Pharm Res
DOI 10.1007/s11095-016-2036-8

RESEARCH PAPER

Novel Fish Oil-based Bigel System for Controlled Drug Delivery and its Influence on Immunomodulatory Activity of Imiquimod Against Skin Cancer

Khurram Rehman [1,2] • Mohd Hanif Zulfakar [2]

Received: 5 July 2016 / Accepted: 1 September 2016

ABSTRACT

Purpose To characterize bigel system as a topical drug delivery vehicle and to establish the immunomodulatory role of imiquimod-fish oil combination against skin cancer and inflammation resulting from chemical carcinogenesis.

Methods Imiquimod-loaded fish oil bigel colloidal system was prepared using a blend of carbopol hydrogel and fish oil oleogel. Bigels were first characterized for their mechanical properties and compared to conventional gel systems. *Ex vivo* permeation studies were performed on murine skin to analyze the ability of the bigels to transport drug across skin and to predict the release mechanism via mathematical modelling. Furthermore, to analyze pharmacological effectiveness in skin cancer and controlling imiquimod-induced inflammatory side effects, imiquimod-fish oil combination was tested *in vitro* on epidermoid carcinoma cells and *in vivo* in Swiss albino mice cancer model.

Results Imiquimod-loaded fish oil bigels exhibited higher drug availability inside the skin as compared to individual imiquimod hydrogel and oleogel controls through quasi-Fickian diffusion mechanism. Imiquimod-fish oil combination in bigel enhanced the antitumor effects and significantly reduced serum pro-inflammatory cytokine levels such as tumor necrosis factor-alpha and interleukin-6, and reducing tumor progression via inhibition of vascular endothelial growth factor. Imiquimod-fish oil combination also resulted in increased expression of interleukin-10, an anti-inflammatory cytokine, which could also aid anti-tumor activity against skin cancer.

Conclusion Imiquimod administration through a bigel vehicle along with fish oil could be beneficial for controlling imiquimod-induced inflammatory side effects and in the treatment of skin cancer.

KEY WORDS bigels · drug delivery system · epidermoid carcinoma · fish oil · imiquimod · immunomodulatory · skin cancer

ABBREVATIONS

HPV	Human papilloma virus
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
VEGF	Vascular endothelial growth factor
ELISA	Enzyme-linked immunosorbent assays
IL	Interleukin
TNF	Tumor necrosis factor
FO	Fish oil
IMQ	Imiquimod
IMQ-FO	Imiquimod-fish oil
PBS	Phosphate buffer solution
SC	Stratum corneum
EPD	Epidermis and dermis
DMBA	7,12-dimethylbenz(a)anthracene
IFN	Interferon

INTRODUCTION

Skin has been the site of interest for the administration of therapeutic drugs via transdermal and topical drug delivery systems. Epidermoid carcinoma is a type of skin cancer that originates in the epithelial layer of the skin, and is also referred to as squamous cell carcinoma. It arises from uncontrolled multiplication of epithelium and it could also occur due to

✉ Mohd Hanif Zulfakar
hanifzulfakar@ukm.edu.my

[1] Lahore Pharmacy College (A Project of Lahore Medical and Dental College), Tulspura Canal Bank, Lahore 53400, Pakistan

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

Published online: 12 September 2016

over exposure to sunlight (1), viral infection by pathogens such as human papilloma virus (HPV), and also untreated chronic inflammation (2, 3). Epidermoid carcinoma appears as an erythematous papule or plaque and could metastasize to other portions of body (4). Imiquimod is an immune response modifier currently used against skin diseases such as actinic keratosis, basal cell carcinoma, neoplasia, and external genital warts in adults (5–9). It is also known for its inflammatory-like side effects such as severe skin inflammation, presenting as psoriasis-like lesions (10, 11).

In an earlier work, we demonstrated via computational molecular modelling an interaction between fish oil and imiquimod. A possible complex formation between imiquimod and eicosapentaenoic acid (EPA) through a π-σ interaction between the aromatic ring of the drug and a long chain fatty acid was confirmed (12). Such interactions play an important role in biological systems and molecular recognition (13, 14) and along with controlled release of imiquimod through bigels may also help in reducing drug-related side effects (12). Bigels are defined as an intimate hydrogel/oleogel colloidal mixture. Described relatively recent in the pharmaceutical industry, they were first prepared to minimize the drawbacks of conventional hydrogels and oleogels (15, 16). They possess the characteristics of both gel systems, and are currently a focus of interest in the development of topical formulations (17, 18).

The aim of this study was to characterize bigel system as a topical drug delivery vehicle and to establish an immunomodulatory role of imiquimod-fish oil combination against skin cancer and inflammation resulted from chemical carcinogenesis. In this study, 5% w/w imiquimod-loaded bigels were formulated from a mixture of fish oil oleogel and carbopol hydrogel at varying w/w ratios. Fish oil as a source of omega-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) was added to enhance the topical permeation of the drug (19–23), and to reduce the inflammatory side effects of the drug (12). Formulations were first physically and mechanically characterized and then subsequently their effects were studied *in vitro* in epidermoid cancer cell lines. In the later part of the study, animal skin cancer models (Swiss albino mice) were used to determine the therapeutic effectiveness of imiquimod-loaded fish oil bigel compared with imiquimod commercial cream formulation as a control. Physical observations through Dino-lite® microscope, along with histopathological studies and enzyme-linked immunosorbent assays (ELISA) for vascular endothelial growth factor (VEGF), interleukin-6 (IL6), interleukin-10 (IL10), and tumor necrosis factor-alpha (TNF-α) were conducted to better understand the effects of delivering imiquimod from the developed delivery system.

MATERIALS AND METHODS

Materials

Blackmores Fish Oil (42% EPA and 21% DHA) was purchased from Blackmores (Warriewood, Australia). Benzalkonium chloride and carbopol 940 were purchased from ACROS (New Jersey, USA). Triethanolamine was obtained from Friendemann Schmidt (Parkwood, Australia). Beeswax was obtained from R & M chemicals (Selangor, Malaysia). Cetrimide, Sudan stain, butylated hydroxyanisole, and imiquimod powder were purchased from Sigma-Aldrich (St. Louis, USA). Dulbecco's modified Eagle's medium (DMEM, high glucose, with L-glutamine and phenol red, no HEPES, no sodium pyruvate), fetal bovine serum (FBS), trypsin-EDTA 0.25% with phenol red, penicillin-amphotericin B (Pen-Strep 1000 U/mL), phosphate buffer solution (PBS 1X), and Alamar® blue were obtained from Gibco ® (Malaysia). Imiquimod (for cell culturing) and trypan blue were purchased from Invivogen (USA). Corning cell culture flasks, 24- and 96-well plates, disposable pipettes, dimethylsulfoxide (DMSO) hydrochloric acid, acetone, absolute alcohol, and ethanol were obtained from Fisher Scientific (Waltham, USA). Human epidermoid carcinoma cell line A431 (ATCC® CRL-1555) were obtained from American Type Culture Collection (ATCC) (Singapore) 7,12-dimethylbenz(a)anthracene, isoflurane, xylene, haematoxylin and eosin (H&E) stain, and 10% buffered formalin solution (BFS) were purchased from Sigma-Aldrich (USA), eBioscience Procarta® Affymetrix assay kit was obtained from Prima Nexus (Malaysia). All reagents and chemicals were of analytical grade or equivalent unless otherwise stated.

PREPARATION OF BIGEL

Carbopol hydrogel and fish oil oleogel were first individually prepared (Fig 1a). Carbopol 940 (3% by weight) was added to the aqueous phase (water) and dispersed under continuous mechanical stirring (500 rpm) for 2 h at room temperature. Subsequently, the mixture was neutralized by the addition of triethanolamine whilst being stirred at a speed of 1000 rpm for 15 min. For fish oil oleogel, imiquimod (equivalent to 5% of total formulation weight) was dispersed in fish oil and later beeswax (10% by weight) was added into the fish oil under continuous magnetic agitation (300 rpm) at 70°C for 15 min until all the beeswax has melted. After a homogenous mixture was achieved, the mixture was removed from heat to allow solidification of the oleogel. Finally, colloidal mixtures of carbopol hydrogel and fish oil oleogel (bigels) were prepared by homogenizing the hydrogel and oleogel phase together (Fig 1a). Each bigel was prepared by mixing different ratios of hydrogel and oleogel (CB1 90:10, CB2 = 70:30, and

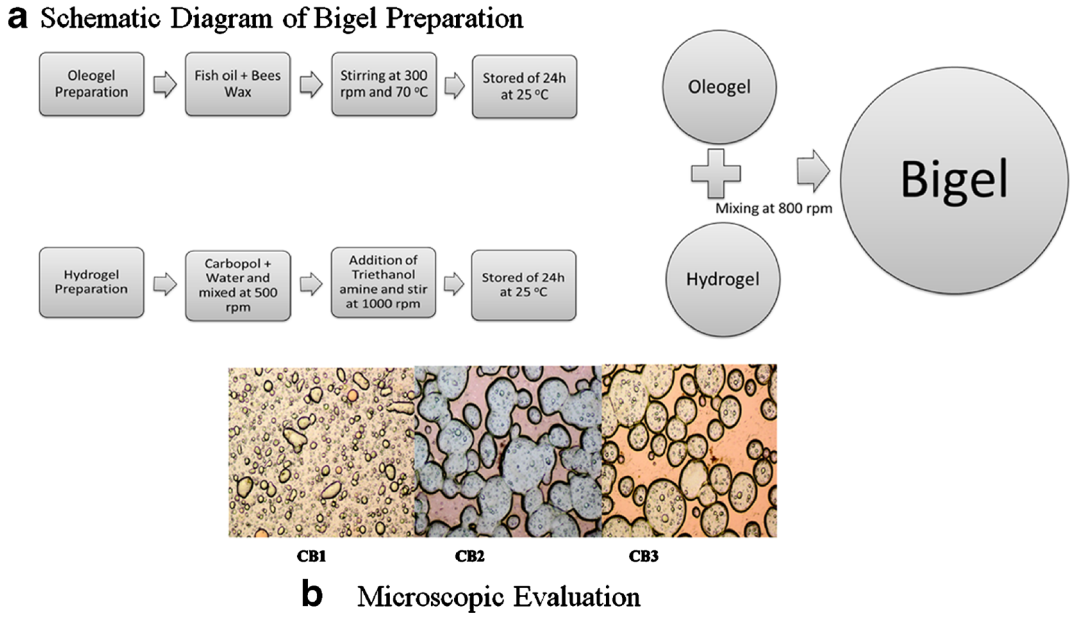

Fig. 1 **(a)** Schematic diagram of bigel formulation **(b)** Microscopic observation of bigels after formulation

CB3 = 50:50) using mechanical stirring (800 rpm) for 15 min at room temperature. After formulation, all the bigels were monitored under optical microscope to observe their homogeneity. They were stained with Sudan stain to differentiate the oleogel region from the aqueous region (Fig 1b). The formulations appeared to be thoroughly mixed where spherical shaped hydrogel regions could easily be differentiated from Sudan stained oleogel region. All the formulations were stored at 5 ± 2°C before experimentation.

FOURIER TRANSFORM INFRARED SPECTROSCOPY

Fourier transform infrared spectroscopy is a qualitative method used to characterize the properties of the different bigels. FTIR-ATR studies were conducted using a Perkin Elmer FTIR-ATR (USA) instrument and samples were scanned from 4000 to 500 cm^{-1} to determine the lipophilic and hydrophilic nature of the colloidal mixtures.

RHEOLOGICAL CHARACTERIZATION

Rheological measurements of bigels were performed using a Bohlin Gemini HR nano-rheometer (Malvern Instruments Ltd, Worcestershire, UK) equipped with a cone and plate measuring system. The experiments were performed using a cone with a diameter of 20 mm and an angle of 2°. The shear rate was increased to 500 s^{-1} in 3 min, followed by a decrease to zero in a same time interval. The average apparent viscosity for each sample was obtained from the maximum shear rate of 500 s^{-1}. All the analysis was performed in triplicates and the mean values of formulations were reported, and the linearity of viscoelastic properties was verified for all samples.

TEXTURE ANALYSIS

Texture analysis was conducted using a Pro CT3 10K (Brookfield Engineering Laboratories, USA) texture analyzer with a 10 Kg load cell. A back extrusion test fixture (TA-DEC) was used with a cylindrical probe (34 mm diameter), and a compression test was performed to determine the textural properties of the bigels. The compression extrusion test involved application of force to the product until it flowed through the space between the probe and the container. This test provides a good indication of the gel strength and consistency. 25 g of the test formulations were placed onto a standard container of the base table (TA-BT-KIT) and the probe was pressed down until it penetrated a distance of 5 mm under the gel surface at a speed of 2 mm/s and then redrawn, for 2 cycles. Adhesiveness, hardness, and peak stress values were then calculated.

EX VIVO PERMEATION STUDY

Swiss albino mice skin sections measuring 2x2 cm, excised from the dorsal area, were used for the permeation studies. Hair from the area was carefully removed using an electric hair clipper. The skin sections were rinsed with PBS and

carefully wiped with a cotton swab dipped in isopropyl alcohol to remove adhering fats, and mounted on glass Franz-type diffusion cell (volume 3.5 mL, contact area 0.95 cm^2) with the stratum corneum facing the donor compartment for *ex vivo* analysis. The receptor compartment was filled with cetrimide solution (30 mg/mL) that acted as a receptor medium, maintained at 37°C, and stirred at 300 rpm. The complete sets of cells were allowed to equilibrate for 1 h prior to dosing. After equilibration, samples (containing 2.5 mg of imiquimod) were dosed on the skin and the receptor fluid periodically collected and replaced with pre-warmed fresh medium at predetermined time intervals up to 48 hrs for subsequent analysis. Imiquimod content in the fluid was analyzed using a reversed phase HPLC (Shimadzu LC-20AT, Shimadzu Corp., Kyoto, Japan) with λ set at 254 nm equipped with Altima C8 column and a mobile phase composed of acetonitrile and water (25:75) adjusted to a pH of 2.7 using phosphoric acid, delivered at a flow rate of 1.5 mL/min. The cumulative amount of imiquimod that had permeated through the surface area (μg/cm^2) of the membrane and the rate of drug released or flux rate (μg/cm^2/h) for the formulations was determined.

MATHEMATICAL MODELING

Drug permeation data were fitted to different mathematical models (Korsmeyer-Peppas, Higuchi, Hixson-Crowell, zero order, and first order models) and finding the best fit to determine the release mechanism. The correlation coefficients were determined from regression plots and this mathematical modeling of release kinetics would highlight the drug transport mechanism involved in controlled release. The equations of different kinetic release used in this study are given below.

$$\text{Zero order equation} : Q = Q_0 - K_0 t \quad (1)$$

$$\text{First order equation} : \text{Log}Q = \text{Log}Q_0 - K_1 t \quad (2)$$

$$\text{Higuchi equation} : Q = K_2 t^{1/2} \quad (3)$$

$$\text{Hixson-Crowell} : Q_0^{1/3} - Q_t^{1/3} = K_s t \quad (4)$$

$$\text{Korsmeyer-Peppas equation} : Q/Q_0 = Kt^n \quad (5)$$

In the equations, K_0 to K_2 are release constants, Q/Q_0 represents the fraction of drug release at time t, and n is diffusion constant that indicates the general release mechanism. The "n" value from the release exponent of Korsmeyer-Peppas model could be used to characterize the release mechanisms.

DEPTH PROFILING OF IMIQUIMOD

Depth profiling of imiquimod was used to estimate the amount of drug retained inside the examined skin layers. Skin sections used in the previous permeation experiment were carefully removed and the remaining portion of the formulation adhering to the skin was wiped with a tissue. To determine the amount of drug retained in stratum corneum (SC) and the remaining portion of the skin, tape stripping technique was employed to separate the stratum corneum (SC) from the epidermis and dermis(24, 25). Ten strips were taken from the skin by applying adhesive tape and removing it with a pair of forceps. The first 2 strips were discarded and the subsequent strips were placed into a jar containing a suitable extraction solvent. Imiquimod was extracted from skin samples using a 7:3 (v/v) methanol:acetate buffer (pH 4.0)(26, 27) solution by mechanically stirring overnight in a water bath (BW-20G, Lab Companion, USA), ultracentrifugation (Beckman Coulter. California, USA) for 20 minutes (12000 rpm at 10°C), and sonication for 2 h at room temperature. The amount of imiquimod was then analyzed using the HPLC conditions as described earlier.

IN VITRO EPIDERMOID CARCINOMA CELL VIABILITY

Cell viability studies were conducted using epidermoid carcinoma cells (A431). Cells were maintained in DMEM supplemented with 10% FBS and seeded in 96-well plates at a density of 2.5 x 10^4 cells/mL. Cells were left to incubate overnight at 37°C (and 5% CO_2) and subsequently treated with series of concentrations of imiquimod (IMQ), fish oil (FO) and imiquimod-fish oil combination, IMQ-FO (50:50 ratio by weight) for 24 h. Concentration-dependent cell viability response was determined by Alamar® blue assay. After 24 h, alamar® Blue (20 μL/well) was added to medium and again incubated for 4 h. Absorbance of each well was then measured in UV–vis spectrophotometer and percent cell viability was calculated.

IN VIVO SKIN CANCER STUDIES

Animals

The study was conducted on 6–8 weeks old healthy female Swiss albino mice (average body weight 25 ± 2 g). The animals were housed under controlled conditions at a temperature of 22 ± 1°C and12 hr light–dark cycle. The animals were fed with standard mouse pellet diet and water ad libitum. Animals were allowed to acclimatize to laboratory conditions for 14 days before the study commenced. Three days before

start of the experiment, hairs from the back of mice were removed with the help of a hair clipper. The study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Universiti Kebangsaan Malaysia. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universiti Kebangsaan Malaysia (Approval Number: FF/2014/HANIF/24-SEPT.16/612-OCT.-2014-FEB.-2015).

Tumor Induction

Skin tumors were induced on the female Swiss albino mice using multiple application of 7,12-dimethylbenz(a)anthracene (DMBA)(28). The animals were divided into five groups of six animals, with Group A serving as an untreated control. Groups B, C, D, and E received topical application of 100 µL DMBA in acetone (2 mg/mL) three times a week for a period of 2 weeks. All groups were observed for 8 weeks. Tumor growth and papilloma greater than 1 mm in diameter was considered as a solid tumor (29). After confirmation of solid tumor growth, test formulations were applied on the groups. Group B was treated with commercial imiquimod cream as a control, and Group C was treated with imiquimod-loaded fish oil bigel. Group D was administered with blank fish oil bigel as a vehicle control, and Group E served as DMBA (carcinogen) control. Treatment was repeated daily for 4 weeks, and serum samples were collected for immunoassays at the end of the treatment.

Physical Observation

Mice were observed under Dino-Lite ® microscope (Courage and Khazaka, Germany) at 50X magnification. Parameters observed were progression of tumor and microscopic comparison of tumor appearance. Physical observations were conducted throughout the study period and observations were also recorded using a digital camera (Nikon, 20.1 megapixels).

Histopathology

The histopathological profile of all the study groups were conducted on excised skin sections using H&E staining and investigated under optical microscope (Olympus BX41TF, Japan).

Procarta ® Multiplex Assay

The plasma concentrations of several cytokines, including IL-10, VEGF, TNF-α, and IL-6, were measured using a mouse Procarta® Immunoassay Kit according to the instructions of the manufacturer. Procarta® Multiplex is a widely used assay which allows simultaneous quantification of multiple protein targets with high sensitivity and reproducibility (30, 31).

Statistical Analysis

Statistical significance of experimental results was analyzed using one-way analysis of variance (ANOVA) and paired sample t-tests with SPSS 20 software (IBM Corporation, USA). A multiple comparison test was used to compare different formulations and p-values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSIONS

Fourier Transform Infrared Spectroscopy

ATR-FTIR analysis was conducted to characterize the colloidal mixture of hydrogel and oleogel. The FTIR spectrum revealed changes in the IR fingerprint with the change in ratio of hydrogel and oleogel phases inside the colloidal mixture (as shown in Fig 2). It was observed that as the oleogel ratio inside the colloidal mixture is increased, the aqueous features of hydrogel phase are minimized. This could easily be observed at the region of 3330 cm^{-1}(representative of –OH group of the aqueous phase), where a reduced transmittance was observed as the oleogel ratio increased from 10 to 50%. The fish oil peaks at the region of 3010–2850 cm^{-1}, which are representative peaks of cis-trans bonds of –CH2 chain inside the fish oil fatty acids, also started to become more prominent as the ratio of oleogel is increased, along with carbonyl group peak at 1734 cm^{-1} and –C-O stretch peak at 1145 cm^{-1}. These peaks indicate an increase in the lipophilic nature of the colloidal mixtures and decrease in its hydrophilicity that could be beneficial in permeation of drugs through the stratum corneum.

Rheological Characteristics of the Bigel Formulations

The viscosity (η) and shear stress (τ) of bigel formulations (polymer-fish oil colloidal mixtures) of different compositions were measured as a function of shear rate (γ). All of the formulations (hydrogel, oleogel and bigel) showed pseudoplastic behaviour (Fig 3). It was observed that control hydrogel CH1 (2.091 ± 0.051 Pa.s) was highly viscous, and the control oleogel (OG1) exhibited the lowest viscosity (0.277 ± 0.052 Pa.s). Changes in hydrogel:oleogel ratio in bigel formulations have a directly proportional effect on the rheological features (Fig 3) and viscosity of the bigel formulations. The hydrogel concentration had a statistically significant ($p < 0.05$, one-way ANOVA, SPSS 20 IBM) influence on the apparent viscosity of the bigel formulations. This could be explained by the fact that there is an increase in the entanglement of polymer chains networks present inside the formulations contributed by the hydrogel phase.

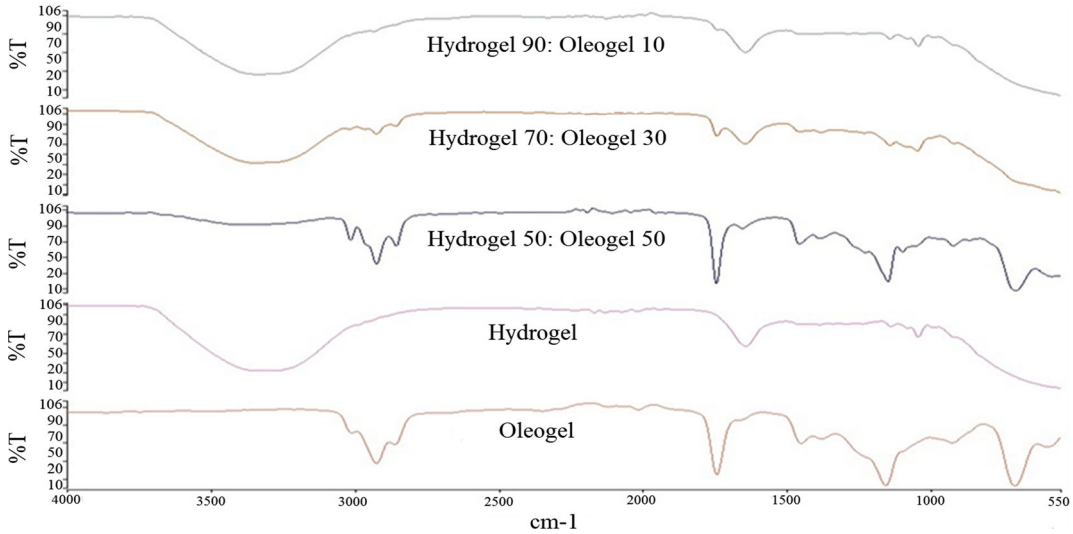

Fig. 2 ATR-FTIR characterization of the hydrogel/oleogel colloidal mixtures of different compositions.

Texture Analysis

Bigels were assessed for their adhesiveness, hardness, and peak stress (Table I). Control hydrogel CH1 showed highest adhesiveness (11.066 ± 1.193 mJ) and the control oleogel OG1 showed the least adhesiveness (4.700 ± 0.721 mJ). All of the bigel formulations had moderate to high adhesiveness depending on the ratio of hydrogel and oleogel phase, which indicates that formulations would adhere to the skin surface to exert desired actions. The firmness of the formulations was determined by hardness and peak stress, which indicate the strength of the intermolecular forces of the gelling network inside the formulations. The hardness of the formulations ranged from 3.066 to 1.676 N and peak stress ranged from 4.337 to 2.423 kPa. The concentration of hydrogel also displayed directly proportional influence on all the texture features of the bigel formulations. The 90:10 hydrogel/oleogel ratio (CB1) showed highest adhesiveness, peak stress and hardness, these values decreased as the concentrations of hydrogel decreased (Table I).

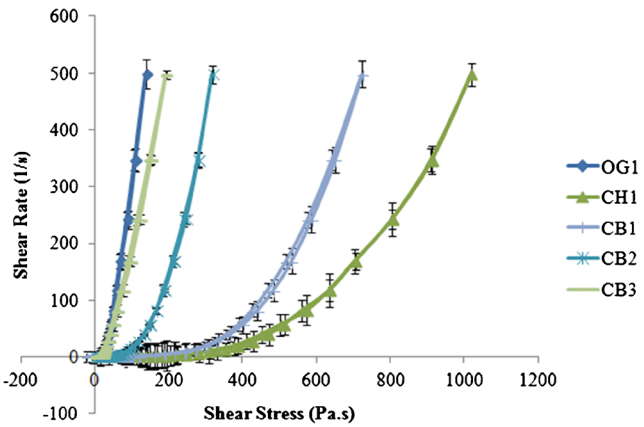

Fig 3 Rheological features of bigel formulations. shear rate vs. shear stress of hydrogel, bigels, and oleogel ($n = 3$, Data expressed as mean ± S.D).

Ex Vivo Skin Permeation

To analyze the drug permeation of bigel formulations as compared to hydrogel and oleogel, *ex vivo* permeation studies were performed (shown in Fig 4a). During ex vivo drug permeation studies, fish oil oleogel (OG1) showed the highest drug permeation (0.3078 ± 0.0023μg/cm^2). The influence of oleogel composition in bigel formulations was also observed; cumulative drug permeation increased as the concentration of oleogel in the bigel formulations increased (Table II) because of the lipophilic nature of the formulation (as investigated in FTIR studies) and so it relaxes the skin permeation barrier. In contrast, the hydrogel control (CH1) showed the least drug permeation through the skin (Fig 4a). This influence of oleogel concentration on the SC and drug permeation is likely being due to the fatty acids (EPA and DHA) present in the fish oil. For imiquimod-loaded fish oil bigels, formulation that was composed of a 50:50 ratio of fish oil oleogel and carbopol hydrogel (CB3) displayed the highest drug permeation (0.3032 ± 0.0124μg/cm^2). The drug permeation of CB3 showed no significant difference to that of OG1 drug permeation (paired sample t-test, $p > 0.05$). The lack of significant differences between OG1 and CB3 may be a result of the synergetic effect of adhesiveness of hydrogel and lipophilic nature of fish oil oleogel, causing increased contact time with

Table I Mechanical properties of hydrogels, oleogel, and bigel formulations ($n = 3$, Data expressed as mean ± S.D)

Formulation	Viscosity (Pa.s)	Adhesiveness (mJ)	Hardness (N)	Peak Stress (kPa)
CB1	1.454 ± 0.048	9.600 ± 1.044	3.066 ± 0.525	4.337 ± 0.742
CB2	0.658 ± 0.054	6.433 ± 0.472	1.970 ± 0.314	2.788 ± 0.450
CB3	0.387 ± 0.014	5.466 ± 0.416	1.713 ± 0.415	2.423 ± 0.584
CH1	2.091 ± 0.051	11.066 ± 1.193	2.990 ± 0.445	4.231 ± 0.632
OG1	0.277 ± 0.052	4.700 ± 0.721	1.676 ± 0.155	2.884 ± 0.823

skin under lipophilic environment and thus resulted into higher drug permeation.

Mathematical Modelling

The kinetic mechanism from the drug release from all the formulations were plotted, and the coefficients of the drug release (R^2) were calculated and are presented in Table III. The mathematical kinetic modeling indicated that the colloidal mixtures of the bigels follow diffusion controlled release mechanism, in which drug permeation was increased as the concentration gradient increased. To confirm the diffusion controlled mechanism the data was fitted into the Korsmeyer's model, and all the formulations showed a fairly good linearity ranging from 0.999 to 0.992. The diffusional coefficient "n" value of the formulations ranged from 0.190 to 0.218, indicating that the diffusion is indeed the dominant mechanism of drug release and is classified as quasi-Fickian diffusion (partial diffusion). This means that diffusion is only partially responsible for the drug release, and there might be other mechanisms involved in the transport of the drug across the membrane.

Drug Depth Profiling

To measure imiquimod retention in SC and the amount of drug that has permeated through it, depth profiling via tape stripping technique was performed. Bigels (CB1-CB3) showed less imiquimod presence in SC and higher availability of drug in the epidermis and dermis (Table II). All bigels produced higher availability of drug inside the skin (epidermis and dermis) as compared to control gels (Fig 4b), and CB3 also displayed a statistically significant ($P < 0.05$, paired t-test, SPSS 20 IBM) low level of drug presence (0.392 mg/cm^2) in the SC compared to oleogel OG1 (0.462 mg/cm^2). This result may be due to the synergetic effect of hydrogel adhesiveness and fish oil fatty acids' (EPA and DHA) ability to alter skin permeability. The effectiveness of bigel formulations (CB1-CB3) to deliver drug through the SC and the availability of imiquimod inside the remaining portions of the skin (epidermis and dermis) was also proven to be higher in depth profiling as compared to hydrogel and oleogel control (Fig 4b). Therefore, it could be concluded that the colloidal mixture of carbopol hydrogel and fish oil oleogel (in bigel formulations) could be beneficial in delivering the drug across the skin membrane due to the synergetic action of the bigel's adhesiveness and lipophilic nature. To investigate the effectiveness of imiquimod-loaded fish oil bigel formulations on skin cancer, CB3 was chosen for the animal studies due to its highest drug permeation through skin.

Cell Viability Assay

Cell viability assay exhibited the effectiveness of IMQ, FO and IMQ-FO. It was observed that IMQ-FO almost in all concentrations tested was more effective in reducing the viability of epidermoid carcinoma cells as compared to IMQ alone

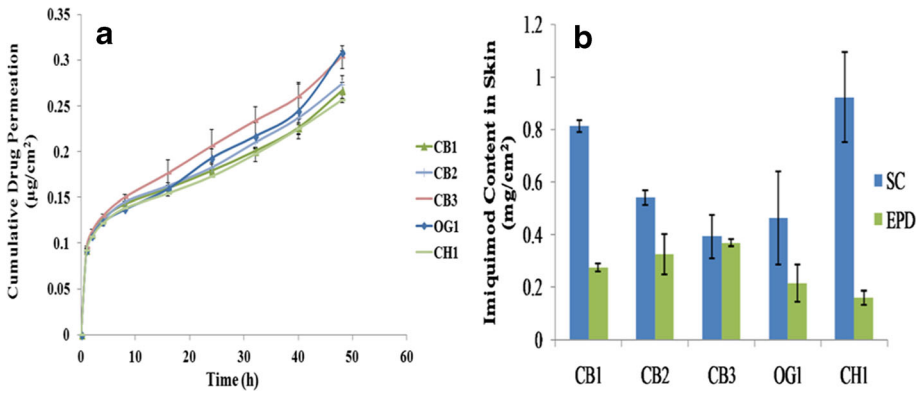

Fig. 4 (**a**) Cumulative permeation of imiquimod from carbopol-fish oil bigels (mean ± SD, $n = 3$) (**b**) Results of imiquimod entrapment in stratum corneum (SC) and epidermis (EPD) from bigel formulations as compared to hydrogels and oleogel ($n = 3$, Data expressed as mean ± S.D).

Table II Cumulative drug release ($\mu g/cm^2$), drug flux ($\mu g/cm^2/h$), of all formulations and drug availability in stratum corneum (SC), epidermis and dermis (EPD) through Swiss Albino mice skin ($n = 3$, Data expressed as mean ± S.D).

Formulation	Mice Skin			
	Cumulative Drug Permeation ($\mu g/cm^2$)	Drug Flux ($\mu g/cm^2/h$)	Drug Availability in Stratum Corneum (mg/cm^2)	Drug Availability in Epidermis and Dermis (mg/cm^2)
CB1	0.2666 ± 0.0091	0.0055 ± 0.0002	0.814 ± 0.024	0.274 ± 0.016
CB2	0.2738 ± 0.0099	0.0057 ± 0.0002	0.539 ± 0.027	0.325 ± 0.077
CB3	0.3032 ± 0.0124	0.0063 ± 0.0003	0.392 ± 0.083	0.368 ± 0.013
CH1	0.2563 ± 0.0026	0.0053 ± 5.4E-05	0.923 ± 0.171	0.159 ± 0.028
OG1	0.3078 ± 0.0023	0.0064 ± 4.8E-05	0.462 ± 0.177	0.213 ± 0.071

(Fig 5). FO itself also has some limited ability to reduce the carcinoma cell growth, which might have contributed in the IMQ-FO induced reduction in cell viability. The inhibition showed by IMQ-FO against epidermoid carcinoma cells was significantly different ($P < 0.05$, one-way ANOVA) from IMQ concentrations. This additional growth inhibition activity of IMQ-FO might be due to the presence of omega-3 fatty acids inside the FO. Thus the combination of imiquimod and fish oil inside the formulations could be proved beneficial against skin cancer.

Physical Observation of Mice Cancer Model

After application of test formulations onto the mice suffering from skin cancer, comparative observation of treatment groups and control groups was conducted. Although mice treated with the commercially available imiquimod cream and imiquimod-loaded fish oil bigel (CB3) did not show a complete absence of tumors, they were significantly smaller in size (diameter, 1.98 ± 1.05 mm and 2.07 ± 1.05 mm respectively) as compared to carcinogen control group (6.48 ± 1.14 mm) and in less severe state. Closer inspection through Dino-lite® microscope revealed that skin lesions resulting from the tumor were beginning to heal, thus moving towards skin repair. The blank bigel formulation (vehicle control, composition equivalent to CB3 but without imiquimod) however was not able to control the tumor growth as efficiently as the imiquimod-loaded fish oil bigel and commercial cream (Fig 6).

Histopathological Study of Mice Cancer Model

Mice treated with carcinogen control exhibited massive tumor size and new blood vessels could be spotted in the tumor histology (Fig 6). Furthermore, squamous cell carcinoma epithelial cells (keratinous pearls) arising from the epidermis and infiltrating into the dermis were also present (Fig 6). Mice treated with both imiquimod-loaded fish oil bigel and commercial cream showed inhibition of tumor growth and blood vessels could not be detected inside the tumor (Fig 6). Although small papillomas were present in mice, they were less severe and chronic in nature as compared to those present in the carcinogen control group. Mice treated with fish oil blank bigel did not exhibit control of the tumor growth as well as the imiquimod-loaded fish oil bigel and commercial cream-treated groups (Fig 5). Chronic inflammation with carcinoma characteristics such as keratinous pearls were still present in mice treated with blank bigel.

Vascular Endothelial Growth Factor Immunoassay

VEGF expression was monitored in serum samples of skin cancer mice model groups (Fig 7). VEGF

Table III Correlation values (R^2) of drug release kinetic equations and "n" value of diffusion constant from Korsemeyer-Peppas model indicating the release mechanism

	Zero order	First order	Higuchi	Hixson-Crowell	Korsemeyer-Peppas	
Formulation	R^2	R^2	R^2	R^2	R^2	n
CB1	0.974	0.932	0.961	0.953	0.992	0.197
CB2	0.982	0.94	0.968	0.96	0.997	0.202
CB3	0.987	0.94	0.978	0.962	0.999	0.218
CH1	0.982	0.946	0.966	0.963	0.995	0.19
OG1	0.979	0.963	0.939	0.976	0.996	0.19

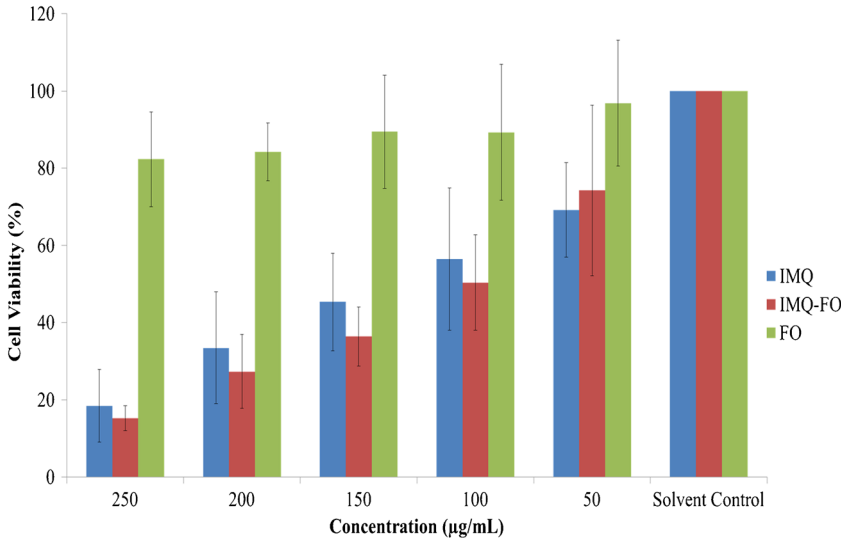

Fig. 5 Epidermoid carcinoma cells were treated with IMQ, FO and IMQ-FO combination at the indicated concentrations for 24 h. Cell viability was determined by Alamar® blue assay ($n = 3$, Data expressed as mean ± S.D).

expression can be utilized as a diagnostic tool for the measurement and progression of skin tumors, especially in the case of squamous cell carcinoma, and displays more intense and widespread expression of tumor cells, which would result in elevated VEGF levels. The carcinogen control exhibited the highest expression ($P < 0.05$, one-way ANOVA) of growth factor (547.14 ± 125.42 pg/ml, $n = 6$) followed by expression in the

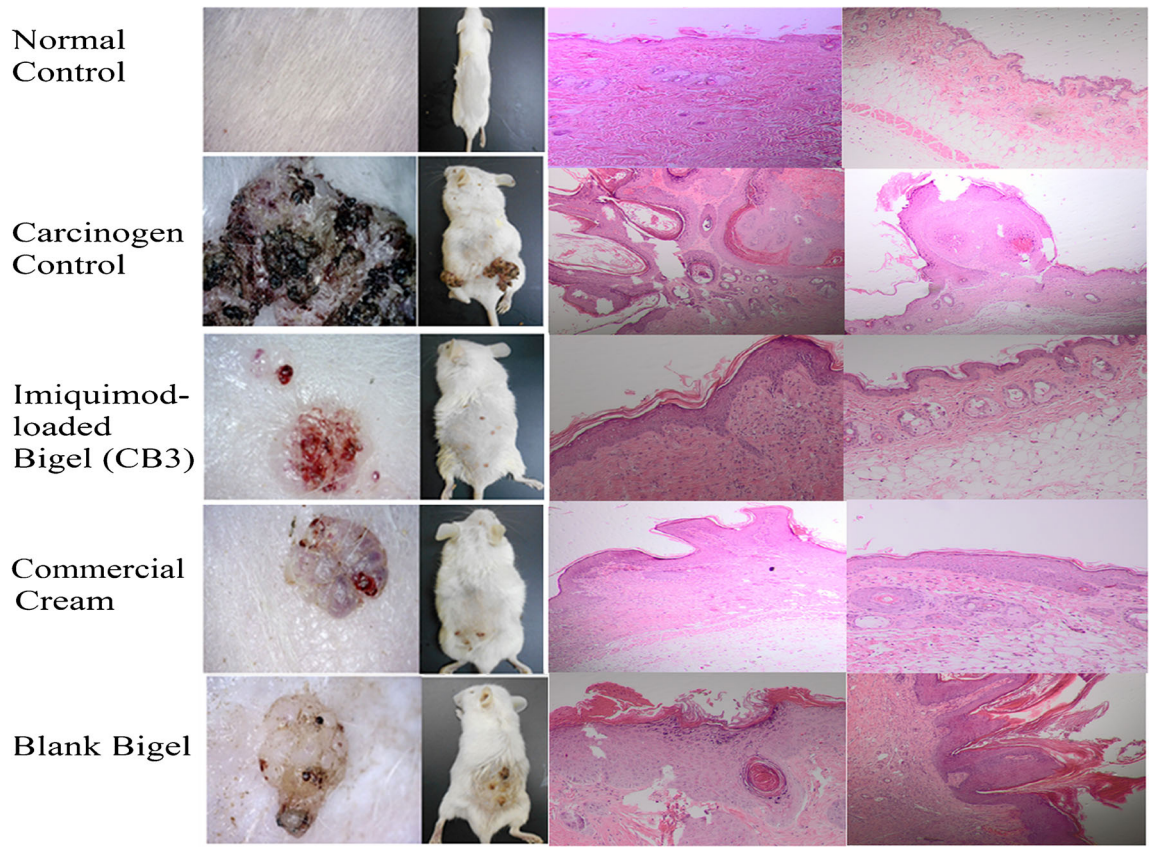

Fig. 6 Physical appearance and histopathology of animal groups. Carcinogen control mice group exhibited non-melanoma carcinoma, solid tumor growth, and blood vessels. Mice treated with imiquimod-loaded bigel exhibited small growth and acute inflammation characterized by thickened epidermis. Mice treated with commercial cream exhibiting small growth and acute inflammation characterized by thickened epidermis. Mice treated with blank bigel (vehicle control) exhibited non-melanoma carcinoma and chronic inflammation characterized by thickened epidermis and massive keratinization.

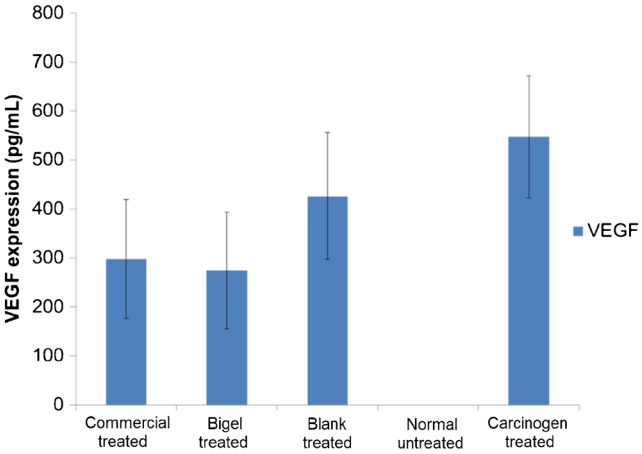

Fig. 7 Vascular endothelial growth factor (VEGF) expression for treatment groups after 12 weeks of study (mean ± SD, $n = 6$). Only the blank bigel treated group showed no significant difference when compared to the carcinogen control group ($P > 0.05$, one-way ANOVA).

commercial cream control group (297.42 ± 121.93 pg/ml, $n = 6$), and the imiquimod-loaded fish oil bigel-treated mice showed the lowest expression (273.819 ± 119.19 pg/mL, $n = 6$). A slight inhibition of growth factor was also obtained with the blank fish oil bigels (425 ± 129.73 pg/mL, $n = 6$). Both commercial cream and imiquimod-loaded fish oil bigel formulations showed a significant decrease in VEGF expression level which highlighted the anti-angiogenic activity of IMQ. This activity of reducing pathological angiogenesis is one of imiquimod's important therapeutic function against skin tumors (32). Imiquimod achieves this anti-angiogenic activity by upregulation of angiogenesis inhibitors and downregulation of pro-angiogenic factors (33, 34). Blank bigels also appeared to exhibit slight growth factor inhibition, which could be due to the presence of fish oil inside the blank bigel. Fish oil is composed of omega-3 fatty acids, which have been studied for their anti-angiogenic activity on different cancer cells (35).

Tumor Necrosis Factor Immunoassay

TNF-α is an important cytokine known for its pro-inflammatory activity and tumor progression. Therefore, inhibition of TNF-α has been a target for cancer and inflammation treatment (36, 37). TNF-α expression was measured in all groups (Fig 8). Mice treated with commercial cream displayed the highest TNF-α value at 170.19 ± 79.07 pg/mL ($n = 6$). Mice treated with imiquimod- fish oil loaded bigel formulation exhibited lesser amounts of TNF-α (78.31 ± 49.39 pg/mL, $n = 6$). Both imiquimod-loaded fish oil bigel and commercial cream showed elevated values of TNF-α ($P < 0.05$, one-way ANOVA) compared to those of the carcinogen control (21.52 ± 6.94 pg/mL, $n = 6$). Blank bigel formulations treatment showed an inhibitory effect and expressed the lowest value of TNF-α (16.49 ± 3.06 pg/mL, $n = 6$). Imiquimod is as an upregulator of the Th_1 cytokine, resulting in increased TNF-α production(32). Therefore, TNF-α expression was high in mice treated with commercial cream compared to that of bigel-treated mice. On the other hand, fish oil also shows its anti-inflammatory effect, as mice treated with blank bigels expressed lower levels of TNF-α than did the carcinogen-treated group (Fig 8). TNF-α is also known to induce the secretion other pro-inflammatory cytokines such as IL6 and IL1(38, 39). Thus imiquimod induced TNF-α production should be controlled and fish oil as an inhibitor could produce such effect, and may result in more antitumor activities and reduced pro-inflammatory effect from TNF-α.

Interleukin-6 Immunoassay

IL6 expression was monitored in all study groups (Fig 9). The carcinogen control group exhibited an IL6 value of 19.89 ± 12.16 pg/mL ($n = 6$) and mice treated with commercial cream expressed very high values of IL6 185.38 ± 82.88 pg/mL ($n = 6$) as compared to imiquimod-loaded fish oil bigel 72.61 ± 11.87 pg/mL ($n = 6$). IL6 is a pro-inflammatory cytokine and is upregulated by imiquimod (32). Imiquimod-loaded fish oil bigel offers a controlled release of imiquimod and omega-3 fatty acid anti-inflammatory influence,

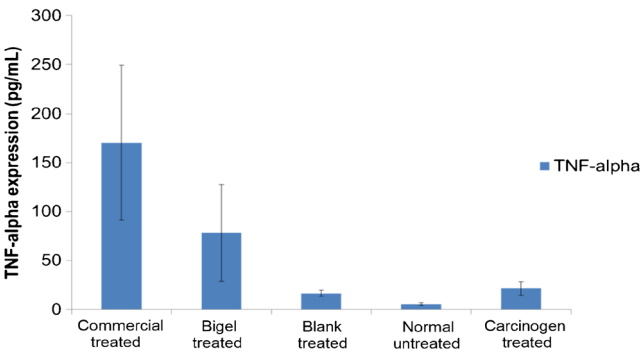

Fig. 8 Tumor necrosis factor (TNF)-α expression for treatment groups after 12 weeks of study (mean ± SD, $n = 6$). Both commercial and imiquimod-loaded bigel treated groups displayed significantly higher TNF-α values than the carcinogen control group ($P < 0.05$, one-way ANOVA).

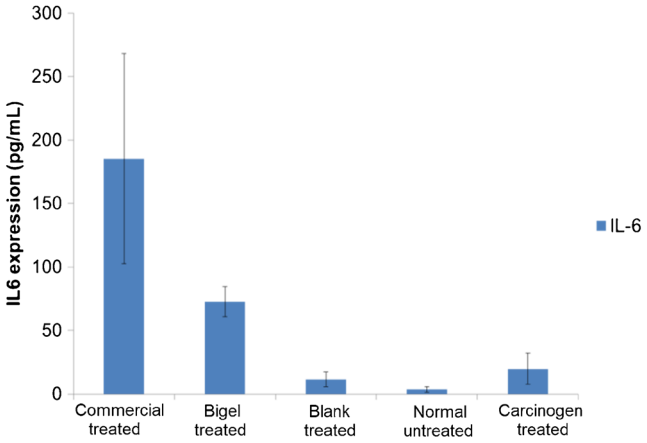

Fig. 9 Interleukin (IL)-6 expression for treatment groups after 12 weeks of study (mean ± SD, $n = 6$). Only commercial cream-treated mice displayed significantly higher IL6 values than the carcinogen control group ($P < 0.05$, one-way ANOVA).

which resulted in lower expression of IL6.Mice treated with blank bigels also exhibited IL6 inhibition (11.6 ± 5.89 pg/mL, $n = 6$) compared to mice in the carcinogen control. Imiquimod-induced production of IL6 may be problematic for skin cancer, because IL6 is known to promote malignant skin growth of squamous cell carcinoma (40) and overexpression of IL6 has also been reported in basal cell carcinoma cells (41). Apart from imiquimod, IL6 production is also enhanced by the presence of TNF-α (38, 39), and as mentioned earlier this could lead to serious chronic inflammation, skin lesions, or more tumor growth. Omega-3 fatty acids and their controlled release from imiquimod-loaded fish oil bigel formulations may help in managing these pro-inflammatory cytokines.

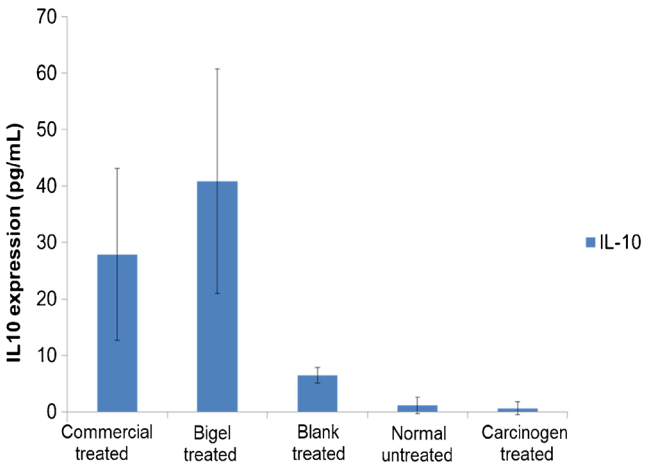

Fig. 10 Interleukin (IL)-10 expression for treatment groups after 12 weeks of study (mean ± SD, $n = 6$). Both the commercial and the imiquimod-loaded bigel groups displayed significantly higher IL10 values than the carcinogen control group ($P < 0.05$, one-way ANOVA).

Interleukin-10 Immunoassay

IL10 is an anti-inflammatory cytokine and is secreted by macrophages, epithelial cells and regulatory T cells (32, 42). Treatment groups were also compared for IL10 expression (Fig 10). Mice treated with imiquimod-loaded fish oil bigels exhibited higher IL10 values (40.86 ± 19.89 pg/mL) as compared to commercial cream (27.82 ± 15.25 pg/mL). IL10 offers an immunoregulatory effect and is known to downregulate pro-inflammatory cytokines such as IFN-γ, IL6, and TNF-α (32), and has also been studied for its antitumor role in skin carcinogenesis (43). Both imiquimod and omega-3 fatty acids are known to upregulate the production of IL10 (32, 44). Mice treated with the blank fish oil formulation also displayed higher IL10 values (6.43 ± 1.39 pg/mL, $n = 6$) than did the carcinogen-treated control group (0.63 ± 1.16 pg/mL, $n = 6$). IL10 can serve as a balance between the inflammatory and immunopathologic activities of other Th_1 cytokines (45). IL10 is known to increase antitumor immunity and inhibits tumor-associated inflammation (42); therefore along with fish oil, it could serve well in controlling skin cancer.

CONCLUSION

Bigels prepared from carbopol hydrogel and fish oil oleogel (CB1–CB3) were shown to be better drug vehicles than conventional hydrogels and oleogel. Bigels exhibited good rheological and mechanical characteristics, higher drug permeation than hydrogels, and approximately equivalent permeation to oleogel (OG1). These characteristics, in addition to low drug retention in SC and high imiquimod availability inside the skin, makes carbopol-fish oil bigels as a competitive candidate for a topical drug delivery system. Imiquimod is a potent drug for skin cancer treatment, but its upregulation of pro-inflammatory cytokines (VEGF, TNF-α and IL6) increases the risk of chronic inflammation. Imiquimod-loaded fish oil bigel also enhances antitumor effectiveness with reduced signs of inflammation. Fish oil could serve as inducers of IL10 and suppressor of VEGF, IL6

and TNF-α which would not only depress tumor growth, but would also serve to alleviate inflammatory side effects of imiquimod. Thus it can be concluded that an imiquimod-loaded fish oil bigels can be beneficial for combined effectiveness against inflammation and skin cancer.

ACKNOWLEDGMENTS AND DISCLOSURES

The authors would like to thank the Ministry of Education, Malaysia for providing research grant ERGS/1/2013/SKK02/UKM/02/3, and Faculty of Pharmacy, Universiti Kebangsaan Malaysia for additional support during this study.

Authors have no conflict of interest to report.

REFERENCES

1. Leibovitch I, Huilgol SC, Selva D, Hill S, Richards S, Paver R. Cutaneous squamous cell carcinoma treated with mohs micrographic surgery in Australia I. Experience over 10 years. J Am Acad Dermatol. 2005;53:253–60.
2. Rogers HW, Weinstock MA, Harris AR. Incidence estimate of nonmelanoma skin cancer of the United States. Arch Dermatol. 2005;146:283–7.
3. Johnson TM, Rowe DE, Nelson BR, Swanson NA. Squamous cell carcinoma of the skin (excluding lip and oral mucosa), Journal of the American Academy of Dermatology. J Am Acad Dermatol. 1992;26:467–84.
4. Vivek VG, Eric MG. Cutaneous squamous cell carcinoma of the head and neck. J Skin Cancer. 2011;2011:3–13.
5. Teicher BA, Ara G, Buxton D, Leonard J, Schaub RG. Optimal scheduling of interleukin-12 and chemotherapy in the murine MB-49 bladder carcinoma and B-16 melanoma. Clin Cancer Res. 1997;3:1661–7.
6. Graells J, Ojeda RM, Garcia-Cruz A. Effect of imiquimod as compared with surgery on the cancerization field in basal cell carcinoma. Actas Dermosifiliogr. 2014;105:53–9.
7. Tarbet EB, Larson D, AB J, Bailey KW, Wong MH Smee DF. Evaluation of imiquimod for topical treatment of vaccinia virus cutaneous infections in immunosuppressed hairless mice. Antiviral Res. 2011;90:126–33.
8. Jin JY, Jung YJ, Choi S, Hwang J, Chung E. Autophagy mediated anti-tumoral of imiquimod in CaCo-2 cells. Biochem Biophys Res Commun. 2009;386:455–8.
9. Hadley G, Derry S, Moore RA. Imiquimod for actinic keratosis: systematic review and meta-analysis. J Investig Dermatol. 2006;126:1251–5.
10. Cantisani C, Lazic T, Richetta AG, Clerico R, Mattozzi C, Calvieri S. Imiquimod 5% cream use in dermatology, side effects and recent patents. Recent Patents Inflamm Allergy Drug Discov. 2012;6(1):65–9.
11. Rosenblatt A. Campos GHGd. Local and systemic adverse effects of imiquimod therapy for external anogenital warts in men: report of three cases. Int J STD AIDS. 2012;23:909–10.
12. Rehman K, Aluwi MFFM, Rullah K, Wai LK, Amin MCIM, Zulfakar MH. Probing the effects of fish oil on the delivery and inflammation-inducing potential of imiquimod. Int J Pharm. 2015;490:131–41.
13. Kadir R, Stempler D, Liron Z, Cohen S. Delivery of theophylline into excised human skin from alkanoic acid solutions: a "push-pull" mechanism. J Pharm Sci. 1987;76:774–9.
14. Chirstopher PT, Jamie P, Thomas T, Heard MC. Probing the skin permeation of fish oil/EPA and ketoprofen: 1. NMR spectroscopy and molecular modelling. Int J Pharm. 2007;338:207–12.
15. Almedia IF, Fernandes AR, Fernandes L, Ferreira MRP, Costa PC, Bahia MF. Moisturizing effect of oleogel/hydrogel mixtures. Pharm Dev Technol. 2008;13:487–94.
16. Rhee GJ, Woo JS, Hwang SJ, Lee YW, Lee CH. Topical oleohydrogel preparation of ketoprofen with enhanced skin permeability. Drug Dev Ind Pharm. 1999;25:717–26.
17. Vinay KS, Indranil B, Tarun A, Krishna P, Mrinal KB, Kunal P. Guar gum and sesame oil based novel bigels for controlled drug delivery. Colloids Surf B: Biointerfaces. 2014;123:582–92.
18. Rehman K, Zulfakar MH. Recent advances in gel technologies for topical and transdermal drug delivery. Drug Dev Ind Pharm. 2014;40(4):433–40.
19. Zulfakar MH, Abdelouahab N, Heard MC. Enhanced topical delivery and ex vivo anti-inflammatory activity from a betamethasone dipropionate formulation containing fish oil. Inflamm Res. 2010;59:23–30.
20. Chirstopher PT, Heard MC. Probing the skin permeation of eicosapentaenoic acid and ketoprofen 2. Comparative depth profiling and metabolism of eicospentaenoic acid. Eur J Pharm Biopharm. 2007;67:156–65.
21. Huri MFD, Ng SF, Zulfakar MH. Fish oil-based oleogels: physicochemicals characterisation and invitro release of betemethasone dipropionate. Int J Pharm Pharm Sci. 2013;5:458–67.
22. Rehman K, Tan CM, Zulfakar MH. Development and in-vitro characterization of fish oil oleogels containing benzoyl peroxide and salicylic acid as keratolytic agents. Adv Drug Res. 2014;64:159–65.
23. Nugteren DH, Hazelhof CE. Van dbA, Houtsmuller UMT. Metabolism of linoleic acid and other essential fatty acids in the epidermis of the rat. Biochim Biophys Acta. 1995;834:429–36.
24. Lademann J, Jacobi U, Surber C, Weigmann HJ, Fluhr JW. The tape stripping procedure–evaluation of some critical parameters. Eur J Pharm Biopharm. 2009;72(2):317–23.
25. Coderch L, Oliva M, Pons M, Maza ADI, Manich AM, Parra JL. Percutaneous penetration of liposomes using the tape stripping technique. Int J Pharm. 1996;139:197–203.
26. Paula DD, Martins CA, Bentley MV. Development and validation of HPLC method for imiquimod determination in skin penetration studies. Biomed Chromatogr. 2008;22:1416–23.
27. Rohrbach R, Iversen OH, Riede UN, Sandritter W. Effects of cellophane tape stripping of mouse skin on epidermal growth regulators (chalones). Beitr Pathol. 1977;160:175–86.
28. Xiaolu L, Johanthan E, Rajendra S, Chenlu M, Krystyna F. Interleukin-1α Up-Regulation in Vivo by a Potent Carcinogen 7, 12-Dimethylbenz(a)anthracene (DMBA) and Control of DMBA-induced Inflammatory Responses. Cancer Res. 2002;62:417–23.
29. Ko JH, Jung YS, Lee BJ. Inhibitory effects of interferon-gamma plasmid DNA on DMBA-TPA induced mouse skin carcinogenesis. Cancer Gene Ther. 2011;18:646–54.
30. Hussain Z, Katas H, Amin MCIM, Endang K. Efficient immunomodulation of TH1/TH2 biomarkers in 2,4 dinitroflurobenzene induced atopic dermatits: Nanocarrier mediated transcutaneous co-delivery of anti-inflammatory and antioxidant drugs. Plos One. 2014;9:1–17.
31. Orbach R, Gurevich M, Achiron A. Interleukin-12p40 in the spinal fluid as a biomarker for clinically isolated syndrome. Multiple Sclerosis J. 2014;20(1):35–42.
32. Kemeny L. Non-surgical treatment of keratinocyte skin cancer. Berlin-Heidelberg: Springer; 2010.

33. Li VW, Li WW, Talcott KE. Imiquimod as an antiangiogenic agent. J Drug Dermatol. 2005;4:708–17.
34. Gabriella C, Fiorella DN, Simona G, Elisabetta P, Simona S, Nicola M, *et al.* n-3 PUFAs reduce VEGF expression in human cancer cells modulating the COX-2/PGE2 Induced ERK-1 and −2 and HIF-1alpha induction pathway. Carcinogenesis. 2004;25: 2303–10.
35. Kelly EJ, Traci AW. Multiple roles of VEGF in non-melanoma skin cancer: angiogenesis and beyond. J Skin Cancer. 2012;2012:1–6.
36. Wu Y, Zhou BP. TNF-α/NF-kB/Snail pathway in cancer migration and invasion. Br J Cancer. 2010;102:639–44.
37. Fiorella G, Patrizia F, Raffaele P, Ilaria P, Vincenzo F, Mario R. TNF/VEGF cross-talk in chronic inflammation-related cancer initiation and progression: an early target in anticancer therapeutic strategy. In vivo. 2007;21:147–62.
38. Neil AT, Romana SM, Philip W, David JOR, Stephen GB, Karen EP. Mechanism of TNF-α -induced IL-1α, IL-1β and IL-6 expression in human cardiac fibroblasts: Effects of statins and thiazolidinediones. Cardiovasc Res. 2007;76:81–90.
39. Kumiko T, Rie MN, Shinobu Y, Hiroki L, Shuji D, Osamu K. Mechanisms of tumor necrosis factor-a-inducd interleukin-6 synthesis in glioma cells. J Neuroinflammation. 2010;7:16–24.
40. Lederle W, Depner S, Schnur S, Obermueller E, Catone N, Just A, *et al.* IL-6 promotes malignant growth of skin SCCs by regulating a network of autocrine and paracrine cytokines. Int J Cancer. 2011;128:2803–14.
41. Shiou-Hwa J, Shing-Chuan S, Hsien-Ching C, Wei-Ling T, Kuo ML. Overexpression of interleukin-6 in human basal cell carcinoma cell lines increases anti-apoptotic activity and tumorigenic potency. Oncogene. 2001;20:198–202.
42. Michele WLT, Philip KD, Mark JS. Stable IL10: a new therapeutic that promotes tumor immunity. Cancer Cell. 2011;20:691–3.
43. Karin L, Jenny A, Maik V, Marietter M, Sandra B, Thomas S, *et al.* IL10 controls ultraviolet-induced carcinogenesis in mice. J Immunol. 2007;179:365–71.
44. Anke J, Michael W, Bernhard W, Christina N, Alexander R, Alfered L, *et al.* Evaluation of suppressive and pro-resolving effects of EPA and DHA in human primary monocytes and T-helper cells. J Lipid Res. 2013;54:923–35.
45. Yone VNC, Maria CAB, Juliana KDALN, Jose CF, Valeria RAP. Role of TNF-alpha, IFN-gamma, and IL-10 in development of pulmonary tuberculosis. Pulmonary Med. 2012;2012:1–10.