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ORIGINAL ARTICLE **OPEN ACCESS**

In Vivo Evaluation of Cubosomes Loaded Gel of Tretinoin for Actinic Keratosis

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ABSTRACT

Actinic keratosis (AKs), also referred to as senile keratosis or solar keratosis, are benign intra-epithelial neoplasms which is often associated with chronic sun exposure, individuals with AKs may present with irregular, red, scaly papules or plaques on sun-exposed regions of the body. Cubosomal gels are the cubosomal dispersion of hydrogel. Gels consist of two-phase system in which inorganic particles are not dissolved but merely dispersed throughout the continuous phase and large organic particles are dissolved in the continuous phase, randomly coiled in the flexible chains. Mouse models most notably those employing the SKH-1 hairless immunocompetent mouse, proved to be useful and allowed the identification of critical molecular and biologic changes that trigger skin tumour development. Mice treated with a UV-B protocol are recognized as the most relevant models of human skin diseases since they develop AK-like lesions and SCC resembles those seen in humans, although differences exist, such as the thickness of the skin, which is much thinner in mice than in humans. A 1.0 % TCu-gel was given to mice with early-stage AK and intermediate-stage AK, respectively and a profound reduction in the lesions was found.

Keywords: *Actinic keratisis, Tritinoin, Cubosomal gel*

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INTRODUCTION

Actinic keratosis (AKs), also referred to as senile keratosis or solar keratosis, are benign intra-epithelial neoplasms which is often associated with chronic sun exposure, individuals with AKs may present with irregular, red, scaly papules or plaques on sun-exposed regions of the body. If left untreated, AKs may evolve into invasive squamous cell carcinoma, which underscores the importance of early detection and development of a treatment plan [1] [2]. The sun's ultraviolet radiation effects the entire sun exposed skin which results in a field of clinical and subclinical lesions [3] [4]. Actinic keratosis varies in appearance.

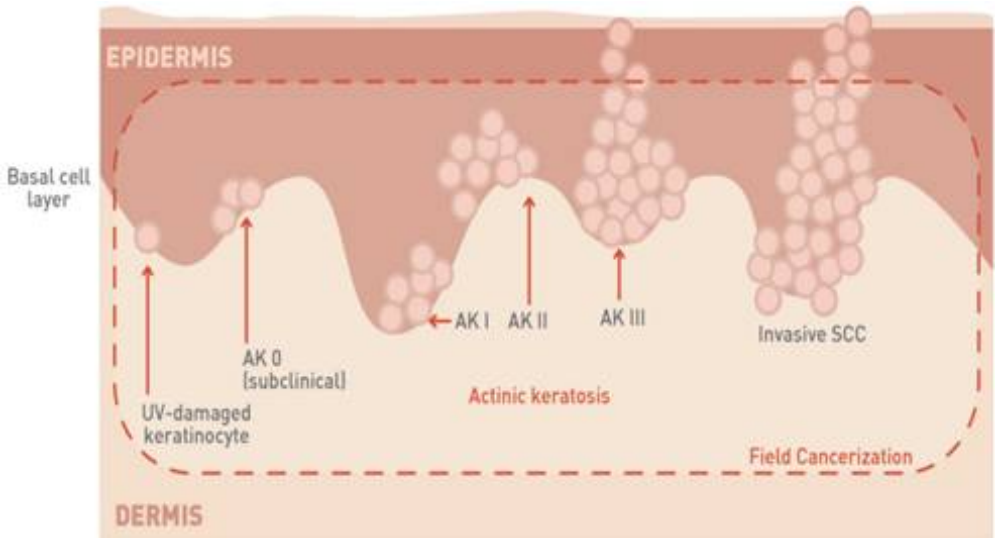

Figure 1: Schematic Representation of AK

Actinic keratosis are predominantly located on chronically sun-exposed areas of the body such as the face, scalp (bald or thinning), back of the arms, and dorsal aspect of the hands, especially in older people who have accumulated decades of sun exposure [5]. The risk factors for AKs are fair skin (burns easily and rarely tans), naturally red or blond hair, light-colored eyes, sun exposure (seldom protected your skin from the sun), 50 years of age or older, tanning bed use, organ transplant recipient, weakened immune system, albino skin, Xeroderma pigmentosum, Rothnord-Thomson syndrome, or Bloom syndrome.

Cubosomal gels are the cubosomal dispersion of hydrogel. Gels consist of two-phase system in which inorganic particles are not dissolved but merely dispersed throughout the continuous phase and large organic particles are dissolved in the continuous phase, randomly coiled in the flexible chains [6]. The optimized batch was further converted into gel by utilizing Carboxy methyl cellulose as gelling agent. The prepared gel was evaluated for appearance, pH, viscosity, in-vitro permeation study. Finally, the stability studies of cubosomal gel were also performed. The cubosomal gel was obtained by addition of weighted amount of carbomer (1% w/w) in distilled water and kept for half day forgetting to swell of carbomer and then add triethanolamine drop by drop up to pH 6.8. Propylene glycol is added to adjust the consistency. The obtained gel was then diluted with an appropriate amount of cubosomes dispersion in the ratio between the dispersion and the gel was 2:1 w/w [7].

MATERIAL AND METHODS

Materials

- Ether for anaesthesia (isoflurane)
- Hematoxylin, eosin and saffron dye for histopathology
- Standard drug (Diclofenac cream) and Test Formulation (Cubosomal gel of Tretinoin)

Methods

Chronic UV-B Exposure in Mice

SKH-1 mice in individual housing (one mouse/ cage) were exposed to UV-B every day for 14–15 weeks, in a dedicated cabinet which were specifically designed to provide UV-B exposure of all cages simultaneously. Medium wave UV-B lamps T-40 M run from 280 to 320 nm with an energy peak at 312 nm. The irradiation time of a single UVB exposure depends on each lamp's UV-output and this may decrease slightly over time (UV degradation). To manage this, each lamp was internally calibrated before experiment initiation, using a UVB-specific photo radiometer to adjust the irradiation period. To generate AK lesions and to prevent the risk of skin burn, gradual exposure was performed as follows: 10 days at 0.05 J/cm^2/day, 10 days at 0.055 J/cm^2/ day and then the MED (minimal erythemal dose) was applied for the 50 to 80 following days.

Topical Application

After the UV-exposure period, mice developing actinic keratosis were arranged randomized into treatment groups, each consisting of mice with observable skin lesions of comparable number and size. Treated areas were tattooed to be easily identified. Mice were then treated topically using an occlusive method, employing a ø19 mm chamber system fixed upon treated areas with Tegaderm™ film, Omnifix elastic and Elastoplast tape. All treatment and photographing were performed under isoflurane (1.5%) mixed with air/oxygen (80/20) gas anaesthesia.

Histology and Immunohistochemistry

Three or four µm thick sections of formalin-fixed and paraffin-embedded skin biopsies were prepared and stained with hematoxylin, eosin and saffron for histopathological analyses. Immunohistochemical analyses were performed using anti-CD3, anti-Ki67, and anti-p53 antibodies. Tissue sections (3 µm thick) were cut out, deparaffinized, and were placed in 10 mmol/l citrate buffer (pH 6.0) (for Ki67 and CD3 staining) or placed in EDTA buffer (pH 9.0) (for p53 staining), and boiled at 96˚C for 20 min. Endogenous peroxidase activity was blocked by incubating the sections in 0.3% H_2O_2 for 15 min at room temperature (RT). After washing three times with 50 mmol/l phosphate buffer (pH 7.6), the sections were preincubated with 3% bovine serum albumin 5% goat serum in PBS for 1h at RT and were then incubated with the primary antibodies; anti-CD3 (1:100), anti- Ki67 (1:100) and anti-p53 (1:750) for 1h at RT. Subsequently, the sections were incubated with peroxidase-labelled polymer-conjugated secondary antibodies against the primary antibodies for 45 min at RT (1:400 Diagomics D13-D18, Blagnac, France) followed by incubation with 3, 3'-diaminobenzidine DAB using the DAKO Liquid DAB Substrate-Chromogen System for 5 min at RT. The sections were then counter stained with hematoxylin and finally dehydrated and cover slipped [8-9].

RESULTS

SKH-1 mice were UV-B-irradiated daily and skin lesions were sampled at different times. The samples were then studied for histology and immunohistochemistry.

Histological Study

Normal murine skin showed mainly a thinner epidermis and a higher number of proliferative cells in the basal layer. Murine skin lesions, sampled on days 67–76 post UV-B irradiation corresponded mainly to early-stage AK-like (Table 1, figure 1). On day 130 post UV-B irradiation, mouse skin lesions clearly represented advanced-stage AK. Overall, the histological analysis showed that AK modelling in mice reflects the clinical situation. Murine lesions frequently showed acanthosis already in the early stages of AK development. Larger nuclei and an increase in the number of mitosis, as compared to normal skin were observed. The advanced stages of AK were characterized in murine cases by a higher number of atypical nuclei and by the presence of mitosis in all layers of the epidermis. Parakeratosis, dyskeratosis or focal points of squamous differentiation and acanthosis were also observed in the advanced forms of murine skin lesions. Overall, the characteristics of the various stages of AK development in the UV-B-irradiated mouse model were very close to those of human AK, with an increase in the size of nuclei, the number of atypical nuclei and mitoses, a modification of keratinocyte distribution in skin layers (Table 1, figure 1).

Immuno-histological Study

p53 staining was observed on keratinocyte nuclei, in all murine skin lesions, particularly in the basal layer but also in the other layers of the epidermis, especially in the advanced forms (Table 1, Figure 2). A heterogeneous pattern of expression, in terms of staining intensity was more frequently observed in the early-to-intermediate human and murine AK cases. Furthermore, as AK progressed to advanced stage or to SCC, a higher number of keratinocytes showed positive p53 staining in murine lesions. Overall, the majority of murine skin lesions showed a higher number of proliferating keratinocytes in the basal layer and in some cases in the supra basal layer of the epidermis than in normal skin. A Ki67 score of ++ or +++ was also recorded in 8 of the 11 murine skin lesions. Because the AK immune infiltrate mainly consists of T cells, the CD3 marker was used to characterize immune infiltration in AK and SCC specimens. CD3-positive T-cells were detected in a lesser number in murine cases. In fact, very few murine skin lesions showed a positive CD3 infiltrate. A CD3 score of ++ was recorded in 2 of the 12 cases of inflammatory T cell infiltration.

Table 1: Histological immunohisto-chemical analysis of mouse UV-B induced AK lesion

Mouse ID	Stage /H & E	P53	Ki67	CD3
A	Normal mouse skin	**-**	**+/-**	**+/-**
B	D67-early-stage AK Acanthosis, thickening of the stratum granulosum Dyskeratotic cells in the mucous body	heterogenous + to +++	+++	++
C	D130 Advanced-stage AK + SCC Acanthosis, atypical keratinocytes and mitoses in the various epidermis layers	+++	++ (zones +++)	+ (zones++)

Scoring: +/-: rare positive cells, +: less intense staining and about 15% positive cells, ++: intense staining and about 30% positive cells, +++: intense staining and about 50% positive cells

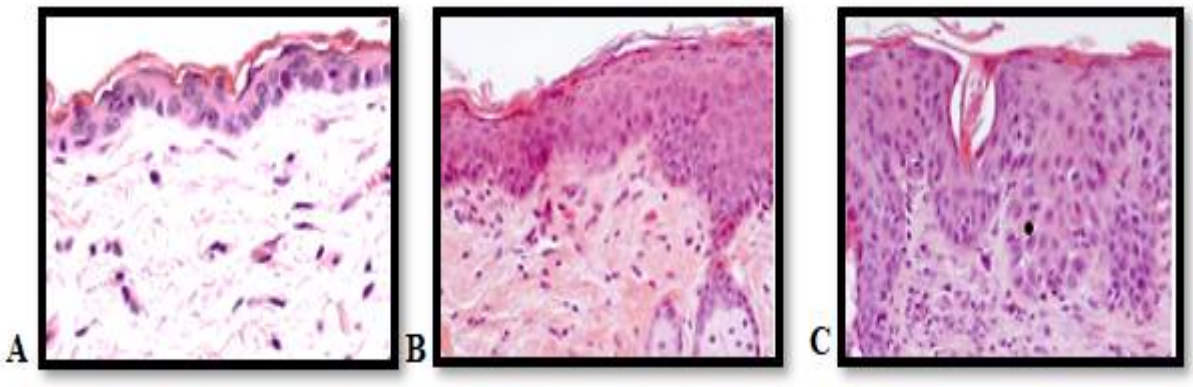

Figure 2: Mouse normal skin (A), Mouse early-stage AK (B), Mouse advanced-stage AK (C)

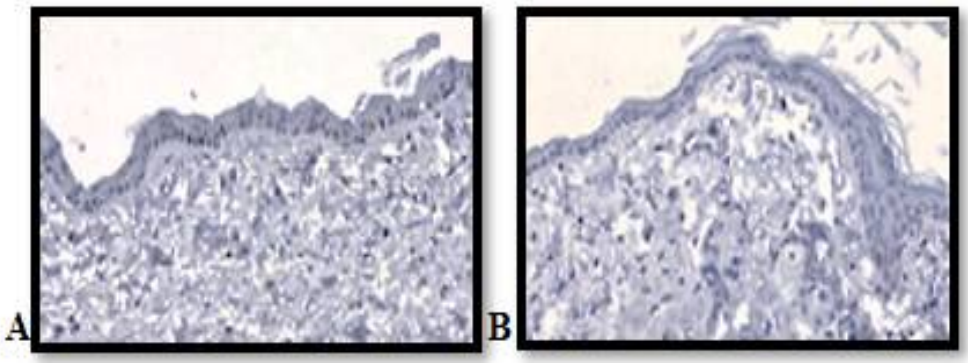

Figure 3: Mouse normal skin, p53 staining (A) and Ki67 staining (B)

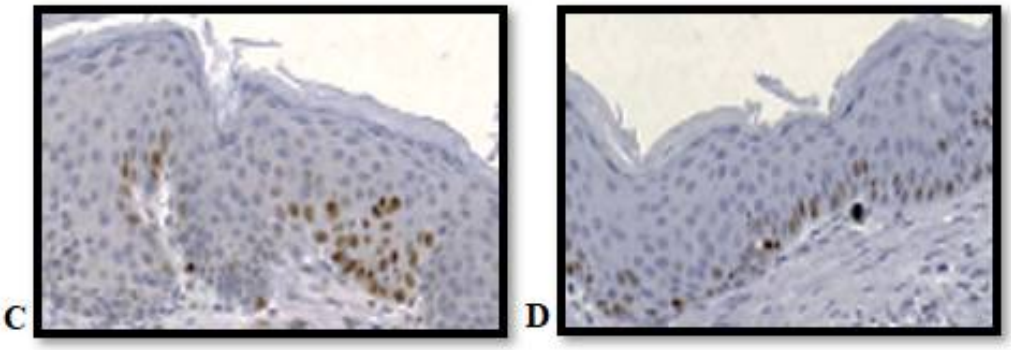

Figure 4: Mouse, early-stage AK, p53 staining (C) and Ki67 staining (D)

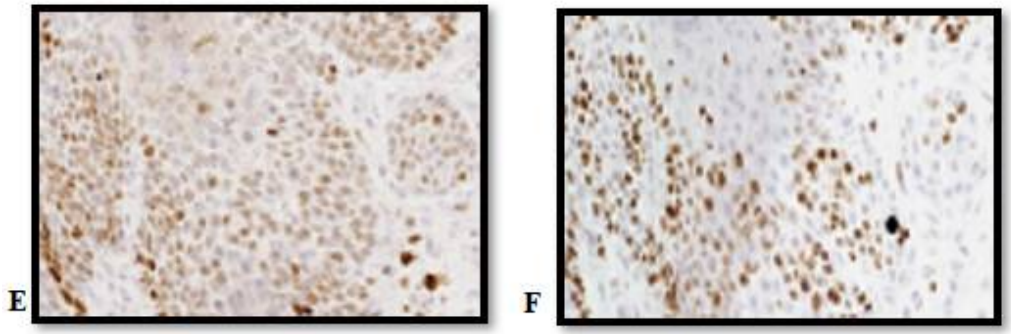

Figure 5: Mouse, advanced-stage AK, p53 staining (E) and Ki67 staining (F)

Evaluation of Cubosomal Gel

Appearance

About 1 week after preparation, the dispersions were visually assessed for optical appearance (e.g., colour, turbidity, homogeneity, presence of macroscopic particles) [10].

pH

pH of all formulations is determined by using digital pH meter by immersing the electrode in gel formulation and pH was measured [11].

Viscosity

Viscosity measurements were performed by Brookfield viscometer (AMETEK Brookfield**, Germany)**. The tested formulations were placed in the sampler tube using spindle no. 4. The spindle was lowered vertically into the centre of the formulation and rotates at a speed of 50 rpm for 10 min. All measurements were carried out in triplicate and the mean value was recorded ± SD [6].

***In-vitro* Release Study**

The *in-vitro* release of Tretinoin from the cubosomal gel was performed. Prior to testing, a piece of cellulose membrane (Molecular weight cut off 12,000–14,000 Da, Spectra/Pro, Spectrum Laboratories, Inc., USA) was soaked in pH 6.8 phosphate buffers for about 12 h. Then, the membrane was fixed in position using rubber band to cover one end of a top-cut plastic syringe acting as a dialysis tube of 1.9 cm internal diameter. An accurately weighed quantity of the test preparation (equivalent to 10.0 mg Tretinoin) was placed in the designed release assembly. The tube enclosing the test sample was then attached to the shaft of a dissolution apparatus I (Hanson Research, California, USA). The dialysis tube was carefully adjusted to a position so that the membrane just touched the surface of the release medium

(pH 6.8 Phosphate buffer). A volume of 50 ml of pH 6.8 phosphate buffer (pH 6.8) was used for the study. The temperature was maintained at 37°C ± 0.5°C and the stirring speed was adjusted to 100 rpm. Two ml aliquots of the release medium were withdrawn at 1, 2, 3, 5, 8 and 12 h time intervals, and replaced with 2 ml fresh medium to maintain the volume. The samples were filtered with micropore 0.22 µm syringe filter and analyzed for Tretinoin content using U.V visible spectrophotometer (Nano Drop Model 1000, Thermo Fisher Scientific, DE, USA). The mean cumulative amount of Tretinoin released (n=3, ± SD) was plotted as a function of time [12].

Evaluation of Tretinoin Cubosomes Gel (TCu-Gel)

The Tretinoin cubosomes gel was checked for appearance, pH, viscosity and in-vitro permeation study. The results of evaluation parameters were depicted in table 1.9.

Table 2: Evaluation Parameters for Optimized Tretinoin Cubosomes Gel Formulation (TCu-gel)

S. No.	Parameter	Inference
1.	Appearance	Homogeneous
2.	pH	7.4
3.	Viscosity	145 cP
4.	In- vitro permeation study	91.948±0.011% in pH 6.8 Phosphate buffer

***In-Vitro* Permeation Study of Optimized Tretinoin Cubosomes Gel Formulation (TCu-gel)**

The in-vitro release results are depicted in Figure 6.

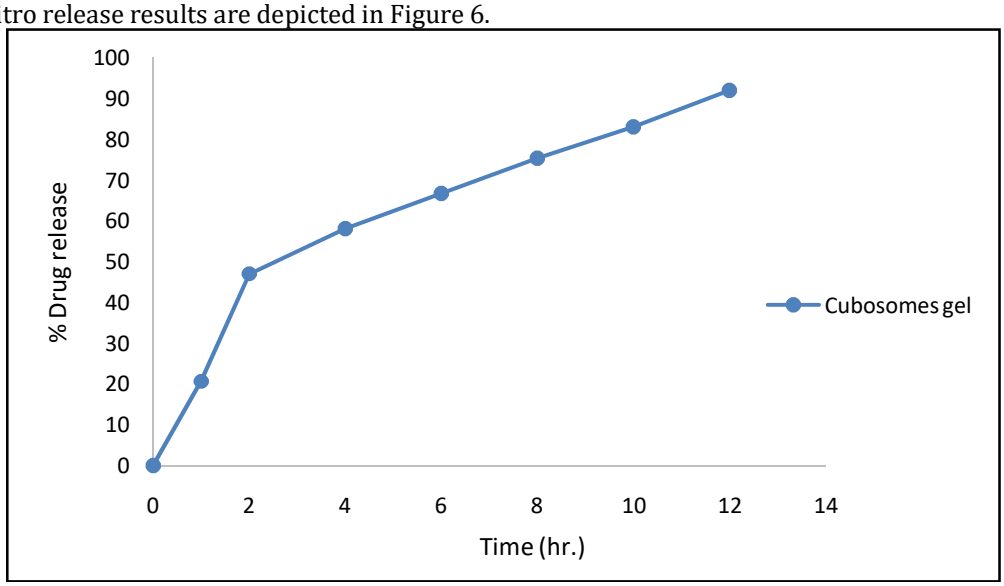

Figure 6: In-Vitro Permeation Study of Optimized Tretinoin Cubosomes Gel Formulation (TCu-gel)

Effects of Tretinoin Cubosomes Gel Formulation (TCu-gel) on Mouse AK

Assessment and preclinical diagnosis of UV-B-induced mouse lesions are mainly based on morphological observation using photographs and histological analysis. The use of calibrated digital dermatoscope through a pharmacological study was illustrated for assessing the effects of Tretinoin Cubosomes gel formulation (TCu-gel), one of the well-known reference topical treatments for AK, on mouse UV-B induced skin lesions. Mice were treated topically with 1.0 % Tretinoin Cubosomes gel formulation for two cycles of 30 h and number of lesions decreased after application of the Cubosomal gel was noted down. A 1% Tretinoin Cubosomes gel decreased the number of AK lesions by 66%, as assessed by analysis of photographs on day 28, whereas the number of skin lesions in the non-treated mice group increased by 25%. Results were presented in table 2.

Table 3: Effect of 1% Tretinoin Cubosomes Gel Formulation on UV-B Induced AK Lesions

		Before Treatment		After Treatment	
		Skin lesion No.	Mean ± SEM	Skin lesion No.	Mean ± SEM
Control	1-1	9	7.8±0.15	10	10.4 ± 0.21
	1-2	4		8	
	1-3	9		11	
	1-4	12		12	
	1-5	5		11	
1% TCu-gel	5-1	8	8.8 ± 0.28	4	2.8 ± 0.11
	5-2	11		2	
	5-3	9		5	
	5-4	7		3	
	5-5	9		0	

Assessment of the number of skin lesions in control vehicle and 5-FU treated mice, before and after treatment (on day 28 after treatment initiation) Experiments involved five mice per experimental group.

DISCUSSION

AKs are skin lesions which are precursors to SCCs on sun-exposed areas through genomic perturbations due to UV radiation and may develop progressively to invasive SCCs. AK treatment is therefore mandatory. Understanding human neoplasia relies on, but has also been limited by, the experimental models used to study tumour initiation and progression. Therefore, correlation of experimental observations made in murine models and in human tissue is of prime importance for demonstrating relevance to human disease. Mouse models, most notably those employing the SKH-1 hairless immunocompetent mouse, proved to be useful and allowed the identification of critical molecular and biologic changes that trigger skin tumour development. Mice treated with a UV-B protocol are recognized as the most relevant models of human skin diseases since they develop AK-like lesions and SCC's resembling those seen in humans, although differences exist, such as the thickness of the skin, which is much thinner in mice than in humans. In this study, we characterized and compared various stages of skin lesion development in UV-B irradiated mice. Three grading scales were established: early-stage AK, intermediate-stage AK and advanced-stage AK, which often included SCC areas. In fact the coexistence of AK lesions and SCC on the same individual is frequently observed in 72 to 92% of cases in the clinical situation, and it is commonly admitted that 60% of cutaneous carcinoma arise from AKs. A 1.0 % TCu-gel were given to mice with early-stage AK and intermediate-stage AK, respectively and a profound reduction in the lesions was found.

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