modulates COX-2 regulation in the viable epidermis. Pomegranates have potential as a novel approach in ameliorating the inflammation and pain associated with a range of skin conditions, including cold sores and herpetic stromal keratitis.

1. Introduction

There is a clinical need for more and improved anti-inflammatory products. Many disease states are associated with inflammation. This is a natural response by the body's defences and is essential in the tissue repair process; however, chronic inflammation is associated with pain and discomfort, and has been implicated as a preliminary stage in life-threatening conditions such as cancer and cardiovascular disease [1]. The immunologic basis for skin inflammation diseases such as psoriasis, allergic contact dermatitis and atopic dermatitis, with emphasis on potentially effective targets for novel anti-inflammatory drugs, was recently reviewed [2].

The processes involved in skin inflammation occur in the viable epidermis, which is largely composed of functional keratinocytes. During inflammatory challenge to the skin the keratinocytes respond by releasing cytokines and activating arachidonic acid metabolism along the COX-2 and lipoxygenase (LOX) pathways [3]. COX-2 is also rapidly upregulated upon chemical or mechanical tissue injury, signalling the arachidonic pathway to produce prostaglandin inflammatory mediators. The upregulation of COX-2 and LOX is transient with a short half-life and therefore is useful as a marker to determine the level of inflammation within skin at

Abbreviations: COX-2, cyclooxygenase-2; DAB, 3,3′-diaminobenzidine; DPX, distyrene, plasticizer, xylene; EDTA, ethylenediaminetetraacetic acid; FDC, Franz diffusion cells; HEPES, n-[2hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; HRP, horseradish peroxidase; HSV, *Herpes simplex* virus; IHC, immunohistochemistry; LOX, lipoxygenase; PRE, pomegranate rind extract; RIPA, radioimmunoprecipitation assay buffer; TFF, tannin-free fraction; TPT, total pomegranate tannins; WB, Western blotting; 96WP, 96 well (microtitre) plate.

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particular time points. Analysis of the level of COX-2 expression in skin *ex vivo* provides a comparative model for the anti-inflammatory anti- (or pro-) inflammatory responses of topically applied xenobiotics, and has been used to study for example n-3 fatty acids, ketoprofen and an extract of *Harpagophytum procumbens* have shown this [4,5].

The pomegranate, fruit of the *Punica granatum* L. tree, has been used since ancient times to treat a wide range of ailments. Pomegranate rind extract (PRE) is obtained from the pericarp (rind), which contains the highest concentration of phytochemicals, principally polyphenolic flavonols and ellagitannins, including ellagic acid and punicalagin. It has recently been shown that the innate antimicrobial activity of PRE is significantly potentiated by the co-administration of zinc (II) ions with up to 7-log reduction potency observed against *Herpes simplex* virus (HSV) - the causative microorganism of anogenital herpes and oral coldsores [6]. HSV type 1 and 2 lesion eruptions cause a significant level of inflammation to the localised area, resulting in erythema, swelling and pain, particularly in the later stages of infection. Furthermore, other HSV-related conditions, such as herpetic stromal keratitis, are also associated with upregulated COX-2 expression [7].

Although challenging the virus is the primary concern when treating such conditions, a substantial viral load reduction may take time to feedback into a reduction of inflammation. Thus there would be distinct advantages if a medication could simultaneously address this inflammation-mediated discomfort and appearance directly. In terms of a pomegranate-based product, this notion is underpinned by a growing body of work including two reviews that illustrate the beneficial effects of pomegranate extracts as potential inflammation treatments [8,9]. The positive effect of the consumption of pomegranate extracts and juice has been demonstrated in relation to inflammation in the gastrointestinal tract, where ellagic acid was stated as the responsible agent [10,11]. Polyphenolics of differing structure regulate inflammation-involved pathways in different ways while attenuating colitis [12]. The flavanol quercetin, was found to suppress the expression of COX-2 mRNA in the pouch exudates cells of a rat paw, indicating that the anti-inflammatory action of quercetin may partly due to suppressing the up-regulation of COX-2 [13]; pomegranate aqueous extract was found to inhibit COX expression in mice [14] and reduce inflammatory processes in patients with type-2 diabetes [15].

The purpose of this study was to probe the effect of topically applied PRE, purified tannins (TPT) and $ZnSO_4$ on COX-2 expression following their individual application, or in combination, on porcine skin *ex vivo*. Porcine ears have been used extensively as an acceptable model for human skin in the percutaneous penetration of xenobiotics [16,17]. Anti-inflammatory effects were assessed using Western blotting and immunocytochemistry to determine the modulation of endogenous epidermal COX-2 levels. Skin penetration depth profiles were also determined for punicalagin and Zn (II) by tape stripping.

2. Materials and methods

2.1. Materials

Pomegranates, of Spanish origin, were obtained from a local supermarket. Radioimmunoprecipitation assay buffer (RIPA buffer, comprised of 50 mM tris-HCl (pH 7.4), 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride serine protease inhibitor), Hanks balanced salt solution (HBSS), gentamycin sulphate, Ponseau S, PBS + 0.05% TWEEN, aprotinin, leupeptin, anti-actin antibody, sodium azide, SDS 10%, paraffin wax pellets, phosphate buffer saline pH 7.4 (PBS), methyl green, distyrene/plasticizer/xylene (DPX mountant) and λ-carrageenan were all purchased from Sigma-Aldrich, Poole, UK. Zinc sulphate ($ZnSO_4$), potassium hydrogen phthalate, trifluoroacetic acid and HPLC-grade solvents were obtained from Fisher Scientific (Loughborough, England). Primary COX-2 antibody (#4842) was purchased from Cell Signalling Technology (Boston, USA). Horseradish peroxidase (HRP)-labelled anti-rabbit polymer, DAB chromagen plus substrate was purchased from Dako UK (Ely, England). Western blocking reagent was from Roche Diagnostics, GmbH (Mannheim, Germany). Rainbow Marker (10–250 Kd), *anti*-mouse HRP, (HRP)-linked antibody were from Amersham Biosciences Ltd (Amersham, UK). Dura substrate was from Perbio, Cramlington, UK, and 3,3′-diaminobenzidine (DAB) chromagen-AB substrate was from Abcam, Cambridge, UK. Freshly excised porcine ears were obtained from a local abattoir prior to steam cleaning and immersed in iced Hanks buffer before being subjected to laboratory experimentation within 1 h. As these were excised from freshly slaughtered pigs their use was not subject to ethical approval.

2.2. Preparation of pomegranate rind extract (PRE)

The rinds of 6 pomegranates were excised by scalpel and cut into thin strips, blended in deionised H_2O (25% w/v) and boiled for approximately 10 min. The crude solution was then transferred into 50 mL screw-capped tubes and centrifuged at 10,400*g* at 4 °C for 30 min using a Beckman Coulter Avanti J25 Ultracentrifuge and vacuum filtered through a Whatman 0.45 μm nylon membrane filter (Fisher Scientific, Loughborough, UK) before being freeze-dried and stored at −20 °C until required. The PRE was reconstituted in pH 4.5 phthalate buffer by adding 2 mg to 10 mL buffer and sonicated for 10 min at 50–60 Hz until fully dissolved - this particular buffer and pH were used as it provided the optimum activity against HSV [6]. Punicalagin content was analysed by HPLC (Section 2.8) and consisted of 20% w/w of the freeze-dried PRE. The chemical structure and a chromatogram of punicalagin showing its composition of two anomers is shown in Fig. 1.

2.3. Preparation of total pomegranate tannins (TPT) and tannin-free fraction (TFF)

Total pomegranate tannins (TPT) is the purified form of PRE which has been stripped of its non-polyphenolic constituents and was prepared by column chromatography using a glass column slurry packed with 75 g of Amberlite XAD-16 resin in H_2O [18]. The resin was washed with 300 mL methanol then 100 mL H_2O and left for 12 h to equilibrate. A mass of 5 g PRE (dry weight) was loaded onto the column - the optimal loading volume 40 ± 5 mL per 75 g of preconditioned XAD-16 resin per column and was eluted with 300 mL of H_2O until the pale yellow tannin-free fraction (TFF) was fully removed. The remaining tannins were eluted with 100 mL MeOH to yield a dark red TPT solution, which was dried under vacuum to yield TPT (freeze dried yield 1.3 g, 26%) and TFF (freeze dried yield 3.4 g, 68%). It was determined that this fraction contained 1.09 g of punicalagin (84% of the TPT fraction), and 0.009 g of ellagic acid (0.69%). These findings are in accordance with the literature which states that punicalagin was equivalent to 80–85% and ellagic acid 1.3% of the total pomegranate tannin content [18]. HPLC revealed no evidence of tannins present within the TFF fraction (not shown).

2.4. Ex vivo skin preparation and topical delivery

The ears were excised immediately post mortem and immersed in iced Hanks buffer, before arriving in the laboratory within 1 h. The ears were subsequently cleaned, hairs trimmed and dorsal full