Minimization of Local and Systemic Adverse Effects of Topical Glucocorticoids by Nanoencapsulation: *In Vivo* Safety of Hydrocortisone–Hydroxytyrosol Loaded Chitosan Nanoparticles

MUHAMMAD IRFAN SIDDIQUE,[1] HALIZA KATAS,[1] MOHD CAIRUL IQBAL MOHD AMIN,[1] SHIOW-FERN NG,[1] MOHD HANIF ZULFAKAR,[1] FHATAHEYA BUANG,[1] ADAWIYAH JAMIL[2]

[1]Centre for Drug Delivery Research, Faculty of Pharmacy, Universiti Kebangsaan Malaysia, Kuala Lumpur 50300, Malaysia
[2]Faculty of Medicine, Universiti Kebangsaan Malaysia Medical Centre, Kuala Lumpur 56000, Malaysia

Received 30 June 2015; revised 5 September 2015; accepted 9 September 2015

Published online 8 October 2015 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24666

ABSTRACT: Hydrocortisone (HC) is a topical glucocorticoid for the treatment of atopic dermatitis (AD); the local as well as systemic side effects limit its use. Hydroxytyrosol (HT) is a polyphenol present in olive oil that has strong antimicrobial and antioxidant activities. HC–HT coloaded chitosan nanoparticles (HC–HT CSNPs) were therefore developed to improve the efficacy against AD. In this study, HC–HT CSNPs of 235 ± 9 nm in size and with zeta potential +39.2 ± 1.6 mV were incorporated into aqueous cream (vehicle) and investigated for acute dermal toxicity, dermal irritation, and repeated dose toxicity using albino Wistar rats. HC–HT CSNPs exhibited LD_{50} > 125 mg/body surface area of active, which is 100-fold higher than the normal human dose of HC. Compared with the commercial formulation, 0.5 g of HC–HT CSNPs did not cause skin irritation, as measured by Tewameter®, Mexameter®, and as observed visually. Moreover, no-observed-adverse-effect level was observed with respect to body weight, organ weight, feed consumption, blood hematological and biochemical, urinalysis, and histopathological parameters at a dose of 1000 mg/body surface area per day of HC–HT CSNPs for 28 days. This *in vivo* study demonstrated that nanoencapsulation significantly reduced the toxic effects of HC and this should allow further clinical investigations.

Keywords: chronic dermal toxicity; corticosteroids; chitosan; nanoparticles; nanotoxicology; nanoencapsulation; skin; targeted drug delivery; biodegradable polymers

INTRODUCTION

Over the last three decades, the incidence of atopic dermatitis (AD) has increased twofold to threefold, affecting 18% of children and 5% of adults,[1,2] and the evidence suggests that its pervasiveness continues to increase.[3] AD is a common, chronically relapsing inflammatory skin disease characterized by itchiness, vesiculation, exudation, scaling, and lichenification of the skin that significantly impairs the patient's quality of life.[4] As AD skin is colonized by *Staphylococcus aureus* (*S. aureus*), the bacterium releases cytolytic toxins. These toxins induce mast cell degranulation and trigger production of specific immunoglobulin E molecules,[5] which exacerbates the severity of AD.

Synergistic effects of biologically active naturally occurring and synthetic compounds expand potential treatment strategies for many diseases. Hydroxytyrosol (HT), also known as phenyl ethyl alcohol, 2-(3,4-dihydroxyphenyl)ethanol is one of the most abundant natural phenolic components of olive oil.[6,7] HT is obtained from enzymatic or chemical hydrolysis of oleopurine during the maturation of olives.[8,9] A significant amount, about 100–600 mg/kg, of phenolic compounds is present in extra virgin olive oil, approximately half of which is HT and/or HT derivatives.[10,11] Red wine is another source of HT, comprising about 1.8–3.1 mg/L HT.[12] HT possesses antioxidant,[13,14] anti-inflammatory,[15,16] cardio-protective,[17] and antimicrobial activities, especially against *S. aureus*.[18] The skin of 90% of patients with AD is colonized by this bacterium.[19,20] HT has gained particular attention owing to its antimicrobial[21,22] and bactericidal activities against gram-positive and gram-negative bacteria.[23]

Topical glucocorticoids are the mainstay of drug therapy for AD because of their broad immunosuppressant and anti-inflammatory effects.[24,25] However, topical steroids have unwanted local side effects, including dermal atrophy, acneiform eruption, striae, and telangiectasia, as well as risks of systemic effects, such as hypothalamic–pituitary–adrenal (HPA) axis suppression[26] and glaucoma development because of increased intraocular pressure.[27,28] Thinning of the skin associated with topical glucocorticoid treatment enhances water permeability, thereby increasing transepidermal water loss (TEWL).[29] Long-term use of topical steroids may cause adrenal insufficiency.[30] These unexpected adverse effects have created a "steroid phobia" among patients.[31] The safety of drug or chemical entity could be evaluated by analyzing parameters recommended by Organization for Economic Co-operation and Development (OECD) guidelines. For example, the systemic adverse effect of glaucoma caused by topical glucocorticoids can be monitored by measuring blood glucose level because significant percutaneous absorption of topical glucocorticoids may cause hyperglycaemia[32] and exacerbate it when absorbed systemically.[33] High level of cortisol in the body is responsible

Abbreviations used: AD, atopic dermatitis; HC, hydrocortisone; HT, hydroxytyrosol; CSNP, chitosan nanoparticle; NOAEL, no-observed-adverse-effect level; TEWL, transepidermal water loss.

Correspondence to: Haliza Katas (Telephone: +603-92897971; Fax: +603-26983271; E-mail: haliz12@hotmail.com)

This article contains supplementary material available from the authors upon request or via the Internet at http://wileylibrary.com.

Journal of Pharmaceutical Sciences, Vol. 104, 4276–4286 (2015)

for the suppression of HPA axis and adrenal corticothyroid hormone (ACTH), which leads to systemic adverse effects such as adrenal atrophy osteoporosis (inhibition of growth). These findings prompted the development of a drug delivery system that could codeliver HC–HT to the skin layers of patients with AD with minimal systemic absorption leading to fewer adverse effects. HC, a class-IV low-potency topical glucocorticoid, was formulated into biodegradable polymeric nanoparticles of chitosan. Codelivery of HC–HT using chitosan nanoparticles (CSNPs) has been previously investigated by our group to alleviate the signs and symptoms of AD.[34,35] The results obtained suggested that both HC and HT were accumulated at the dermis and epidermis layers and thus minimizing systemic absorption.

The safety of orally administered HT has been previously reported elsewhere.[36] Despite the availability of safety information, the toxicological profile of HT when used as topical formulations, particularly those containing other active ingredients and a carrier (HC–HT CSNPs), is unknown. Therefore, we have evaluated the use of CSNPs as a carrier for HC–HT and the effects on skin irritation. Acute and repeated dose dermal toxicity on healthy albino Wistar rat skins were also measured in an attempt to gain a better understanding of the adverse effects caused by local delivery of HC–HT incorporated into CSNPs.

MATERIALS AND METHODS

Materials

Hydroxytyrosol, CS (MW, 70 kDa; deacetylation degree, 85%), HC (base form), and phosphate-buffered saline were purchased from Sigma–Aldrich Chemicals Company Ltd. (St. Louis, Missouri). Pentasodium tripolyphosphate (TPP) was purchased from Merck KGaA Company Ltd. (Darmstadt Germany). Isoflurane (inhalation anesthetic) was obtained from Medigen Pharma Limited (Kuala Lumpur, Malaysia). A commercial cream containing 0.5% HC (DermAid 0.5%; Ego Pharmaceuticals Pvt. Ltd., Selangor, Malaysia) was used as a positive control. Aqueous (AQ-cream) (a gift from Xorix Pharmaceuticals Pvt. Ltd., Dungun, Terengganu, Malaysia) was used as vehicle base for compounding nanoparticle formulation.

Methods

Preparation of Nanoparticles

Hydrocortisone–HT CSNPs were prepared by ionic crosslinking of CS with TPP ions (ionic gelation). A mixture of the two active ingredients (HC and HT, 1 mg/mL each in 30:70 ratios of ethanol and water) was added to CS solution (0.2%, w/v, in 1%, v/v, acetic acid) and left to achieve equilibrium for 30 min. The pH of the mixture was adjusted to 5.0 by adding 1 M sodium hydroxide (NaOH) solution. Coloaded CSNPs were formed spontaneously by dropwise addition of TPP solution to CS solution containing both HC and HT at a CS to TPP mass ratio of 5:1 with constant magnetic stirring at 700 rpm. Coloaded CSNPs were collected by ultracentrifugation using an Optima L-100 XP Ultracentrifuge (Beckman-Coulter, Brea, California) with a NV 70.1 Ti rotor (Beckman-Coulter) at 72,002*g* for 30 min. The nanoparticles were then lyophilized at −40°C for 24 h.

Physicochemical Characterization

Size, Zeta Potential, and Morphology. Laser Doppler anemometry (Nano-Zetasizer, Malvern, UK) was used to measure particle size, polydispersity index, and zeta potential. These measurements were carried out in triplicate using disposable polystyrene cuvettes at 25°C. Data are presented as mean ± standard deviation (SD). The morphology of nanoparticles was analyzed under a transmission electron microscope (TEM; Hitachi H-7000 TEM, Japan). Prior to the analysis, freeze-dried nanoparticles were suspended in distilled water and sonicated for 3 min. A drop of suspension was then deposited onto a glow discharged carbon-coated microscopy grid and allowed to dry.

Drug Encapsulation Efficiency. Encapsulation efficiency (%EE) of the drugs into the nanoparticles was determined as follows.[37] A calibration curve was obtained by reverse-phase (RP-HPLC) (Waters: Millford, Massachusetts) of various standard solutions of HC and HT. The %EE for each drug was calculated as:

$$\mathrm{EE}(\%) = \mathrm{Wt} - \left(\frac{\mathrm{Wf}}{\mathrm{Wt}} \times 100\right)$$

where Wt is the total initial amount of HC or HT and Wf is the amount of free drug in the supernatant after ultracentrifugation. All measurements were performed in triplicate and are reported as mean ± SD.

Drug Analysis. A RP-HPLC analysis was established for measuring the loading amount and efficiency of HC and HT within the nanoparticles. The HPLC system (Waters) was composed of autosampler (2707), and photodiode array detector (2998), operated with a software empowers with C_{18} column (LiChroCART® 250–4.6 mm, 5 μm; Merck KGaA Company Ltd.). The mobile phase was consisting of acetonitrile–methanol–water (27:15:58, v/v) with a flow rate of 1.0 mL/min. Eluted compounds were detected at 248 nm for HC (retention time at 9.58 min) and 280 nm for HT (retention time at 3.34 min).

In Vivo Study

Animals. All experimental procedures involving animals were reviewed and approved by the UKM Animal and Ethics Committee (FF/2014/HALIZA/16-JULY/595-AUG-2014-OCT-2014). Male and nonpregnant or nulliparous female Albino Wistar rats weighing between 150 and 250 g were obtained from the UKM animal house. Their overt signs of health were checked, especially their skin. Animals were housed in polypropylene cages with wood shavings (Lignocel, type 3/4; Rettenmaier, Rosenberg, Germany) under standard laboratory conditions (temperature = 22 ± 1°C, relative humidity = 70%, 12 h light:12 h dark cycle). The animals were acclimated for 7 days prior to start the experiment. All animals had free access to water and standard laboratory diet.

Experimental Design. Acute dermal toxicity. The experiment was carried out according to the Organization for Economic Cooperation and Development Guidelines, OECD-402.[38] The day before testing, a dorsal area of the trunk (5 cm^2) was clipped with professional pet clippers (Codos CP-3800) carefully to