Egyptian Dermatology Online Journal
Volume 11 Number 2

Reference of Circadian Rhythm in the Treatment of Psoriasis: A Novel Insight

G.V. Amruthavalli[1], Gayathri Rajagopal[1], Ranganathan.S[1], Sriram .V[2],

1 JRK's Siddha Research and Pharmaceuticals Pvt., Ltd., Kundrathur, Chennai-600 069.
2 Aurous Health Care Research and Development Pvt., Ltd., G1 and G2, RR Villa, No.180/109, Rangarajapuram Main Road, Koddambakkam, Chennai - 600 024.

Corresponding author: G.V. Amruthavalli
Email: amruthavalli_gv@jrksiddha.com

Egyptian Dermatology Online Journal 11 (2): 6, December 2015

Abstract

Objective

The objective of the present study was to establish the need for different products in the treatment of psoriasis with reference to circadian rhythm. The events like proliferation of keratinocytes and elastase activity are influenced by circadian rhythm. The proliferation of the keratinocytes occur more during early hours of the day, however the enzymatic activities occur post to that.

Materials and methods

Psorolin ointment and Psorolin oil were studied for the cell proliferation inhibition of the keratinocytes and elastase and LDH inhibition separately.

1. In vitro assay on growth inhibition of keratinocytes by MTT,
2. Cytotoxicity assay by measuring LDH,

3. Tryphan blue exclusion assay,
4. Elastase inhibition assay,
5. Hyaluronidase inhibition assay.

Results

Findings of the study showed that Psorolin oil inhibited the proliferation of keratinocytes in a non-cytotoxic manner as proved by LDH assay and Tryphan blue staining.

The Psorolin ointment effectively inhibited elastase activity.

Considering the time specificity, cell division and enzymatic activity are separate, warrant the need of both Psorolin oil and Psorolin ointment for the treatment of psoriasis.

Conclusion

The present study confirm the need of two separate drugs namely Psorolin oil and Psorolin ointment for the treatment of psoriasis not due to their mechanism action was different but due to the effect of circadian rhythm in the pathogenesis of psoriasis.

Introduction

The effect of light in the growth, development and the behavioral aspects of all animals and plants are known [1]. Such an effect in plants is called as photoperiodism [2]. Several animals have evolved as either nocturnal, diurnal, crepuscular (active in early morning hours) etc. only due to the effect of light [3]. Collectively such an effect of light on life is called circadian rhythm (day and night cycle) [1].

Light is one of the well-recognized stimuli that activate the endocrinal functions [4]. The hypo and or hyper production and release of various hormones in our body such as serotonin, bradykinin, oxytocin, melatonin etc. are largely influenced by light [4]. The hormones, besides enzymes do play a significant role in the bio-chemical, physiological and division & differentiation of various cells [5]. This means that the proliferation and differentiation rate of different cells in our body are time specific i.e., happening during different hours of either the day or night [6].

The keratinocytes in the skin proliferates and differentiates significantly during late night and early morning hours whereas the cells in the dermis proliferate more during day hours [6]. Even the treatment of some type of cancers is linked with circadian rhythm [7].

Psoriasis is an auto- immune disorder of the skin wherein the keratinocytes before attains its maturity, proliferate rapidly and differentiate, thus the cells formed offering no functional benefit to the skin as expected.

Even today the real cause of psoriasis is although enigmatic [8], however two distinct causes have been identified based on the clinical manifestations namely,

1. Defects in the keratinocytes
2. Immune stimulation

The primary manifestation is due to the erratic and eccentric proliferation & differentiation of cells and also due to the role of certain enzymes (known to play a role in the manifestation and severity of Psoriasis) especially Elastase and Hyluronidase [9,10].

Therefore the treatment of psoriasis must include the possible influence of circadian rhythm as well. Psoriasis manifests mainly in two forms; inflammatory stage and non-inflammatory (remission) stage. If the treatment is target- specific towards either inhibiting keratinocyte proliferation, or inhibiting the key enzymes along with some immune factors, then we can expect the treatment success to be higher. However, all such specific treatments are not as effective as they are expected to be may be because of the mismatch in the treatment time versus the circadian rhythm.

Psorolin oil and Psorolin ointment are proprietary Siddha formulations of Dr. JRK's Siddha Research and Pharmaceuticals Pvt., Ltd., Chennai. The Psorolin oil is composed of extracts of Wrightia tinctoria, Indigofera tinctoria and Indigofera asphalathoids. The Psorolin ointment is composed of Wrightia tinctoria and Cynodon dactylon.

Aim of the work

In the present study, we discussed a novel treatment approach by selective targeting of Psorolin oil and Psorolin ointment linking with circadian rhythm. The proof of concept is being tested by invitro assays and the details are included in the paper.

Materials and methods

In vitro assay on growth inhibition of keratinocytes by MTT

The cell proliferation is based on the ability of mitochondrial succinate-terazolium reductase system to convert 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to a blue colored formazan [11]. The test denotes the survival cells after exposure to the test samples.

The two separate batches of normal neonatal keratinocyte cell lines were used for the above study. The cell lines was maintained and sub cultured in 25mm2 tissue culture flasks using 5 ml of minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 3% L-glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), Amphotericin B (20 µg/ml), phenol red. The pH of the medium was adjusted to 7.2-7.4 with 7.5% sodium bicarbonate and all flasks were incubated at 37 ˚C in a humidified 5% CO2 / 95% O2 incubator.

Cells were grown and seeded at concentrations of 5 × 104 cells per ml of media in a 24 well plate. The cells were allowed to stand for 12 hours to adhere and then treated with different concentrations of Psorolin oil and Psorolin ointment separately and incubated for 48 hours at 37 °C with 5 % CO2. The concentrations tested were 100,200, 300, 400 and 500μg/ml.

After 48 hours of treatment, the cells were treated with 10% MTT for 4 hours in 37 °C, 5 % CO2. Media was then aspirated and the adherent cells with formazon product was dissolved in DMSO, centrifuged at an RPM of 5000 for 15 minutes to remove debris and the spectrophotometric absorbance of the sample was measured using microplate reader. The wavelength used to measure the absorbance of formazan product was 570nm and the percentage reduction in cell proliferation was determined.

Cytotoxicity assay by measuring LDH

Lactate Dehydrogenase (LDH) is an oxidoreductase which catalyzes the inter-conversion of lactate and pyruvate. When disease or injury affects tissues containing LDH, the cells release LDH into the blood stream, where it is identified in higher than normal levels. Therefore, LDH is most often measured to evaluate the presence of the tissue or cell damage [12].

The non-radioactive colorimetric LDH assay is based on the reduction of tetrazolium salt MTT in a NADH-coupled enzymatic reaction to a reduced form of MTT which exhibits an absorption maximum at 565nm. Intensity of the purple color formed is directly proportional to the enzyme activity.

The LDH (Lactate Dehydrogenase) assay was performed to understand the damaging effect of Psorolin oil and Psorolin ointment on normal keratinocyte cells. The dying cells and cells that suffer from

membrane damage would release LDH. Higher rate of release of LDH is indicative of cell death/membrane damage.

Tryphan blue exclusion assay

The intact cell wall does not penetrate the entry of dye and thus appear colorless whereas the dead cells takes up the stain and hence would appear in blue color [13].

The cells were treated with Psorolin oil and Psorolin ointment separately. The concentration of the drugs used for the above experiment was 100, 200, 300, 400 and 500μg/ml. The untreated cells were maintained as control.

Elastase inhibition assay

Elastase enzyme activity was determined by monitoring the release of p-nitroaniline by measuring its absorbance at 410nm.The porcine pancreatic elastase was assayed spectrophotometrically using SANA as a substrate. Psorolin oil and Psorolin ointment at the concentrations of 100, 200, 300, 400 and 500μg/ml were incubated in a mixture of 800 of 200mM Tris buffer (pH 8.0) + 1 unit of enzyme for 15 minutes. The enzyme reaction was initiated by the addition of the substrate and then incubated for further 15minutes at 37°C. The absorbance was measured at 410nm using UV spectrophotometer.

Hyaluronidase inhibition assay

The effect of Psorolin oil and Psorolin ointment on hyaluronidase was studied based on the spectrophotometric measurement of the chromophore reaction with para-dimethyl amino benzaldehyde and is measured at 585nm. This was determined by measuring N-acetylglucosamine splicing from sodium hyaluronate and bovine hyaluronidase was used as a source of the enzyme [14]. Fifty unit of the enzyme was dissolved in 100mM acetate buffer (pH 3.5) and was incubated for 20 minutes with appropriate concentrations of Psorolin oil and Psorolin ointment. The concentration of Psorolin oil and Psorolin ointment used for the study was 50, 75, 100, 150 and 200μg/ml. The enzyme reaction was initiated by addition of the substrate and was incubated for 30 minutes. 50μl of NaOH and 100μl of 0.2M sodium borate were added to the reaction mixture and then incubated in the boiling water bath for 3 minutes. After cooling to room temperature 1.5 ml of para-dimethyl amino benzaldehyde was added and again incubated for further 20 minutes at 37oC. The developed colour was read at 585nm and % inhibition was determined.

Results

Psorolin oil

MTT Assay

The effect of Psorolin oil in inhibiting the proliferation and differentiation of keratinocytes was dose dependent. A maximum inhibition of 63.78% was observed when cells were treated with Psorolin oil at 500μg/ml **(Table 1)**.

Sample (µg/ml)	OD at 570nm			Average of Batch I,II & III	% Inhibition	% SD
	Batch I	Batch II	Batch III			
Control	0.5891	0.6512	0.6222	0.6208		
100	0.6689	0.5912	0.6433	0.6345	-2.2	6.24
200	0.4865	0.5012	0.4621	0.4833	22.15	4.09
300	0.3561	0.36941	0.3625	0.3627	41.58	1.8
400	0.2564	0.3251	0.3625	0.3147	49.31	17
500	0.2213	0.2512	0.2021	0.2249	63.78	11

Table 1: Cell proliferation inhibition effect of Psorolin oil on normal neonatal keratinocytes

Trypan Blue Exclusion Assay

Irrespective of the dose of Psorolin oil used in the treatment of cells, the treated cells did not take up the tryphan blue dye suggests that Psorolin oil has not caused any cellular damage.

LDH assay

There was no release of LDH by the cells treated with Psorolin oil was noticed irrespective of the dose of Psorolin oil.

Elastase inhibition assay

Psorolin oil did not show any significant effect in inhibiting elastase activity despite its concentration **(Table 2)**

Sample(µg/ml)	OD at 410nm	C-T	C-T/C	% Inhibition
Control				
100	2.16	3.21	2.3	1.91
200	4.13	6.11	4.31	3.1
300	6.98	5.13	4.88	5.19
400	11.15	10.91	9.15	12.17

Table 2: Elastase Inhibition Assay- Consolidated data

Hyaluronidase Inhibition Activity

Psorolin oil did not show any significant effect in inhibiting Hyaluronidase activity despite its concentration **(Table 3)**

Sample (µg/ml)	OD at 585 nm	C-T	C-T/C	% Inhibition
Control	0.488			
50	0.491	-0.003	-0.61475	0.61
75	0.474	0.014	2.868852	3.68
100	0.469	0.019	3.893443	4.93
150	0.487	0.001	0.204918	0.2
200	0.459	0.029	5.942623	6.42

Table 3: Hyaluronidase Inhibition Activity.

Psorolin ointment

MTT Assay

The effect of Psorolin ointment in inhibiting the proliferation and differentiation of keratinocytes was insignificant irrespective of its dose**(Table 4)**.

Sample (µg/ml)	OD at 570nm				%	%
	Batch I	Batch II	Batch III	Average	inhibition	SD
Control	0.6453	0.5643	0.5666	0.5921	Nil	7.8
100	0.7012	0.6972	0.6654	0.6879	-10.81	2.9
200	0.6754	0.7825	0.5671	0.675	-8.73	16
300	0.6855	0.5769	0.5563	0.6062	2.35	11.5
400	0.6321	0.5911	0.5612	0.5948	4.19	6
500	0.3218	0.3092	0.5643	0.3984	35.82	36.1

Table 4: Cell proliferation inhibition effect of Psorolin ointment on normal neonatal keratinocytes.

Trypan Blue Exclusion Assay

Irrespective of the dose of Psorolin ointment used in the treatment of cells, the treated cells did not take up the tryphan blue dye suggests that Psorolin ointment has not caused any cellular damage.

LDH assay

There was no release of LDH by the cells treated with Psorolin ointment was noticed irrespective of the dose of Psorolin ointment.

Elastase inhibition assay

Psorolin ointment showed a significant effect in inhibiting elastase activity and which was dose dependent. The maximum percentage inhibition of elastase observed for Psorolin ointment was 41.62% at 400 μg/ml **(Table-5)**.

Sample µg/ml	% of inhibition				AVG	SD
	Batch I	Batch II	Batch III	Batch IV		
Control						
100	8.96	-8.29	1.4	5.61	1.92	7.48
200	-1.13	5.41	15.37	12.1	7.94	7.33
300	26.99	16.73	20.28	22.1	421.52	4.27
400	41.25	43.71	39.45	42.1	41.62	1.77

Table 5: Elastase Inhibition Assay- Consolidated data

Hyaluronidase inhibition assay

Psorolin ointment did not show any significant effect in inhibiting Hyaluronidase activity despite its concentration **(Table-6)**

Sample (µg/ml)	OD at 585 nm	C-T	C-T/C	% Inhibition
Control	0.439			
50	0.435	0.004	0.911162	0.91
75	0.431	0.008	1.822323	1.82
100	0.411	0.028	6.378132	6.37
150	0.408	0.031	7.061503	7.06
200	0.391	0.048	10.93394	11

Table 6: Hyaluronidase Inhibition Activity

Discussion

The proliferation and differentiation rate of keratinocytes do differ significantly during the day and night and such differences in the behavior of keratinocytes in human system strongly indicates the role of circadian rhythm in the biology of keratinocytes.[6] Therefore the drugs that target keratinocytes proliferation inhibition must be used prior to the readiness of keratinocytes to proliferate and differentiate.

If the treatment is perfectly linked and synchronized with the proliferating time of keratinocytes, the drug can contain the psoriatic cells effectively. The cells would be highly vulnerable during their early phase of division than after they having divided.

Psoriasis is an autoimmune disorder that manifests as collection and deposition of immature, non-functioning hyper proliferated keratinocytes. Keratinocytes are believed to be defective and are also believed to be due to existence of some compelling reasons (co-stimulatory molecules, antigen theory) the proliferation and differentiation rate of keratinocytes reduces from 28 days to 3 days. This rapid turnover of cells is continuing due to the trigger of co-stimulatory molecules in psoriatic skin may be without a definite reason.

The present study clearly reveals that the treatment of psoriasis must be performed with greater understanding about the effect of circadian rhythm so that great solace to the patients and treatment success with the drug of choice can be achieved.

In most occasions it may not be the drug that is ineffective but their targeted delivery may not be happening hence they are offering poor relief to the patients.

Our present study shows that Psorolin oil is effective in inhibiting the proliferation and differentiation of keratinocytes. Interestingly the inhibitory effect of Psorolin oil is non- cytotoxic in action. To confirm the above we treated the keratinocytes pretreated with Psorolin oil, with tryphan blue dye.[13] The percentage of live cells out scores the dead cells. This suggests that cell wall damage had not occurred and as a result of the above, the cells have excluded the dye. To reconfirm the non-cytotoxic action of Psorolin oil, we performed LDH assay. [12] Once again it was proved that the release of LDH was insignificant as the cells were intact.

The elastase inhibition effect of Psorolin oil was however insignificant. Whereas, the Psorolin ointment retarded the activity of elastase enzyme.

Elastases are matrix degrading enzymes involved in the tissue homeostasis and are mainly produced by the epithelial cells in the skin, lungs and neutrophils etc. The neutrophil-derived elastases play a major role in the regulation of vascular injury and inflammation, such as ischemia-reperfusion injury. Elastases are available both as membrane bound and intracellular forms. Intracellular elastases break down the foreign proteins, whereas the extracellular elastases released by neutrophils and mostly bound to the neutrophil plasma membrane, assists neutrophil migration to the inflammation sites by degrading various host proteins, such as extracellular matrix proteins. [9]

Elastase does play a significant role in cell damage, cell ageing and other barrier functioning of the skin. Elastase is also known to play a significant role in the pathophysiology of psoriasis. Therefore inactivation of elastase must be included in the treatment approach of psoriasis. Under normal condition the elastase enzyme is under the control of endogenous inhibitors like elafins α1-antitrypsin (α1-AT), secretary leukocyte proteinase inhibitor and α2- macroglobulin. However, large amounts of oxygen radicals and proteases released by leukocytes are recruited to the site of inflammation and that can inactivate these endogenous inhibitors leading to severe inflammatory flare-ups and injuries to the tissues. Hence potential elastase inhibitors could serve as targets in the anti-inflammatory therapy.

The other important target organ or elastases are the matrix proteins in skin which impart the structural and functional integrity to it. During the process of chronological ageing, the metabolic events like formation of advanced glycation end products in the skin, draws inflammatory infiltrates leading to the formation of wrinkles. Hence the elastase inhibitors could serve as multiple treatment options for various skin problems including Psoriasis. In the above context the elastase inhibiting effect of Psorolin ointment assumes greater significance.

Similarly, Hyaluronic acid (HA) is a mucopolysaccharide, occurring naturally in all living organisms. This constitutes an important extra-cellular matrix in various tissues like skin, lungs, ligaments etc. Some of the biological functions of HA include maintenance of the elasto-viscosity of liquid connective tissues such as joint synovial and eye vitreous fluid, control of tissue hydration and water transport, supramolecular assembly of proteoglycans in the extra-cellular matrix and numerous receptor-

mediated roles in cell detachment, binds to water to lubricate the movable parts such as joints and muscles, play a role in mitosis, migration of cells, tumour development, metastasis, and inflammation. HA is found primarily in the extra-cellular matrix, peri-cellular matrix and also present intra-cellularly. HA also plays a major role in imparting volume to the dermis of skin by virtue of its affinity to water and thus it gives an overall appearance to the skin. The enzyme hyaluronidase degrades the HA. Throughout the body, the enzyme - hyaluronidase is found in various forms, intracellularly and in serum. By catalyzing the hydrolysis of HA, a major constituent of the interstitial barrier, hyaluronidase lowers the viscosity of hyaluronic acid, thereby increases the tissue permeability and absorption of drugs as additional benefits.[14]

Interestingly neither the Psorolin oil nor Psorolin ointment affected the hyluronidase activity, and the above findings strongly suggest that both the Psorolin oil and Psorolin ointment are safe and effective for psoriasis.

The findings of the above study suggest the need for wide choice of drugs for the treatment of psoriasis along with the time of treatment. If the consideration of the time is not duly recognized, however effective the drug may be, the drug need not offer the required level of clinical effect to the patient. The study suggest that beyond testing the efficacy of the drugs, the treatment time that affect the microcosm of the cells also need to be considered.

References

1. Derk-jan Dijk,Simon N Archer. Light, sleep and circadian rhythms: together again. PLOS Biology 2009; 10: 1371- 7.

2. Ruth Bastow, Caroline Dean. The molecular basis of photoperiodism. Developmental cell 2002; 3: 461- 2.

3. Sara J Shettle worth. Animal cognision and animal behavior. Animal behavior 2001; 61: 277- 86.

4. Yoshimura T, Soyasuo S, Watanabem, Iigo M, Yamamuri T,Hirunagi K,Ebihara S. Light induced hormone conversion of T4 to T3 regulates photoperiodic responses of gonads in birds. Nature 2003; 426:178-81.

5. Pike Mc,Dahoush L, Spicer DV, Press MF. Estrogen, Progesterone, Normal brest cell proliferation and brest cancer risk. Epidemological reviews 1993; 15: 17- 35.

6. Reinberg A, Koulbains C, Soudant E.Day night differences in effects of cosmetic treatments on facial skin. Effects on facial skin appearance. Chronobiology international 1990; 7: 69- 79.

7. Iversen OH, Iversen UH. A diurnal variation in the tumorogenic effect response of mouse epidermis to a single application of the strong short acting chemical carcinogen methyl nitrosourea. A dose response study of 1, 2 and 10 mg. Invivo 1995; 9: 117- 32.

8. Tony Burns,Stephen Srekthnach, Neilcox, Christopher Griffiths. Rooks text book of Dermatology, Vol.4. USA: Black well publishing Ltd; 2010.

9. Yang X, Yang H, Zhai Z, Hao F, Ye Q, Zhong B. Neutrophill elastase promotes proliferation of HaCaT cell lines and transwell psoriasis organ culture model. International journal of Dermatology 2010; 49: 1068- 74.

10. Necas J, Bartosikova L, Brauner P, Kolar J. Hyluronic acid(Hyaluronan): A Review. Veterinarni Medicina 2008; 53: 397- 411.

11. Loeffler M,Potten CS, Wichmann HE. Epidermal cell proliferation II A comprehensive mathematical model of cell proliferation and migration in the basal layer predicts some unusual properties of epidermal stem cells. Virchowsarch.B cell Pathology 1987; 53: 286-300.

12. Photakas G,Timbrell JA, Invitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicology letters 2006; 160: 171- 7.

13. Warren strober, John wiley and sons, Inc. Tryphan blue exclusion test of cell viability. Current protocols in immunology 2001; 21: 3B:A.3B.1-A.3B.2.

14. Karl Meyer. The biological significance of hyaluronic acid and hyaluronidase. Physiological reviews 1947; 273: 335- 59.