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The effects of betamethasone dipropionate and fish oil on HaCaT proliferation and apoptosis

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ABSTRACT

The current work examined the effect of fish oil (FO) and betamethasone dipropionate (BD) on the growth of immortalized HaCaT keratinocytes. HaCaT cells were grown and treated with FO and/or BD, and proliferation determined using the MTT method. The cells were further probed by immunocytochemistry (ICC) techniques for apoptosis using Cleaved Caspase-3 Asp175, and inflammatory processes using cyclooxygenase-2 (COX-2). The addition of FO increased the inhibition of HaCaT cells by 27.2%, from 43.15% to 70.35% compared to BD alone (p 0.034). FO alone appeared to induce expression of Asp175 and the effect was greater in combination with BD. The net effect, however, were less than BD alone. Similar observations were seen with regards to COX-2 inhibition. The added benefits of FO to the effect of BD on the inhibition of cell growth, induction of apoptosis and inhibition of inflammation have now been demonstrated on a cellular level. Each of these activities supports beneficial effects in hyperproliferative skin disorders, such as psoriasis.

1. Introduction

Corticosteroids have been a mainstay in topical therapy of mild to moderate psoriasis for some time (Stein, 2005), and in the United States, corticosteroids are among the most commonly prescribed topical agents (Feldman et al., 2000). This class of drug exerts its anti-psoriatic effect via multiple processes including immunosuppression, anti-proliferation, and anti-inflammation (Elenkov and Chrousos, 2002; Valencia and Kerdel, 2003). In general, corticosteroids penetrate poorly into the skin and derivatives such as betamethasone dipropionate (BD) and betamethasone valerate, are typically used topically and classed according to their potency, ranging from superpotent (Class 1) to the least potent (Class 7) (Callis and Krueger, 2003).

However, there has been considerable interest in the use of naturally occurring products, including those for improving the symptoms of psoriasis. One of the most documented and popular natural treatments involve the use of bioactive fatty acids and lipids from oils derived from sources such as fish. Certain omega-3 fatty acids, in particular eicosapentaenoic acid (EPA), are associated with anti-inflammatory activity, resulting from the formations of less potent eicosanoids compared to those derived from omega-6 fatty acids, mainly arachidonic acid (Gil, 2002). Topical application of EPA results in incorporation of this fatty acid into cell membranes and subsequent competition with arachidonic acid in the inflammation cascade or the arachidonic acid cascade (Calder, 2001). Trials using fish oil (FO) (Nugteren et al., 1985) and EPA (Kojima et al., 1991; Escobar et al., 1992; Riku et al., 1993) have shown that it could be beneficial in alleviating symptoms related to psoriasis, although no direct evidence was provided to prove EPA delivery to viable epidermis (Zulfakar et al., 2007). Apart from this benefit in arresting inflammatory processes, it has also been established that EPA can act as a permeation enhancer in topical formulations containing fish oil and a non-steroidal anti-inflammatory drug (NSAID) (Thomas and Heard, 2005).

FO was found to enhance the topical delivery of BD when applied to ex vivo porcine ear skin, as well as enhancing the anti-inflammatory activity of BD (Zulfakar et al., 2010). In the current work, the effects of these compounds were examined in cultures of the immortalized keratinocyte cell line, HaCaT. Their effects on apoptosis were investigated in terms of Cleaved Caspase-3 (Asp175), an important marker for the execution-phase of apoptosis (Jänicke et al., 1998; Gown and Willingham, 2002). The effects of FO and BD on the modulation of cyclooxygenase-2 (COX-2) enzyme expression as an inflammation marker were also studied.

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2. Materials and methods

2.1. Materials

Gibco® Dulbecco's modified Eagle medium 1× (DMEM, high glucose, with L-glutamine and phenol red, 1.8 mM Ca^{2+}, no HEPES, no sodium pyruvate), fetal bovine serum (FBS), trypsin 0.05% with EDTA·4Na 1× were obtained from Invitrogen (Paisley, UK). Penicillin (10,000 units)-streptomycin (10,000 μg) mixture were purchased from Lonza Group Ltd. (Basel, Switzerland). Ethanol (EtOH), formaldehyde, phosphate buffered saline (PBS) sachets, and sodium chloride, Corning cell culture flasks, 24- and 96-well plates, dimethylsulfoxide (DMSO) were all obtained from Fisher Scientific (Loughborough, UK). Betamethasone dipropionate (BD), Dulbecco's phosphate buffered saline (DPBS), Tween 20 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma–Aldrich (Poole, UK). EnVision+ System HRP Labeled Polymer Anti-Rabbit, liquid DAB+ substrate Chromagen System were from Dako (Ely, UK). Antibodies for Cleaved Caspase-3 Asp175 (#9664) and COX-2 (#4842) were both from Cell Signaling Technology, New England Biolabs (Hitchin, UK). Fish oil capsules (Boots n-3 Fish Oil 1000 mg high strength) were obtained from a local store.

2.2. Routine cell culture

The HaCaT cells were grown in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin mixture (100 U mL^{-1} and 100 μg mL^{-1} respectively). Aliquots of 50 mL of the medium were prepared from the stock. Inactivation of the FBS was achieved by placing the serum at 56 °C for 30 min. The temperature of the incubator was set at 37 °C and flow of CO_2 at 5%. The medium was replenished every 2 days, until the monolayer cultures reached 70–80% confluency, and then passaged or split to maintain cells in exponential growth. For passaging, the cells were then washed with 2 changes of DPBS, followed by incubation with trypsin-EDTA at 37 °C for 5 min or until all the cells have detached. The trypsin was then deactivated by addition of a small amount of DMEM and the cell suspension transferred to a sterile centrifuge tube and centrifuged at 1000 rpm for 5 min. The resulting supernatant was removed and 8–10 mL fresh medium added, followed by cell re-suspension. The suspension was then ready to be used to seed cells in new flasks; frozen down for storage; used for experimental work; and for cell counting.

2.3. HaCaT cell growth study

HaCaT growth curves were determined and used to obtain lag time, population doubling time, and saturation density (Mather and Roberts, 1998). Cells were seeded by preparing a cell suspension of 1×10^4 cells mL^{-1} in medium and placing 1 mL of the suspension in each well of a 24-well plate, which had a surface area of 2 cm^2. This gave a cell density of 5×10^3 cells/cm^2. Two plates were prepared for the study. The plates were then placed in the incubator at the setting mentioned earlier (37 °C, 5% CO_2) and allowed to settle for 24 h before the first counting was performed. For each sampling time, 2 wells were counted by first aspirating the medium, washing with DPBS and 1 mL of trypsin pipetted into the wells. The trypsin was allowed to dislodge the cells from the well surfaces for 10 min at 37 °C, before being neutralized with an equal amount of medium. The resulting suspensions were then sucked out, placed in a microcentrifuge tube and counted. Cell counting was carried out using a Neubauer haemocytometer. The cells were counted every 24 h to a maximum of 8 days, with the medium being replenished every 2 days. The number of cells versus the sampling time was plotted to construct the growth curve. The lag time and cell doubling time were determined from the growth curve. The subsequent assay and immunocytochemistry (ICC) staining were carried out according to these times.

2.4. MTT cell viability assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay is a common assay used to determine cell viability. HaCaT cells were seeded into 96-well plates at a density of 5×10^4 cells/mL. Each well had a surface area of 0.16 cm^2 and working volume of 200 μL. After seeding, the wells were left overnight in the incubator.

Stock solution of BD was prepared by diluting in DMSO and mixing it with DMEM (containing 10% v/v FBS) to provide a series of concentrations ranging from 0.78125 mg mL^{-1} to 2 mg mL^{-1}. The stock solution was prepared with care taken to limit the final DMSO concentration in the medium at 0.5% v/v. Mixing of the solution was done aseptically by filtering the stock BD solution with a syringe attached with a 0.2 μm filter before addition to DMEM. Four wells were filled for each concentration (at 200 μL/well), with a further 4 wells filled with DMEM containing 0.5% (v/v) DMSO (without BD) as vehicle control and 4 wells with only DMEM and 10% (v/v) FBS as control.

The cells were allowed to grow within the treated medium for 48 h in the incubator. After this period, the medium was removed from all the wells, and replaced with 20 μL of MTT in DPBS at a concentration of 5 mg mL^{-1}. The plates were incubated for a further 4 h; then all the wells were aspirated, leaving only the formazan precipitate at the bottom. 100 μL of DMSO was then pipetted into each well to dissolve the formazans, and the absorption of each well was read at 550 nm using a spectrophotometer (Sunrise™, Tecan Trading, Switzerland) after 20 min, preceded by a gentle shaking of the plates for 15 s to completely dissolve the formazans. The number of viable cells was calculated as a percentage of the absorbance of the test wells over the vehicle control. The percentage inhibition was obtained by subtracting the number from 100. The results were then plotted as a dose response curve (% inhibition versus $\log_{10}$ BD concentration). The GI_{50} value for BD, which will be used in all further cell experiments, was determined by extrapolating the concentration of BD which resulted in 50% growth inhibition in the dose response curve.

The assay was also repeated using a combination of BD at GI_{50} and fish oil. It was found that the maximum amount of fish oil that can be dissolved in the medium was limited by the amount of EtOH allowed in cell medium. Typically, this was kept at 0.5% v/v or lower. Therefore, the fish oil was dissolved in EtOH at a 2:1 ratio, and the final concentration of the fish oil solution in the medium was 1% v/v (the EtOH concentration in the final medium solution kept at 0.5% v/v). At this maximum concentration, there were no observable inhibitions of cellular growth (no reduction in cell count), therefore we were unable to determine GI_{50} value of FO alone and focused on the combination of BD at GI_{50} and 1% v/v FO: EtOH. The medium containing BD at GI_{50} was dosed into 5 × 8 wells), and an equal number of wells were dosed with medium containing BD at GI_{50} and 1% v/v FO:EtOH (equating to 0.66 mg mL^{-1} EPA in each well of a 96-wells plate). As control, 2 × 8 wells were dosed with medium containing DMSO and EtOH, the vehicles used to dilute BD and FO at 0.5% v/v each.

2.5. Cell seeding and treatment

HaCaT cells were seeded at a density of 1×10^5 cm^{-2} onto a TESPA-coated cover slip inside tissue culture treated dishes with a culture area of 8 cm^2. Post-seeding, the cultures were left overnight to allow the cells to attach to the surface of the cover slip. The following day, the medium in each dish was replaced with medium

containing the test compounds. The incubation time was 24 h at 37 °C and 5% CO_2. The treatments used were BD (in DMSO) at 0.25 mg mL^{-1}, a 2:1 mixture of FO: EtOH at 1% v/v, a combination of 0.25 mg mL^{-1} BD and the FO: EtOH mixture (also at 1% v/v) and control containing the vehicles (0.5% v/v DMSO, 0.5% v/v EtOH) and 10% v/v FBS in medium. For the study of COX-2 expression, the cells were exposed to UV light for 5 min prior to treatment to elicit an inflammatory response.

2.6. Fixation and ICC staining

Cell fixation was carried out at the end of the incubation period using formalin saline (4.5 g NaCl, 50 mL formaldehyde in 450 mL water) by first removing the medium and replacing it with 1 mL of formalin saline and leaving it for 10 min. This was followed by 1 mL of 100% EtOH for 5 min, and 2 changes of PBS at 5 min each. At each rinse, the cover slips were lifted up with forceps to ensure complete washing and to release trapped medium beneath the cover slips. The dishes were then transferred to a humidity chamber to avoid desiccation of the cover slips. The slips were given another rinse of PBS, this time with the addition of a few drops of PBS-Tween for 15 s as a blocking reagent. Prior to incubation with primary antibody of the desired proteins, the blocking solution was completely removed. The primary antibodies were prepared in PBS at 1 in 100 dilutions for Cleaved Caspase-3 (Asp175) and 1 in 50 for COX-2. 75–80 μL of the antibody were applied on top of each cover slip and incubated at room temperature and incubation time of 1 h for Asp175 and overnight for COX-2.

Post-incubation, the cover slips were rinsed with 3 counts of PBS for 5 min each, followed by PBS Tween. The cover slips were then incubated with Dako Envision+ secondary antibody at 25 °C for 90 min. The cover slips were then washed with PBS for 3 min followed by PBS Tween (2 min × 5 min). Visualization of the protein was achieved by incubating the cover slips with 75 μL of DAB chromogenic HRP substrate (Dako) for 10 min at room temperature. The cover slips were then rinsed with deionized water (3 min × 2 min) and counter-stained using an aqueous solution of 0.5% methyl green solution for 35 min. The cover slips were rinsed several times with deionized water and then left in an oven set at 40 °C to dry overnight. They were then mounted onto pre-cleaned microscope slides using DPX and dried at 60 °C before inspection and imaged under a microscope (Nikon Optihop, Tokyo, Japan) equipped with image capture facilities (Axiovison LE, Carl Zeiss Ltd., Welwyn Garden City, UK). The staining of the cells with Asp175 or COX-2 at the different treatments was compared.

2.7. Statistical analysis

Mann–Whitney tests were carried out using Instat 3 for Macintosh GraphPad Software, Inc. (Hercules, CA, USA). A p-value of <0.05 was considered significant.

3. Results

3.1. Growth curve for HaCaT cells

A typical growth curve pattern for the HaCaT cells was achieved (Fig. 1). From the linear section of the plot, the cell doubling time was determined to be 22.1 h, while the lag time was 20.6 h.

3.2. MTT assay–BD dose response curve and GI_{50} determination

The dose response curve for BD (Fig. 2) revealed that the GI_{50} value was 0.22 mg mL^{-1}. To facilitate preparation of test solutions, the concentration was rounded to 0.25 mg mL^{-1}, and all subsequent studies were conducted using this value. The low solubility of fish oil made it impossible to obtain the GI_{50} value for fish oil. Initial growth inhibition assay at the maximum fish oil concentration allowed, only resulted in an average of 7.63 ± 3.66% growth inhibition. All subsequent assays involving fish oil (either alone or in combination) were then performed at the maximum possible concentration.

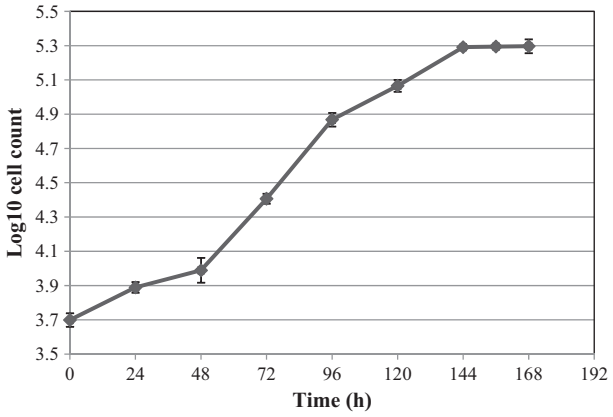

Fig. 1. HaCaT cell growth curve. Cell doubling time determined from the linear portion of the curve is 22.1 h.

3.3. Growth inhibition of HaCaT cells by BD and effects of FO addition

The treatment of HaCaT cells with BD at GI_{50} led to 43.15 ± 3.14% growth inhibition. There was a significant difference between the treatment of the HaCaT cells with BD compared to the control (p 0.0001). Fig. 3 shows that the addition of FO to BD increased cellular growth inhibition to 70.35 ± 3.74%. A significant difference in the growth inhibition of HaCaT cells was seen between the treatment of BD and the treatment of BD with FO (p 0.034).

3.4. Cleaved Caspase-3 (Asp175)

Fig. 4 shows the ICC staining for Asp175. The staining of nuclei to brown indicates the positive presence of the protein and the number of positive nuclei was a direct indication of the extent of cellular apoptosis in each slide. The cells were counted visually under the microscope, and those which showed brown staining were determined as a percentage of the total. The BD treated cells showed approximately 75% of nuclei positive for Asp175, compared to the almost complete absence in the control slide. In cells treated with

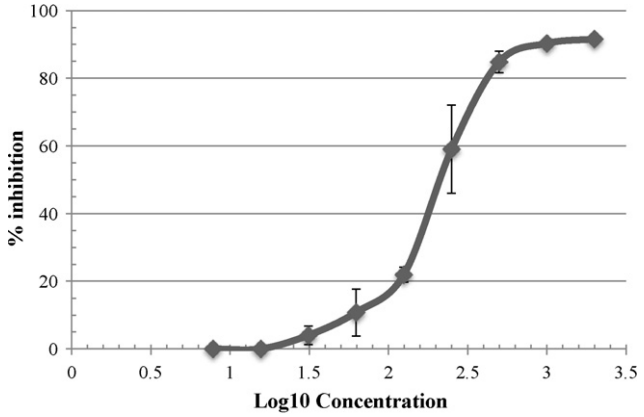

Fig. 2. Dose response curve of BD. GI_{50} was determined to be at 0.22 mg mL^{-1}.

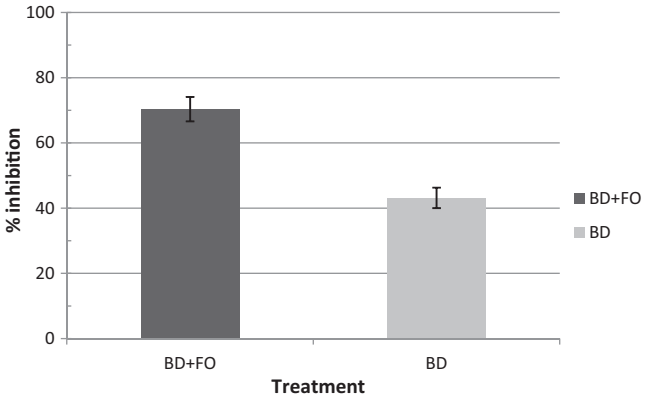

Fig. 3. Growth inhibition of HaCaT cells following addition of BD and FO, $n = 4 \pm$ SD. The addition of FO increased the inhibition of HaCaT cells by 27.2%, from 43.15% to 70.35% compared to BD alone (p 0.034).

FO, ~25% were positive, while it was increased to ~50% when combined with BD at the same concentration as the first treatment. However, the number of positive nuclei for the combination of FO and BD was less than BD alone.

3.5. Modulation of COX-2 expression

Fig. 5 shows representative images for COX-2 staining. Again, the presence of brown staining indicates the positive presence of COX-2 in the cytoplasm. The control slide indicates that COX-2 was expressed, although at a very low level. Treatment with BD, FO and combined BD + FO resulted in reduced staining, which indicated inhibition on the COX-2 expression. The greatest inhibition of COX-2 expression was seen with the combined BD + FO formulation.

4. Discussion

The mechanisms by which BD exerts its activity in the treatment of psoriasis include anti-proliferative effects via disruption of DNA synthesis (Gottlieb, 2005). In this study, the addition of FO to BD increased cellular growth inhibition, suggestive of a synergism in inhibitory action between BD and FO. The exact mechanism of this synergism is unknown. Although at the maximum FO concentration used in this study, growth inhibition was only less than 10%, the anti-proliferative effects of FO and its constituent fatty acids have been repeatedly demonstrated in many cell lines, particularly tumor cells (Chen and Auborn, 1999; Kapoor, 2009). The effect of EPA, one of the primary fatty acids found in fish oil, on the growth of cultured keratinocytes has also been reported (Riku et al., 1993).

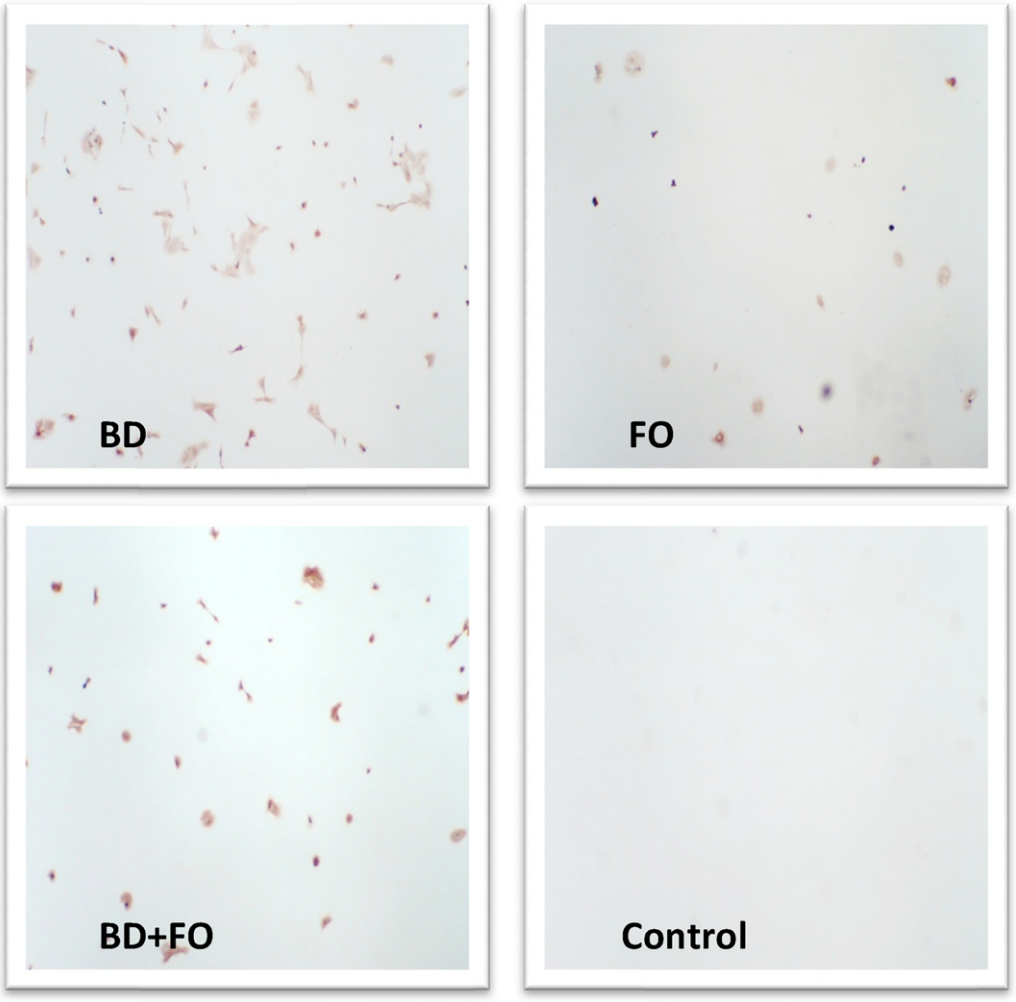

Fig. 4. ICC staining for Cleaved Caspase-3 (Asp175). BD = BD 0.25 mg mL^{-1}, FO = 1% FO-EtOH, BD + FO = BD 0.25 mg mL^{-1} + 1% FO-EtOH. BD treated: ~75% of nuclei positive for Asp175, compared to the almost complete absence of Asp175 in the control slide. FO-treated: ~25% were positive, while it was increased to ~50% when combined with BD at the same concentration as the first treatment.

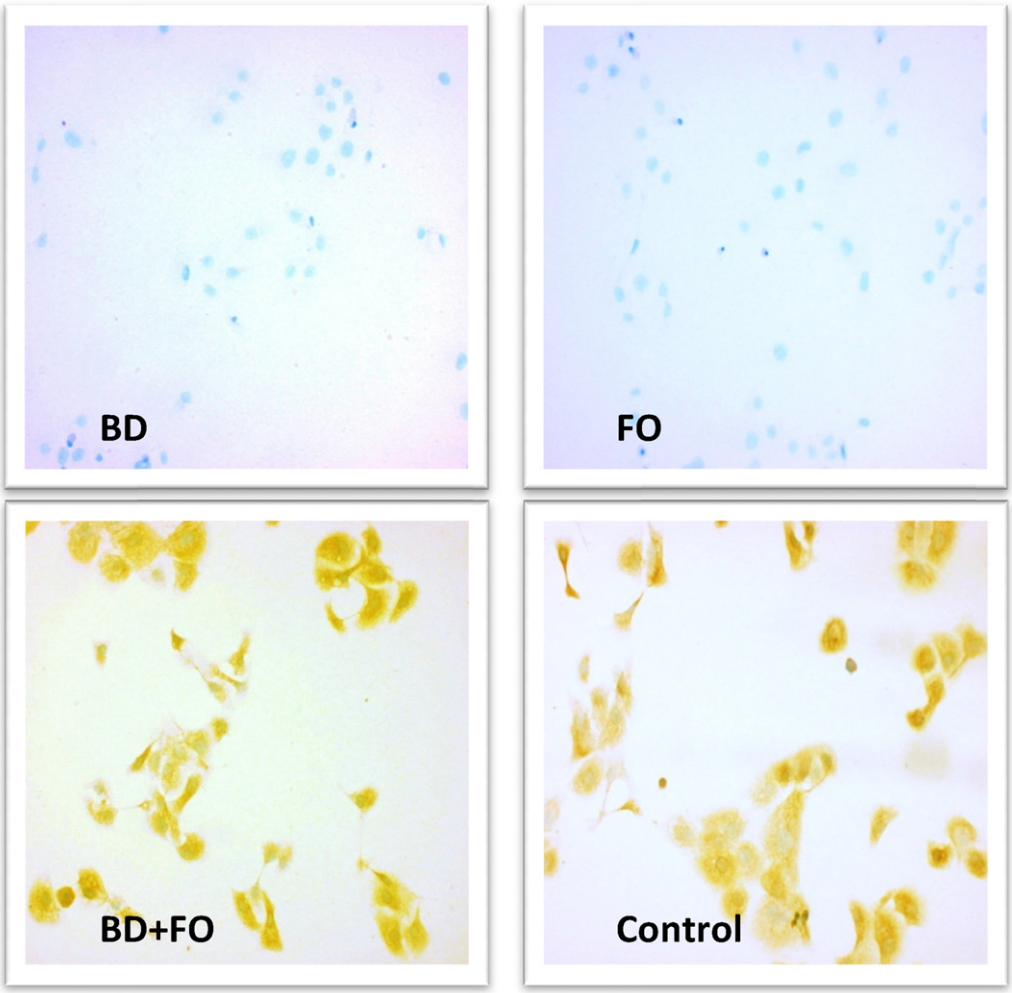

Fig. 5. ICC staining for COX-2. BD = BD 0.25 mg mL^{-1}, FO = 1% FO-EtOH, BD + FO = BD 0.25 mg mL^{-1} + 1% FO-EtOH. Control slide indicate COX-2 expression at a very low level. Treatment with BD, FO and combined BD + FO resulted in reduced staining, indicative of inhibitory effects on COX-2 expression.

This inhibition is believed to be attributed to the ability of EPA to modulate the various steps of signal transduction by growth factors (Terano et al., 1997).

The efficacy of corticosteroids, e.g. betamethasone and other anti-psoriatics, in the treatment of psoriasis depends, among others, on their ability to exert anti-proliferative effects on keratinocytes. Although the mechanism of action varies between the agents, most of the treatments possess the ability to induce apoptosis in keratinocytes (Ceović et al., 2007).

Earlier findings indicated that the expression of Asp175 is unique in the sense that it is expressed in apoptotic, but not normal, cells - a fact utilized in the development of improved Asp175 detection assay in determining cellular apoptosis (Gown and Willingham, 2002). A further report by Allombert-Blaise et al. (2003) however, indicated that Cleaved Caspase-3 is also expressed in continuously renewing tissues. Furthermore, the same study reported the presence of Asp175 in the upper stratum granulosum where cellular degradation of keratinocytes into dead cells takes place. The authors concluded that the apoptotic machinery may play a role in the terminal differentiation of such cells. Based on these findings, we are unable to confirm whether the expression of Asp175 in our study was due to apoptosis or terminal cellular differentiation without examining other parameters, such as the presence of DNA fragments or cellular blebbing–processes catalyzed by Asp175 in cellular apoptosis (Jänicke et al., 1998).

On the other hand, the moderate concentration of Ca^{2+} in the media (1.8 mM) and the morphology of the cultured HaCaT cells in this study was not suggestive of a differentiated state (absence of cellular tight junction, less compact arrangement), as reported by Deyrieux and Wilson (2007). In both cases (terminal differentiation vs apoptosis) we believe that it may be beneficial in the treatment of psoriasis where dysregulation of cellular growth and differentiation are distinctive features.

The ability of FO/n-3 fatty acids to induce apoptosis in tumor and other cell lines has been well-documented (Siddiqui et al., 2001; Hong et al., 2003; Sanders et al., 2004). It was reported that markers of cellular apoptosis were increased in ultraviolet-B (UVB) irradiated keratinocytes following pre-treatment with EPA compared to the monounsaturated fatty acid, oleic acid (Pupe et al., 2002). The mechanism involved has been identified to be a synergistic overexpression of tumor-necrosis-factor-α (TNF-α) between EPA and UVB irradiation itself. This supports the protective ability of FO in reducing the incidence of skin cancer as a result of UV exposure by inducing death of damaged cells that can potentially induce tumor formation.

In the current study, the ability of FO to induce apoptosis and/or terminal differentiation of cells was again demonstrated in HaCaT cells, an immortalized keratinocyte cell line, again supporting the role of FO in the treatment of psoriasis. The FO used in the study was analyzed in-house and found to contain approximately 85%

fatty acids, 33.5% and 21% of which was EPA and DHA respectively, making the n-3 fatty acids major components of the oil. The remaining fatty acids (less than 7% individually) and other natural components of the oil (tocopherol as antioxidant, triglycerides, and sunflower lecithin) are not believed to affect the biological process under investigation.

COX-2 is one of the main enzymes involved in the production of inflammatory mediators in tissues, including the skin. Product mediators such as the arachidonic acid-derived prostaglandin E2 (PGE-2) is known to be overexpressed in psoriatic lesions (Ikai, 1999; Reilly et al., 2000), which may therefore be potentially reduced in the presence of a COX-2-inhibiting agent. The inhibitory effect of BD, FO and BD-FO combination on COX-2 expression in this study correspond to another study carried out on an ex vivo porcine skin model of inflammation, where the expression of COX-2 was inhibited by BD, and by FO alone (Zulfakar et al., 2010). The greatest inhibition was also observed when the two agents were combined in a single formulation, suggesting a potentiation effect. In the current study (Zulfakar et al., 2010), the effect on COX-2 was more pronounced compared to experiments using excised skin, as the primary cells of interest were directly exposed to the treatments, and not have to diffuse across the stratum corneum barrier prior to reaching the viable epidermis.

Interestingly, the study done by Chêne et al. (2007) found that the expression of COX-2 in a HaCaT cell line was enhanced by both n-3 (EPA) and n-6 (γ-linoleic acid) mediated by peroxisomal proliferator-activated receptor (PPAR)-γ. It was also suggested that the protective/anti-inflammatory effect of n-3 polyunsaturated fatty acids (PUFAs) was in fact dependent on COX-2. This sheds light on the 'duality' of the inducible enzyme, which is generally viewed as a pro-inflammatory enzyme, and the deleterious effects of excess inflammatory mediators produced by COX-2 metabolism in diseases, such as colorectal cancer, is well established (Dommels et al., 2003). However, it was also discovered that, in the resolution phase, products of COX-2 metabolism such as prostaglandin D_2 (PGD_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) possess pro-resolution/anti-inflammatory activity (Bertolini et al., 2001; Gilroy et al., 1999). Considering the activity of COX-2, which encompasses both ends of the spectrum (strongly pro-inflammatory through strongly anti-inflammatory), it is reasonable to assume that induction of COX-2 might prove to be beneficial, or deleterious, depending on the phase of inflammation.

Contrastingly, several studies showed that induction of PPAR-γ by its ligands had an inverse relation with COX-2 expression (Yang and Frucht, 2001; Kulkarni et al., 2008). Both EPA and DHA have been found to activate PPAR-γ (Yang and Frucht, 2001; Li et al., 2005; Allred et al., 2008), and this activation is central to the anti-tumor effects of fish oil. Several publications extolled on the virtues of PPAR-γ ligands as a potential new class of therapeutic agent for psoriasis and psoriatic arthritis, given its action on cell proliferation and inhibition of pro-inflammatory mediators (Ellis et al., 2000; Bongartz et al., 2005; Friedmann et al., 2005). It is in this regard that the induction of COX-2 as reported by Chêne et al. (2007) requires further investigation, particularly in the knowledge that the opposite was observed in the current study involving HaCaT cells and also in ex vivo porcine skin (Zulfakar et al., 2010).

5. Conclusions

The ability of FO to inhibit cell growth, induce apoptosis and inhibit inflammation has now been demonstrated at the cellular level. The results obtained by addition of FO to conventional anti-psoriatic treatment (BD) highlights the potential in combining both agents in a single topical formulation to improve therapeutic outcome and provide further evidence of the beneficial effects of FO in hyperproliferative skin disorders, such as psoriasis.

Acknowledgments

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