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Original article

Protease loaded permeation enhancer liposomes for treatment of skin fibrosis arisen from second degree burn

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ABSTRACT

Cysteine protease (papain) is a plant derived enzyme and due to its collagenolytic activity has potential in fibrosis reduction. However, a major hurdle in its use as fibrosis reducing agent is to overcome stratum corneum skin barrier via topical application, owing to its hydrophilic and high molecular weight and protein nature which is prone to degradation. The aim of the present study was to develop a penetration enhancer incorporated drug delivery system, i.e. propylene glycol (PG) liposomes, loaded with papain for application in fibrosis therapy. Papain loaded PG-liposomes were prepared by the solvent injection method and characterized by size, shape, zeta potential, entrapment efficiency, drug release and stability. Papain conformational changes due to process stress were evaluated by electrophoresis and fluorescence spectroscopy. Biological evaluation was carried out in rodents by skin irritation and percent fibrosis reduction assays following induction of fibrosis arisen due to controlled second degree burn. Papain loaded PG-liposomes had mean vesicle size 180 ± 30.3, zeta potential -25 ± 1, polydispersity index 0.181 and $85 \pm 4.3\%$ entrapment efficiency. Cumulative drug release after 8 h was found to be $74.26 \pm 3.0\%$. SDS-PAGE and fluorescence spectroscopic studies confirmed the stability of papain after incorporation in PG-liposomes. Fibrosis reduction studies in animal models revealed that PG-liposomes incorporated papain improved fibrosis reduction significantly in comparison to conventional liposomes and free papain solution ($p < 0.05$). Data suggest that propylene glycol incorporated liposomal system enhances papain proteolytic and collagenolytic activity along with a reduction in skin irritancy via preventing direct contact of papain with skin, improves papain therapeutic fibrosis reduction potential, an approach that may provide an efficient alternative for protease mediated fibrosis reduction in a variety of demanding circumstances.

1. Introduction

Thermal Burn is a type of tissue lesions to the skin caused by exposure of heated objects such as hot liquids (water, steam, cooking oils, petroleum etc), fire and hot objects (ashes and coals, utensils, soldering equipment, exhaust pipelines etc) [1–3]. Thermal burns [4] have a relatively high incidence and can affect people of all ages, from newborns to the elderly worldwide. It is estimated that every year, around billion people suffered various burns. Prevalence of burns is much higher, particularly in underdeveloped countries [5]. Every year in India, annually estimated burn incidences are around 6.6 million, the second largest group of injuries after road accidents [6]. The early response to thermal injury involves protein denaturation, followed by inflammation and ischemia-induced injury, which causes burns of varying skin depth [7].

Burn wound conversion occurs as the secondary consequences of burn injury i.e. edema, infection along with perfusion changes and the varying degree of cell death [6,8]. Wound healing cascade starts with an orderly chain process of signals and responses includes hemostasis, inflammation, and proliferation (fibrin deposition) and remodeling (collagen crosslinking) and fibrosis maturation [9,10]. The wound healing ability of skin mainly depends upon the dermis, as it contains macrophages, fibroblast, produce growth factor, which regulates epidermal regeneration [11]. Fibrosis is areas of replacement of the normal structural tissue elements of the skin by vague, dysfunctional fibrosis tissue. Many clinical problems are associated with excessive fibrosis formation [12]. Fibrosis tissue overproduction diminishes flexibility of tissues, promotes associated diseases such as Keloids, scleroderma

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etc. Fibrosis tissues are less resistant to ultraviolet light and sweat glands. Hair follicles do not grow back again in this tissue [13,14].

Fibrosis management is based on findings of patient's individual requirements. Many techniques for management of fibrosis are prevalent for example chemical peel, filler injection, dermabrasion, laser treatment, semi-occlusive dressing, radiotherapy, pressure dressing, steroids, surgery, etc, however, some of them have been beaten up by potential studies with ample control groups [15–22]. A number of new therapies presented their remarkable results in small-scale trials but seems poor on large-scale trials for the reason that recurrence rates are high.

Cysteine protease enzyme also commonly known as papain is obtained from the fruit of *Carica papaya* consists of an enzyme mixture, involving essentially a combination of papain and chymopapain [23]. Papain has stronger proteolytic activity as well as large range of action involving in the deprivation of the key protein of myofibrillar muscles [24]. Due to collagenolytic activity, it enhances the skin turnover rate and brings to the surface a layer of smoother, soft skin which removes fibrosis. Furthermore, it facilitates diffusion of hydrophilic substances into the epidermal and dermal layer by exfoliating the stratum corneum thus altering its barrier properties [25]. Unfortunately, two major obstacles circumvent its use in topical administration, firstly, its high molecular weight and second, the barrier function of the stratum corneum. To surmount these hurdles, novel delivery systems are proven to be miraculous tools.

Liposomes are colloidal particles that are composed of concentric bilayers formed from self-assembly of amphiphilic molecules. Synergistic interactions between the components of the liposomes and between the liposomes and skin components are deemed to be accountable for the superior skin permeation enhancement [26]. Permeation enhancer liposomes are flexible lipid vesicles used to enhance delivery of pharmaceuticals into the deeper layer of skin. Due to the presence of propylene glycol [27], they enhance entrapment efficiency and deposition of the drug on the skin, improved solubility, and stability and reduce skin irritation caused by a variety of drugs [28,29].

The objective of the present study was to formulate papain loaded PG-liposomes using propylene glycol as a permeation enhancer and determine its fibrosis reducing potential in an animal model of second degree burns. Thermally induced skin lesions were induced in Sprague Dawley rats via heated aluminum rod and marketed formulation of Aminacrine hydrochloride and Cetrimide (Bernol®) were applied on wounds to prevent infection. After 21 days, fibrosis was developed which was treated with papain loaded PG liposomes and fibrosis reducing potential were measured.

2. Materials and methods

2.1. Materials

Papain was obtained from *Caraya papaya* fruits latex and cholesterol (99.8% Pure) was purchased from Himedia Pvt. Ltd., Mumbai, India. Soy Phosphatidylcholine (PC) was a kindly donated by Lipoid GmBH (Germany). Propylene glycol was obtained from Thermo Fischer Scientific Mumbai, India. Reagents of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), Sephadex G-50, Fluorescein isothiocyanate (FITC)-OVA and Bradford reagent protein assay kit were obtained from Sigma-Aldrich Pvt. Ltd., St. Louis, USA. Aluminum rod, cotton, marketed formulation of Aminacrine hydrochloride and Cetrimide (Bernol®) and diclofenac sodium (Diclomol®) was purchased from local market. Ketamine and xylazine obtained from Neon Laboratories Pvt Ltd and Indian Immunological Ltd. Hyderabad India respectively. All other chemicals were of analytical grade. Double distilled water was used throughout the study.

2.2. Methods

2.2.1. Preparation of PG-liposomes

Uniform-sized liposomes were prepared by solvent injection method. Briefly, 40 mg PC (4% w/v) was dissolved in 1 ml of propylene glycol on the heating magnetic stirrer at 47 °C for 15 min with 750 rpm (REMI Instruments, India). 40 mg papain was dissolved in 9 ml of PBS buffer (5.8 pH, 0.1 M). Papain solution was added with the help of peristaltic pump connected a syringe in streamline flow with continuous stirring for 30–35 min at 55–60 °C and 750 rpm. During the whole process, the container was properly closed with cork and parafilm. The resulting dispersion was then sonicated with a microprobe sonicator for 20 min to obtained final PG liposomes formulation. The PG liposomes was placed on the top of Sephadex G-50 column, centrifuged in cooling ultracentrifuge (REMI Instruments, India) at temperature 4 °C (3000 rpm, 20 min) and finally the drug-loaded liposomes free from unentrapped drug was settled down in bottom of centrifuge tube were collected and lyophilized for future use (5% mannitol as cryoprotectant). The blank PG-liposomes prepared by the same method and experimental parameters were same as described above except that during hydration step phosphate buffer solution was added without any papain content. Conventional liposomes with papain were also prepared as described previously [30].

2.2.2. Characterization of liposomes

Vesicles size, zeta potential, and polydispersity index (PDI) of prepared liposomes were measured by photon correlation spectroscopy using Zeta Nano ZS (Malvern Instruments, UK) with dilution 1:100 using PBS pH 7.0 with an angle of detection 173° at 25 °C. Each sample was measured three times. Liposomes morphology were examined using transmission electron microscopy (TEM) (Joel, JEM-100 CX, Holland). Negative staining of samples was employed, using 1% w/v Phosphotungstic acid at pH 5.2 as a contrasting agent. Viscosity and pH measurement of optimized formulations were performed with the help of Viscometer (Brookfield DV-111p, USA) with spindle LV 63 at 100 rpm and pH meter (Systronic digital pH meter 355, India) respectively. Enzymatic activity of the standard papain formulation was calculated using 5 ml of the buffered substrate, casein; 10 mg/ml, pH 6.0 phosphate-cysteine disodium ethylene diamine tetraacetate buffer, incubated for 60 min at 40 °C. The digestion process of casein was stopped by adding 3 ml 30% w/v Trichloroacetic acid solution and allowed to stand for 30 to 40 min at 4 °C. Digested amino acids were filtered and absorbance was calculated at 280 nm against their respective blanks with the help of UV Spectrophotometer (UV-1800 Shimadzu, Japan) [31].

For the determination of entrapment efficiency, 1 ml liposomal formulation was lysed using 0.1 ml (10% v/v) Triton X-100 and drug content was determined after subsequent dilution and the amount of papain was analyzed by Bradford's method at 595 nm (colorimetric assay) using UV–vis spectrophotometer (UV-1800 Shimadzu, Japan). Each experiment performed in triplicate.

$$\text{Entrapment efficiency (\%EE)} = (\text{Amount in liposomes/Initial drug added}) \times 100$$

In vitro release study of papain containing PG-liposomes and conventional liposomes were monitored by dialysis method. An appropriate quantity of liposomes (papain content, 1 mg/ml) was placed in a dialysis membrane (25 kDa) in a beaker containing 200 ml of Phosphate buffer (PBS pH 5.8) at 37 ± 0.5° C with continuous stirring at 20–30 rpm. Aliquots were removed at 1 h