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Aquaporin 3 (AQP-3) Modulatory Effects of an Herbal Formulated Cream in the Management of Skin Psoriasis.

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Abstract: Aquaporins form a large family of integral, transmembrane water channel proteins that mainly function in transporting water over cellular membranes. Amongst aquaporins, aquaporin-3 (AQP-3) is the predominant skin aquaporin which is localised in the basal layer keratinocytes of epidermis in normal skin, and is found to be important in maintaining balanced skin hydration. In addition, several clinical studies have demonstrated reduced AQP-3 expression in cases of psoriasis, suggesting a strong relationship between AQP-3 expression levels and skin hydration/epidermal water loss in psoriatic skin. In scope of this, the potential to manipulate AQP-3 levels by skin care products designed to address psoriatic skin problems gains pharmaceutical attraction. Therefore, we examined the effects of InnoVision's Psoriderm cream on AQP-3 expression in keratinocytic cell culture model *in vitro*. AQP-3 expression was found to be significantly increased upon treating keratinocytes with the study material. Test substance at concentrations, 350 and 700 µg/mL increased AQP-3 mRNA levels by 0.03 and 0.07 folds, compared to untreated control cells. In summary, it could be illustrated that using InnoVision's Psoriderm in psoriasis care can up regulate AQP-3 expression in psoriatic skin, and thus may play a functional in bettering skin conditions in psoriatic skin lesions.

Key words: Psoriasis; Skin hydraion/dryness; water loss; InnoviSion's Psoriderm; AQP-3 stimulation

Introduction

Skin is a vital organ that plays a major role as a first and foremost line of defense in protecting our body, both physically and biologically. It is a prominent organ bearing composite structure which performs multiple functions ranging from-(i) barrier functioning against mechanical, thermal physical injuries and foreign agents, (ii) as a sensory organ (ii) regulating body temperature (homeostasis) and loss of moisture, (iii) prevention from the harmful effects of UV radiations, (iv) immunological roles in immune surveillance and cellular immunity, (v) endocrine related functions in the metabolic synthesis of vitamin D3 (vi) cosmetic, social and aesthetic associations etc. Simply put, keeping a healthy skin is an important necessity for our survival in many terms in the scope of our overall health and well-being [1-5]. As in the cases of other organs of our body, there are several disease conditions that can affect the health of our skin. Most common ones are related to dry skin disorders including vitiligo, epidermolysis bullosa, psoriasis etc. [6-9].

One clinical type in particular, psoriasis, is a very common inflammatory skin disorder that manifests due to excess buildup of diseased cells focally on the surface of the skin due to keratinocytic hyper-proliferation in the epidermal region of the skin. It is a chronic erythamatous condition of skin clinically characterized by dry, itchy, red scales and plaque like formation on parts of skin anywhere on the body, Especially, on the affected scalp, knee, elbow and torso areas. It is a sustained, long-standing condition that could possibly improve or deteriorate randomly at any given point of time [10-12]. Psoriasis can flare up at a high incidence rate of one in every 50 people with an estimated 250 million people affected worldwide, and is reported to leave the affected patients stressed out mainly with dry and severely itchy skin [13, 14]. Although it is neither an infectious nor a contagious disease to be extremely worried about, it can get serious at times and people suffering from it have often talked about their struggle with it causing social and self-esteem problems.

As of yet, there isn't a comprehensive cure for psoriasis, however, affected individuals have made efforts to control the causative factors by resorting to holistic treatment regimens involving diet, exercise, stress management, medications therapies using skin emolliments, lotions, steroid gel, creams and ultraviolet (UV-B) light exposure therapy [15-16].

At the outset, genetically controlled, auto-immunological pathways related to T-helper cell mediated hyper-proliferation of keratinocytes and the interplay between inflammatory cytokines appear to be the key pathophysiological mechanisms involved in the etiology of psoriasis [17, 18]. Additionally, it has been shown that there are alternate mechanisms in the pathogenesis of keratinocyte derived psoriasis showing the potential involvement of aquaporin-3 (AQP-3), a member of the aquaporin membrane bound water channel proteins, which facilitates the

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movement of water and other molecules such as, glycerol, in ketratinocytes, in psoriasis disease manifestation. There is mounting evidence now to substantiate the defining role of AQP-3 expression in relation to over- and under- hydration of skin. Scientific studies have verified the down regulation of AQP-3 expression confirming the direct involvement of AQP-3 in skin dryness. This might also provide evidence for the functional role of AQP-3 in the context of epidermal water loss and skin dehydration which is characteristically seen in psoriasis-affected skin lesions. Additionally, there are also reports that suggest the role of AQP-3 in regulating the proliferation and differentiation of keratinocytes [19, 20].

Thus, given the degree and pattern of AQP-3 expression in human skin and its clinical relationship, the modulation of AQP-3 gene expression may be a potential target in treating dry skin condition associated with psoriasis. Pharmacological agents, especially herbal ones, which can trigger AQP-3 expression, can be effectively used to target psoriasis [21]. The present investigation deals with the AQP-3 stimulatory attributes of the test substance, InnoVision's Psoriderm cream.

Materials and Methods

Test system HaCat (Human Keratinocyte) cell line has been used as an *in vitro* the test model to evaluate the AQP3 stimulatory effect of the test substance.

Test culture preparation

Cell lines were cultured in DMEM high glucose media supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100µg/ml) and amphotericin B (5µg/ml) in a humidified atmosphere of 5% CO_2 at 37^0C until confluent. The cells will be dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures will be grown in 25 cm^2 culture flasks and all experiments will be carried out in 96 microtitre plates.

Test Product

InnoVision's Psoriderm cream: Each gram of the study/test material contains extracts of: Black mustard (*Brassica nigra Linn.*), Bakuchi (*Psoreala corylforea Linn.*), Neem (*Azadirachta indica Linn.*), Manjistha (*Rubia cordifolia Linn.*), Gotu Kola (*Centella asiatica Linn.*), Licorice (*Glycyrrhiza glabra Linn.*) and Coconut palm (*Cocos nucifera Linn.*).

Preparation of Test Doses

For studies, each weighed test substances were separately dissolved in Media and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration, and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out Cytotoxic studies.

Determination of cell viability by MTT Assay

Principle: The ability of the cells to survive a toxic insult has been the basis of most Cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used.

Procedure: The monolayer cell culture was trypsinized and the cell count was adjusted to 100,000 cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37^o C for 3 days in 5% CO_2 atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37^o C in 5% CO_2 atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilise the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

Gene expression

Culture treatment: The HaCat cells were cultured in 60 mm petridish and maintained in DMEM medium for 24 hrs. The DMEM medium was supplemented with FBS and amphotericin. Twenty-four hours after plating, HaCat cells were treated with test substances of non-toxic concentration i.e., at concentrations of 700 µg/ml and 350 µg/ml, cell control (Culture media) and positive control all-trans retinoic acid (ATRA), incubated for 24 hr. After incubation, the supernatant solution from the cultures was discarded and cultures were processed for total RNA extraction.

RT-PCR Procedure

The mRNA expression levels of AQP-3 carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Briefly, after 24-hour incubation period, Total cellular RNA was isolated from the untreated (control) and treated cells

using Tri-Reagent according to manufacturer's protocol. cDNA was synthesized from total isolated RNA by reverse transcriptase kit according to manufactures instructions (Thermo scientific). Then 20µl of the reaction mixture was subjected to PCR for amplification of AQP 3 cDNAs using specifically designed primers procured from Eurofins India, as an internal control, the house keeping gene GAPDH was co-amplified with each reaction. PCR was carried out in MJ Mini Thermocycler (Bio Rad, U.S.A) and PCR conditions for genes were initial denaturation at 95 °C for 5 min followed by 35 cycles consisting of denaturation at 95 °C for 1 minute, annealing of primers (refer table for temperature) for 1 minute, extension at 72 °C for 1 minute and final extension at 72 °C for 10 minutes.

Details of the primers

Primer type	Oligonucleotide bases	Annealing temperature	Product size (base pairs)
AQP-3	Forw 5' GCTGTCACTCTGGCATCCTG'3' Rev 5'GCGTCTGTGCCAGGGTGTAG'3'	61	150
GAPDH	Forw 5'ACC ACA GTC CAT GCC ATC AC 3' Rev 5'CAC CAC CCT GTT GCT GTA GCC 3'	60	500

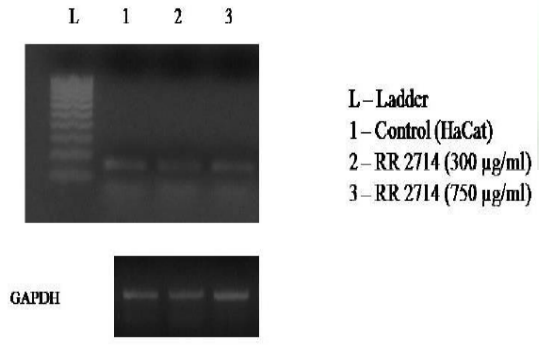
Table 1: Cytotoxic properties of test substance against HaCat cell line

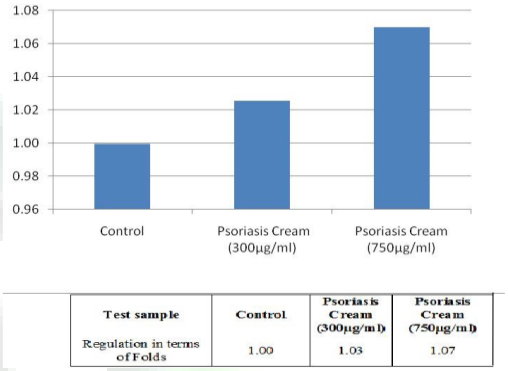
S. No	Name of Test sample	Test Conc. (%)	% Cytotoxicity	CTC_{50} (µg/ml)
1.	RR 2638 (InnoVision's Psoriderm cream)	1000	44.51 ± 2.1	> 1000 µg
		500	30.3 ± 3.4	
		250	23.37 ± 3.7	
		125	13.73 ± 1.4	
		62.5	6.70 ± 1.2	

Results

Results suggested that the test substance showed regulatory effects on the levels of AQP-3 gene expression in keratinocytes. The test substances at concentrations of 350 and 700 µg/ml up-regulated the expression of AQP-3 levels by 0.03 and 0.07 folds, respectively, in comparison to the basal levels of AQP-3 mRNA seen in untreated control HaCat cells (Figures 1and 2). Furthermore, cytotoxicity associated results indicated that the test substance in the concentration range tested for AQP-3 stimulation had insignificant toxicity on keratinocytes with a CTC50 of >1000µg/ml (Table 1/Figure 3), and was found to be safe for usage.

Figure 1: Semi-quantitative RT-PCR profile of AQP-3 from the test substance treated HaCat cell line.





Test sample	Control	Psoriasis Cream (300µg/ml)	Psoriasis Cream (750µg/ml)
Regulation in terms of Folds	1.00	1.03	1.07

Figure 2: Densitometric analysis of gene transcripts. The relative expression of AQP-3 is normalized to GAPDH. Values shown depict arbitrary values.

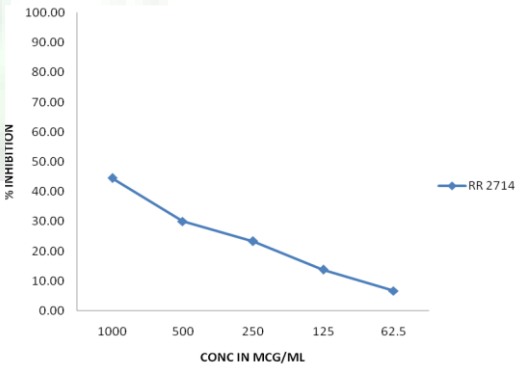


Figure 3: Cytotoxic effects of test substance on HaCat cell line.

Discussion

AQP-3 is a unique membrane bound protein with a distinct structure and function compared to other of the aquaporin members. AQP-3 which is abundantly located in skin keratinocytes serves as membrane

channels that enable transport of water across cell plasma membrane [22]. Altered expression of AQP-3 has been reported in a variety of dermatological disorders with these reports suggesting the contribution of AQP-3 toward water loss and skin dryness that are typically observed in the pathophysiology of the said diseased skin conditions [20]. Amongst them, we highlight psoriasis, a chronic and non-contagious auto-immune, inflammatory disease that affects the skin and joints and other related skin disorders in humans. It has been a consistent observation that patients suffering from psoriasis are not only presented with scaly plaques on the affected parts due to hyper proliferation of keratinocytes and its epidermal build up, but also severe skin dryness and itchiness are distinctly observed in these patients [23, 24]. Although routinely there have been several studies that have focused on investigating the immunopathological mechanisms characterized by T-cell mediated hyper proliferation of keratinocytes resulting in psoriatic skin, studies now have also more thoroughly explored that the physiological mechanisms of skin responsible for skin dryness/hydration are believed to be associated with psoriatic disease processes [20]. Abnormal AQP-3 expression levels have been shown to be significantly involved in psoriatic conditions. Apart from decreased AQP-3 expression levels in psoriatic skin, studies have additionally shown the dislocation of the plasma membrane bound AQP-3 channel proteins into the cytoplasmic region of kerationocytes, thereby suggesting the compromised action of AQP-3 in transporting water/glycerol in these cells during psoriasis [20, 25]. AQP-3 function could thus be highlighted as a considerable causative factor that could be implicated in the pathogenesis of psoriatic skin. In addition, reports are also available that advocate the roles of AQP-3 and its reduced levels in direct or indirect correlation with the keratinocytic proliferation and differentiation event, the deregulation of which, is the mainstay of psoriasis manifestation [26]. In light of these, endogenous stimulation of AQP-3 gene expression becomes an integral part of the pharmacological approach in treating psoriatic skin conditions.

Despite the availability of several classes of synthetic compounds and their derivatives (such as ecdysteroids, xanthenes, ginsenosides, retinoic acid, tocopheryl retinoate, glyceryl glycosides/glucosides etc.) in the market that are capable of increasing AQP-3 levels in skin in order to palliate the symptoms of skin dryness arising due to AQP-3 down-regulation in psoriatic skin, as with any other allopathic medication, the lack of a comprehensive cure, variable efficacy/responsiveness, cost and the

associated disadvantages/side effects with these medicines have paved way for the herbal cosmoceutical industry to get interested and develop safer, more effective and affordable alternative remedies using natural products mainly based on individual herb/plant extracts [27, 20, 19].

The present investigation relates to the antipsoriatic activity in terms of AQP-3 stimulatory effects of an herbal composition formulated as InnoVision's Psoriderm cream, consisting multi-herbal extracts of Black mustard (*Brassica nigra Linn.*), Bakuchi (*Psoreala corylfolrea Linn.*), Neem (*Azadirachta indica Linn.*), Manjistha (*Rubia cordifolia Linn.*), Gotu Kola (*Centella asiatica Linn.*), Licorice (*Glycyrrhiza glabra Linn.*) and Coconut palm (*Cocos nucifera Linn.*). Results disclosed that the study material could act as an effective instigator of AQP-3 gene expression in a dose-dependent manner in human keratinocyte cell line. The active ingredients of the various plant extracts and the corresponding bioactive components making up the composition of test material are the most commonly used herbal agents that are widely reported to possess anti-inflammatory, anti-proliferative and tissue-repair activities. The said activities by themselves may explain the benefits of the multi-herbal formulation of the study material against psoriasis. In addition, since we were also interested in evaluating the effect of study material on AQP-3 activation, and subsequently we could demonstrate the ability of the study material to significantly activate skin AQP-3 as a mechanism for regulating water loss and skin hydration in psoriasis, it further strengthens the antipsoriatic roles of the study material indicating its therapeutic effect in treating psoriasis.

Conclusion

In conclusion, the findings presented herein indicate that the study material, InnoVision's Psoriderm cream, composed of active herbal extracts could induce AQP-3 gene expression promoting cellular hydration, which otherwise is a key aspect affecting skin hydration and epidermal water loss in psoriatic skin. Together taken, this report suggests that InnoVision's Psorider cream might be considered as an effective formulation to address problems related to psoriatic conditions of the skin.

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