UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA
DEPARTAMENTO DE FARMÁCIA GALÉNICA E TECNOLOGIA FARMACÊUTICA

CARRIER-MEDIATED DERMAL DELIVERY FOR PREVENTION OR TREATMENT OF SKIN DISORDERS

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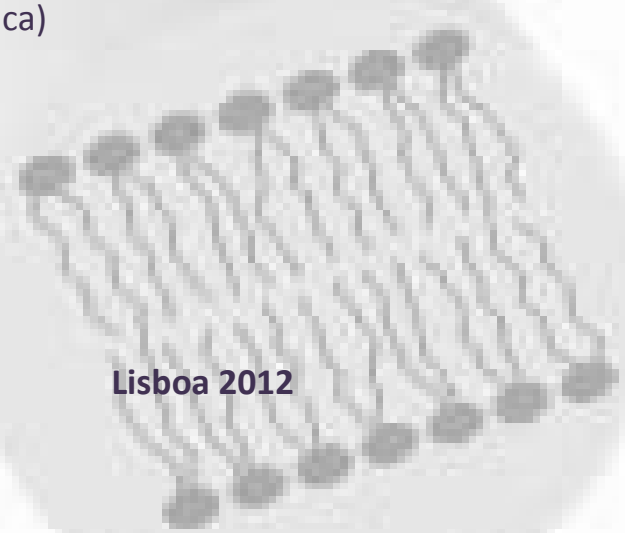
Doutoramento em Farmácia
(Tecnologia Farmacêutica)



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CARRIER-MEDIATED DERMAL DELIVERY FOR PREVENTION OR TREATMENT OF SKIN DISORDERS

(com resumo em português)

PhD thesis supervised by Doctor Sandra Isabel Dias Simões and Prof. Doctor Maria da Conceição Lopes Vieira Santos.

The experiments described in this thesis were performed mainly at the Departamento de Tecnologia Farmacêutica da Faculdade de Farmácia da Universidade de Lisboa (iMed, FFUL) and Departamento de Biologia, Universidade de Aveiro, Portugal.

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"No great discovery was ever made without a bold guess”

Isaac Newton (1643-1727)

Abstract and Keywords

The effects of solar ultraviolet radiation that reaches the Earth surface can be beneficial but also harmful depending on the circumstances. The recent search for new chemical entities against photo-induced skin disorders has received particular focus on phytocompounds investigation. The use of natural compounds, working through different pathways in the cell, in both skin care products and therapeutic purposes may be an effective approach for reducing UV-B generated reactive oxygen species (ROS) mediated photoaging and photocarcinogenesis. In this work, the role of two active molecules is presented and discussed: tretinoin (the acid form of vitamin A) and lycopene (a carotenoid without vitamin A activity) in the context of photoaging and photocarcinogenesis, respectively. Tretinoin is involved in the control of cell differentiation and proliferation in several tissues, particularly in the skin epithelium. It has been demonstrated that photoaging can be ameliorated by topical retinoids, such as tretinoin, and long term effects of their topical application on the skin can be the reduction and redistribution of epidermal melanin, improved ultrastructural characteristics of the epidermis, increased anchoring fibrils, increased deposition of papillary dermal collagen, and increased vascularity in the papillary dermis. On the other hand, the anticancer activity of lycopene has already been demonstrated in both *in vitro* and *in vivo* different tumour models. The mechanisms underlying the inhibitory effects of lycopene on carcinogenesis could involve ROS scavenging, upregulation of detoxification systems, interference with cell proliferation, induction of gap-junctional communication, inhibition of cell cycle progression and modulation of signal transduction pathways.

Dermal delivery after topical application of actives has gained increased interest and development due to the lower risk of systemic side effects. In particular, for antioxidants skin delivery, the search for a new delivery system that, simultaneously, preserves the antioxidant stability and enhances its deposition on the skin, opened a new chapter in drug delivery design. Since epidermal lipids are predominantly found within the penetration skin barrier (*stratum corneum, SC*), topically applied lipid

nanocarriers, allowing lipid interaction between the outermost layer of SC and the carrier, appear promising. In fact, nanocarriers, and among these advanced drug delivery systems, liposomes and cyclodextrins, have successfully enhanced the clinical efficiency of several drugs. Liposomes are typically hollow spheres surrounded by a lipid bilayer. More recently, specially designed carriers have claimed the ability to cross the skin intact and deliver the loaded drugs into the systemic circulation, being at the same time responsible for the percutaneous absorption of the drug within the skin. These carriers were firstly introduced as transfersomes®*, and this denomination as well as deformable vesicles, were used to differentiate them from the more conventional liposomes. The highly flexible membranes are the result of combining into a single structure phospholipids and an edge-active component (surfactant) in order to give to transfersomes the necessary deformability to move spontaneously into the skin, delivering the associated drugs dermal or systemically. Cyclodextrins are cyclic water-soluble, non-reducing and macrocycle carbohydrate polymers. Some derivatives, such as β- and methylated-β-cyclodextrins are usually used for topical formulations.

The **aim** of this thesis was to use cyclodextrins and transfersomes, or their combination, as delivery systems for tretinoin and lycopene, and further investigate the resulting systems behavior in *in vitro* and *in vivo* conditions.

Tretinoin was incorporated in transfersomes (tretinoin-in-dimethyl-β-cyclodextrin-in-ultradeformable vesicles and tretinoin-in-ultradeformable vesicles) in order to fully characterize these new formulations, and further study its dermal delivery.

The need of an appropriate source of lycopene led to the development of a simple and fast process for the extraction of lycopene from tomato. The incorporation of this lycopene-rich-extract in vesicular nanocarriers suitable for topical application was also performed and reported on this thesis. Lycopene was described as the antioxidant most quickly depleted in the skin upon exposure to solar radiation, and consequently, might play a role for protecting skin against UV radiation. To test this hypothesis, the main effects of UV-B irradiation on human keratinocytes (HaCaT cells line) after lycopene exposure were studied.

*Transfersome® is a trademark of IDEA AG, Munich, Germany

Regarding the **methods** used, the tretinoin-cyclodextrin complex was prepared by the kneading process and transfersomes resulted from the combination of soybean phosphatidylcholine and Tween® 80 to make 10 % - 20 % lipid suspension. The resulting suspension was brought to the final mean vesicles size of approximately 100-150 nm, by sequential filtration. In the case of lycopene, an extraction process from dried tomatoes was previously carried for 30 min at room temperature, protected from light. The extracts were analyzed by high-performance liquid chromatography and mass spectroscopy and incorporated in deformable vesicles. The physicochemical characterization of the vesicular formulations was based on the evaluation of the following parameters: mean particle size and polydispersity index measured by photon correlation spectroscopy and atomic force microscopy; zeta potential determined by laser-doppler anemometry; phospholipid concentration determined by an enzymatic-colorimetric test; and drug assay by an adequate high performance liquid chromatography method.

The topical delivery of the tested formulations involved different studies, such as the release and permeation profiles (tape stripping); skin penetration (fluorescence analysis) and induced electrical changes in skin barrier properties. The *in vitro* toxicity was also achieved.

The irritation potential of the developed tretinoin formulation was evaluated *in vivo* using a modified Draize test and compared to a commercially available tretinoin semisolid form. The *in vivo* therapeutic effect of lycopene topical formulations was tested in mice, using the anthralin-induced ear swelling model, being anthralin topical application responsible for the generation of reactive oxygen species in the skin.

Thiazolyl blue tetrazolium bromide (MTT) assay was performed in order to select the UV-B irradiation protocol specific conditions. After lycopene exposure and irradiation experiments, HaCaT cells were analyzed in order to achieve information on the following parameters: genotoxicity/clastogenicity by determining the cell cycle by flow cytometry (FCM) and analyzing apoptosis biomarkers and gene expression by real time

– polymerase chain reaction quantification; apoptosis by performing the Annexin V assay by FCM; and oxidative stress damage by ROS quantification by FCM.

Results of tretinoin-loaded transfersomes revealed that the incorporation efficiency was high for all formulations tested. However, the tretinoin vesicular formulation with 20 % lipid concentration presented better drug stabilization, attending to the higher chemical stability achieved.

Tretinoin-loaded deformable vesicles applied on the skin were able to decrease the skin resistance, suggesting their ability to induce barrier disruption, as theoretically expected. It was also observed by fluorescence microscopy that these vesicles were mainly concentrated in the SC and epidermis layers. Permeation studies showed that no tretinoin is measurable in the receptor phase. In fact, drug retention in the SC and in epidermis/dermis are indicative of topical delivery, whereas the drug quantification in the receptor phase is indicative of transdermal delivery. These results showed that tretinoin-in-transfersomes has a dermal instead of a transdermal delivery, which serves the therapeutic purpose since tretinoin receptors, such as RAR-γ, are mainly located in epidermis. Finally, tretinoin-in-transfersomes formulation was not toxic in *in vitro* and *in vivo* conditions at least at 5×10^{-3} mg/mL and 0.5 mg/mL of tretinoin, respectively.

Regarding lycopene extraction, the little number of steps involved in the extraction process may have improved the yields of lycopene in the extract and exerted a beneficial role in the stability of this carotenoid. The vesicular formulations presented a good incorporation efficiency of lycopene in deformable vesicles (transfersomes and ethosomes). Cytotoxic data demonstrated that extracted lycopene and pure (commercial) lycopene present similar performances of low cytotoxicity, and were similarly affected by environmental conditions. The vesicular formulations containing extracted lycopene were able to inhibit the edema formation *in vivo* on anthralin-induced ear swelling model relative to untreated controls, and without a statistically significant difference relative to the control treated with a model drug.

In vitro photocarcinogenesis studies with UV-B irradiated keratinocytes revealed that lycopene decreased the irradiated cells viability according to MTT and Annexin V results. It was also observed the cell cycle arrest (at G0/ G1 phase and a higher apoptotic Sub-G1 peak), and a higher ROS level.

In summary, according to the obtained results, an effective dermal delivery of actives could be achieved by the use of a carrier-mediated transport. Some aspects regarding the elucidation of drug biological activity behind this delivery system were also successfully explored.

Keywords: Tretinoin; Lycopene; Nanocarriers: Cyclodextrins and Deformable vesicles; Dermal Delivery; Photoaging and Photocarcinogenesis.

Resumo e Palavras-Chave

Os efeitos da radiação solar ultravioleta (UV) que atinge a superfície da Terra podem ser benéficos, mas também prejudiciais, dependendo das circunstâncias. Nos últimos anos, a pesquisa de novas moléculas com actividade na prevenção e/ou tratamento de lesões cutâneas, associadas a uma excessiva exposição à radiação solar, tem focado com especial interesse os fitocompostos. A utilização de compostos naturais que atuem através de diferentes vias celulares, em produtos terapêuticos e de cuidados da pele, poderá ser uma abordagem eficaz ao nível da redução de espécies reativas de oxigénio geradas pela radiação UV-B e envolvidas nos processos de fotoenvelhecimento e fotocarcinogénese. Neste trabalho, é apresentado e discutido o papel de duas moléculas ativas, a tretinoína (forma ácida da vitamina A) e o licopeno (carotenóide sem atividade provitamina A) no contexto do fotoenvelhecimento e da fotocarcinogénese, respetivamente. A tretinoína está envolvida no controlo da diferenciação celular e proliferação em vários tecidos, especialmente no tecido epitelial da pele. Tem sido demonstrado que o fotoenvelhecimento pode ser tratado com retinóides tópicos, como é o caso da tretinoína, e que os seus efeitos a longo prazo, ao nível da pele, passam pela redução e redistribuição da melanina epidérmica, melhoria ultra-estrutural da epiderme, aumento das fibrilhas de ancoragem, aumento da deposição de colagénio da derme papilar e aumento da vascularização na derme papilar. Por outro lado, a atividade anticancerígena do licopeno foi já demonstrada em estudos *in vitro* e, *in vivo*, em diferentes modelos tumorais. Os mecanismos subjacentes aos efeitos inibitórios do licopeno ao nível da carcinogénese poderão envolver a captura de espécies reativas de oxigénio, a supra-regulação de sistemas de desintoxicação, a interferência com a proliferação celular, a indução da comunicação ao nível das junções intercelulares, a inibição da progressão do ciclo celular e a modulação de vias de transdução de sinal.

A veiculação dérmica de moléculas ativas constitui um tema que tem captado cada vez mais interesse e crescente investigação, uma vez que o risco de efeitos colaterais sistémicos é mais reduzido. Particularmente, no que se refere à veiculação de antioxidantes para a pele, a procura de um novo sistema de veiculação que mantenha a

estabilidade da molécula antioxidante e, simultaneamente, aumente a sua deposição na pele, abriu um novo capítulo na área de veiculação de fármacos. Uma vez que os lípidos epidérmicos predominam ao nível da barreira de penetração da pele (estrato córneo, EC), os nanotransportadores lipídicos administrados topicamente, que permitam a interação dos lípidos entre a camada mais externa do EC e o transportador, parecem ser uma abordagem promissora. De facto, a utilização de nanotransportadores, tais como lipossomas e ciclodextrinas, tem sido bem-sucedida ao nível da eficácia clínica de vários fármacos. Os lipossomas são tipicamente vesículas rodeadas por uma bicamada lipídica. Mais recentemente, foram concebidos e desenvolvidos lipossomas deformáveis capazes de atravessar a pele intata, promovendo a absorção percutânea de moléculas ativas e a sua veiculação para a circulação sanguínea. Para os diferenciar dos lipossomas convencionais, foram nomeados de vesículas deformáveis, tendo sido inicialmente introduzidos com a designação de transfersomas*. As membranas altamente flexíveis destas vesículas são o resultado da combinação numa única estrutura de fosfolípidos e um tensioativo, que contribui para a sua deformabilidade e capacidade de se moverem espontaneamente através da pele, libertando as moléculas a eles associadas dérmica e sistemicamente. As ciclodextrinas são polímeros cíclicos de hidratos de carbono não-redutores e solúveis em água. Alguns derivados, tais como β-ciclodextrinas e ciclodextrinas β-metiladas, têm sido geralmente utilizados em formulações tópicas.

Esta dissertação teve como **objetivo** a utilização de ciclodextrinas e transfersomas, ou a sua combinação, como sistemas de veiculação de tretinoína e licopeno e, posteriormente, o estudo *in vitro* e *in vivo* das formulações desenvolvidas.

A tretinoína foi incorporada em transfersomas (tretinoína-dimetil-β-ciclodextrina-transfersomas e tretinoína-transfersomas), posteriormente caracterizadas e usadas nos estudos de aplicação tópica.

A necessidade de obter licopeno de forma economicamente viável, relativamente à oferta do mercado, conduziu ao desenvolvimento de um processo simples e rápido de extração de licopeno a partir do tomate. Posteriormente, esse extrato rico em licopeno foi incorporado em nanotransportadores vesiculares adequados à aplicação tópica.

*Transfersome® é uma marca registada de IDEA AG, Munich, Alemanha

O licopeno tem sido descrito com um dos antioxidantes mais rapidamente eliminados na pele após a exposição à radiação solar, podendo por isso desempenhar um papel de proteção contra a radiação UV. Para testar esta hipótese, foram feitos estudos sobre os principais efeitos da radiação UV-B em queratinócitos humanos (linha celular HaCaT) previamente expostos ao licopeno.

Relativamente aos **métodos** utilizados, a complexação da tretinoína com ciclodextrinas foi obtida por malaxagem e os transfersomas foram preparados através da adição de fosfatidilcolina de soja ao Tween® 80, de modo a obter suspensões com concentrações lipídicas de 10 % a 20 %. A suspensão resultante foi filtrada até obter vesículas com um diâmetro médio de 100-150 nm. O licopeno foi obtido previamente por um processo de extração a partir de tomate seco, durante 30 minutos, à temperatura ambiente e sob proteção da luz. Os extratos de licopeno foram analisados por cromatografia líquida de alta eficiência e por espectroscopia de massa e incorporados em transfersomas e etossomas. A caracterização físico-química das formulações vesiculares foi baseada na avaliação dos seguintes parâmetros: diâmetro médio das vesículas e índice de polidispersão, medidos por espectroscopia de correlação fotónica e microscopia de força atómica; potencial zeta, determinado por anemometria laser-doppler; concentração de fosfolípidos, determinada por um ensaio enzimático-colorimétrico e doseamento do fármaco por cromatografia líquida de alta eficiência.

A aplicação tópica das formulações desenvolvidas envolveu diversos estudos, nomeadamente, os perfis de cedência e permeação, penetração na pele e alterações induzidas ao nível da barreira cutânea. A toxicidade das formulações finais foi testada *in vitro*.

O potencial de irritação da formulação vesicular de tretinoína foi avaliado *in vivo*, utilizando o teste de Draize modificado, e comparado com uma forma tópica comercializada de tretinoína. Em relação às formulações vesiculares de licopeno, foi avaliado o efeito terapêutico *in vivo*, utilizando o modelo de inflamação aguda cutânea,

induzido pela formação de espécies reativas de oxigénio após a aplicação de antralina na orelha de ratinho.

As condições de irradiação dos queratinócitos por UV-B, previamente expostos ao licopeno, foram determinadas através da realização do ensaio MTT. Após a exposição ao licopeno e irradiação UV-B, as células foram analisadas a fim de se obter informação sobre: genotoxicidade / clastogenicidade, através da análise do ciclo celular e de biomarcadores de apoptose e expressão genética, por citometria de fluxo e quantificação da reação da transcriptase reversa em tempo real, respetivamente; apoptose, através do ensaio da anexina V; danos causados pelo stresse oxidativo, através da quantificação de substâncias reativas de oxigénio por citometria de fluxo.

Os **resultados** referentes à formulação tretinoína-transfersomas revelaram que a eficácia de incorporação foi elevada para todas as condições testadas. No entanto, a formulação tretinoína-transfersomas com 20 % de concentração lipídica apresentou maior estabilidade química.

De acordo com os resultados dos estudos de libertação tópica da formulação tretinoína-transfersomas, as vesículas deformáveis diminuíram a resistência elétrica da pele, sugerindo a sua capacidade para induzir a rutura da barreira cutânea, tal como teoricamente seria esperado. Além disso, através da microscopia de fluorescência, foi possível observar uma maior concentração destas vesículas ao nível do EC e epiderme. No entanto, a tretinoína não foi detetada na fase recetora nos estudos de permeação. A maior concentração de fármaco a nível do EC, epiderme e derme é indicativa de veiculação dérmica, ao passo que a presença de fármaco na fase recetora é indicativa de veiculação transdérmica. Deste modo, os resultados demonstraram que se obteve uma veiculação dérmica com a formulação tretinoína-transfersomas, a qual serve o propósito terapêutico, uma vez que os recetores da tretinoína, tais como RAR-γ, estão localizados sobretudo na epiderme. Finalmente, esta formulação não revelou toxicidade em condições *in vitro* e *in vivo*, pelo menos, a 5×10^{-3} mg/mL e 0,5 mg/mL de tretinoína, respetivamente.

Em relação à extração do licopeno, o número reduzido de passos envolvidos neste processo pode ter melhorado o rendimento e exercido um papel benéfico na estabilidade deste carotenóide. Os resultados experimentais mostraram ainda uma incorporação eficiente do licopeno nas vesículas deformáveis. Além disso, tanto o licopeno extraído como o comercializado apresentaram valores reduzidos e semelhantes de citotoxicidade. As formulações vesiculares de licopeno extraído, posteriormente testadas *in vivo,* usando o modelo de inflamação aguda em orelha de ratinho induzida por antralina, promoveram a redução do edema tal como o controlo tratado com um fármaco modelo.

Nos estudos *in vitro* de fotocarcinogénese com queratinócitos irradiados com UV-B, foi possível observar que o licopeno reduziu a viabilidade destas células de acordo com os resultados obtidos nos ensaios de MTT e anexina V. Foi também observada a paragem do ciclo celular (na fase G0/G1 e um pico mais alto Sub-G1 apoptótico) e, ainda, um aumento do nível de espécies reativas de oxigénio.

Em suma, e de acordo com os resultados obtidos, a veiculação dérmica de fármacos pode ser conseguida com sucesso através da utilização de um nanotransportador. Foram ainda identificados, com sucesso, alguns aspetos relacionados com a atividade biológica das moléculas estudadas associadas a este tipo de transporte.

Palavras-Chave: Tretinoína; Licopeno; Nanotransportadores: Ciclodextrinas e Vesículas deformáveis; Veiculação Dérmica; Fotoenvelhecimento e Fotocarcinogénese.

Table of contents

Aims and Organization of the Thesis

The aim of this thesis was to contribute to the understanding of the mechanisms involved in the dermal delivery of active molecules mediated by lipid-based carriers. Thus, the proposed work "CARRIER-MEDIATED DERMAL DELIVERY FOR PREVENTION OR TREATMENT OF SKIN DISORDERS" includes the use of a similar strategy of delivering two entities, Tretinoin and Lycopene, with similar physicochemical characteristics with different therapeutic interests.

In addition to the evaluation of drug dermal delivery, chemical drug stabilization in the formulation was another objective addressed.

The thesis is organized as a collection of chapters, each one with a scientific paper format. The first chapter is introductory to the work, with two review articles containing the state-of-the-art. The following chapters have the structure of research articles. Format differences were kept, intrinsically, according to each journal requirements and some aspects of the introduction, materials and methods are repeated in the different chapters, being these repetitions needed for each research article to be an independent document.

Chapter 1.1 (***Tretinoin and Lycopene as Protectors against UV-Radiation: Photoaging and Photocarcinogenesis***) provides an overview of the role of tretinoin and lycopene for the treatment and/or prevention of skin disorders, such as photoaging and photocarcinogenesis, respectively. In fact, human skin is constantly exposed to the UV irradiation that may induce a number of pathobiological cellular changes. Tretinoin is currently marketed for topical treatment of acne *vulgaris* and has also been used for photoaging prevention and/or treatment by increasing the proliferation of keratinocytes on damaged skin. Lycopene was reported as the antioxidant most quickly depleted in skin upon exposure to solar radiation and may play a role on protecting skin against UV radiation. Lycopene has been implicated in reduced incidence of certain cancers and it was described to act as an antioxidant, act via gap-junction communications, modulate cell proliferation and present apoptotic properties.

Chapter 1.2 (***Topical Delivery of Antioxidants***) provides an overview of reactive oxygen species (ROS), antioxidants and its topical delivery by means of conventional and advanced delivery systems, including cyclodextrins, non-ionic surfactant vesicles, solid lipid nanoparticles and, in particular, liposomes and deformable vesicles. ROS and free radicals have been implicated in a number of diseases and disorders and the skin, for its localization, it is exposed to a large number of environmental insults. Free radical scavengers and antioxidants have thus been proposed as protective or therapeutic agents against ROS-mediated injuries. Topical delivery of antioxidants has gained increasing interest and development, especially by offering better targeting to the upper skin layers. However, topical delivery of antioxidants for dermal action is a challenging field of research since the molecules are, in general, susceptible to degradation. The search for a new delivery system that, simultaneously, preserves the antioxidant stability and enhances its deposition on the skin, opened a new chapter in drug delivery design. Nanocarriers have been successfully used in enhancing the clinical efficacy of several drugs. More recent approaches in modulating through-the-skin delivery led to the development of specialized nanoparticulated systems such as ultradeformable liposomes, cyclodextrins and the combination of both.

Chapter 2 is related to ***Dermal Delivery of Tretinoin Carrier Formulations***: Tretinoin-loaded ultradeformable vesicles or transfersomes, in which the drug is complexed or in the free form.

Chapter 2.1 (***Novel Tretinoin Formulations: a Drug-in-Cyclodextrin-in-Liposome Approach***) reports Tretinoin incorporation in special lipid vesicles. The ultradeformable systems used in this work comprised a mixture of soybean phosphatidylcholine and a surfactant, in a ratio that ensured adequate vesicle adaptability, drug loading and stability. Special attention was paid to maximize drug chemical stability.

Chapter 2.2 (***In Vitro and In Vivo Studies of a Novel Topical Tretinoin Formulation***) presents the releasing profile and skin permeation and penetration of Tretinoin-loaded ultradeformable vesicles. Skin electrical resistance was measured to determine the

changes in its barrier properties in the presence of the formulation. Finally, the evaluation of cellular viability and the potential of skin irritation were also considered.

Chapter 3 is related to the ***Dermal Delivery of Lycopene Carrier Formulations*** in which the extracted lycopene was incorporated into the carrier (transfersomes and ethosomes).

Chapter 3.1 (***Lycopene from Tomatoes: Vesicular Nanocarrier Formulations for its Dermal Delivery)*** presents the development of a rapid, simple and low cost extraction method of lycopene from tomatoes. Extracted lycopene was incorporated in deformable vesicles. The cellular uptake of deformable vesicles and the *in vivo* performance of lycopene formulations were also evaluated.

Chapter 3.2 (***The Effect of UV-B Irradiation on Human Keratinocytes after Lycopene Exposure***) deals with *in vitro* photocarcinogenesis studies in the presence of lycopene, using HaCaT cells to analyze genotoxicity/clastogenicity, apoptosis and oxidative stress.

This thesis ends with **Conclusions** and **Future Perspectives**.

In **Annex** (only in electronic version) to this thesis, there are three research articles published during PhD work period and resulting from Master Thesis work plan. Most of the studies published in these articles are the background of tretinoin experiments performed in the PhD thesis work.

This thesis was performed according to "**Despacho n.º 4624/2012, Diário da República, 2.ª série — N.º 65 — 30 de Março de 2012 and Deliberação n.º 1062/2008, *Diário da República, 2.º série — N.º 70 — 9 de Abril de 2008*".** I also declare that all experiments resulted from original research work.

Chapter 1

Introduction

Chapter 1.1 Tretinoin and Lycopene as Protectors against UV-Radiation: Photoaging and Photocarcinogenesis

Chapter 1.2 Topical Delivery of Antioxidants

Chapter 1.1

Tretinoin and Lycopene as Protectors against UV-Radiation: Photoaging and Photocarcinogenesis

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Tretinoin and Lycopene as Protectors against UV-Radiation: Photoaging and Photocarcinogenesis

Abstract

Background: The search for new anti-skin cancer drugs is particularly focused on natural compounds. Tretinoin (acid form of vitamin A) is involved in the control of cell differentiation and proliferation in several tissues, particularly in the skin epithelium. Lycopene (carotenoid with non vitamin A activity) has been proposed to play a critical role on anticarcinogenic action at different levels in both *in vitro* and *in vivo* tumour models.

Scope of the review: This review focuses on the effect of UV-radiation on skin, and the photoprotection conferred by two different active molecules: tretinoin and lycopene against photoaging and photocarcinogenesis, respectively. Regarding this context, skin cancer (non-melanoma and melanoma cancer) is addressed as well as other current therapeutic strategies.

Major Conclusions: UV irradiation may induce a number of pathobiological cellular changes, including photoaging and photocarcinogenesis. Tretinoin can be used for photoaging treatment by different mechanisms, including the inhibition of the activated protein-1 and blocking dermal matrix degradation followed by sun exposure. The photoprotective properties of lycopene remain unclear. Some studies point out a positive and others a negative effect both in *in vitro* and *in vivo* models. Currently, researchers recognize that crucial gaps endure in our understanding of the role of carotenoids as effective modulators of apoptosis, cell cycle dynamics and/or of their *in vivo* behavior as cellular antioxidants. On other hand, it remains to confirm if these compounds, supplied through a suitable diet combining different natural antioxidants, contribute to basal protection and improve skin defense against UV light-mediated damage to skin.

General Significance: The development of novel preventive and therapeutic strategies for skin disorders depends on our understanding of the molecular mechanism of UV damage on skin cells. The use of several effective phytocompounds, working through different (preventive and/ or corrective) pathways in the cell, may be an effective approach for reducing UV-B generated damage mediated photoaging and photocarcinogenesis.

Keywords: Photoaging; Photocarcinogenesis; Lycopene; Tretinoin

Introduction

The effects of solar ultraviolet radiation (UVR) on most living organisms and, particularly, on human health are well known. Some of the beneficial and most detrimental effects of UVR on human health have been recently reviewed by Norval *et al*. (1). In order to cope with the consequences of UVR exposure, both plants and animals possess various protective small molecules and enzymatic defences such as the families of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). If the concentration of free radicals increases significantly and reaches a critical level, the generated oxidative stress can destroy cells and cell compartments and, consequently, origin premature **skin aging** and also **skin cancer** (2).

The search for new anti-cancer drugs is particularly focused on natural compounds, such as botanical antioxidants, available in the regular human diet. These compounds efficiently regulate a wide range of cellular pathways involved in carcinogenesis, and rarely exhibiting severe side-effects (3). **Botanical antioxidants** can be divided in different chemical groups, such as: polyphenols, phenols, flavonoids, isoflavonoids, phytoalexins, anthocyanidins and carotenoids. They can work in preventive ways (sunscreen and antioxidant effects) and/or in corrective ways (redox regulation of signal transduction pathway) (4, 5). The use of effective phytocompounds in skin care products may be an approach for reducing UV-B generated reactive oxygen species (ROS) mediated photoaging and photocarcinogenesis (4).

Carotenoids are protective pigments at the complex I and II in plants photosynthetic systems, and are in abundant quantities in several crops (6, 7). Carotenoids defence properties in human cells may involve their potent antioxidant activity and their ability to induce cellular protective responses (8). In fact, the influence of carotenoids and/or their metabolites on the expression of certain genes and on the inhibition of regulatory enzymes has already been discussed regarding its cancer preventive properties (9).

In this review, a special focus will be given to the two carotenoid related compounds tretinoin and lycopene in the context of their protective effects of photoaging and photocarcinogenesis, respectively.

Tretinoin is a natural-occurring acid of retinol, an active metabolite of vitamin A, and is an agent involved in the control of cell differentiation and proliferations in several tissues, particularly in the skin epithelium. It binds to and activates retinoic acid receptors (RAR), inducing changes in gene expression that leads to cell differentiation, decreased cell proliferation, and inhibition of tumourigenesis. It has been demonstrated that photoaging resulting from UV-B radiation can be treated by retinoid formulations. Pretreatment of human skin with tretinoin blocks dermal matrix degradation (followed by sun exposure) inhibiting the induction of the activated protein-1 (AP-1) transcription factor and AP-1 regulated matrix-degrading metalloproteinases (MMP). Tretinoin is largely used in cosmetics, and is commercially available worldwide under several brand names.

Lycopene is a non-provitamin A carotenoid that is responsible for the red to pink colors seen in tomatoes, pink grapefruit, and other foods (10). Lycopene has been recognized as the most effective singlet oxygen quencher among carotenoids (11) and it is currently receiving considerable scientific attention (3). Epidemiological, tissue culture, and animal studies provide convincing evidence supporting the role of lycopene in the prevention of chronic diseases. Human intervention studies are now being conducted to validate epidemiological observations and to understand the mechanisms of action of lycopene in disease prevention (12).

The anticancer activity of lycopene has already been demonstrated in both *in vitro* and *in vivo* tumour models (13). The inhibitory effects of lycopene on carcinogenesis may be due to different mechanisms, such as: ROS scavenging, upregulation of detoxification systems, interference with cell proliferation, induction of gap-junctional communication (GPC), inhibition of cell cycle progression and modulation of signal transduction pathways (13). Some of these mechanisms will be discussed in this review within the photocarcinogenesis context.

1. UV-Radiation and its Effects on Skin

Although both the environment and genetics may play a role in the development of skin cancer, UVR is considered to be one of the most efficient skin carcinogens and mutagens as well as immunosuppressive agent. The UV spectrum is composed by: UV-C (200-280 nm), UV-B (280-320 nm) and UV-A (320-400 nm) (**Table 1**). The UV-C has the higher energy radiation, but it is (almost) blocked by the ozone layer and the UV-B and UV-A represent about 5 % and 90 % of UVR, respectively. Although there are beneficial effects of UVR on skin, such as the generation of vitamin D and therapy of some skin diseases (atopic eczema, psoriasis), there are also acute and chronic harmful effects especially if the UVR exposure is too prolonged. The main effects of acute exposure include "sunburn" or erythema, acanthosis (i.e. increase of epidermal thickness), desquamation, immunosuppression (increase of interleukins IL-10 and IL-12) and immediate pigmentation darkening. The effects of chronic exposure are the solar elastosis (i.e. the accumulation of elastin in the superficial dermis), degradation of collagen and skin cancer (4, 11).

UV-A and UV-B radiation can cause photoaging and photocarcinogenesis through lipid peroxidation, protein cross linking and DNA damage. (14).

Table 1 - Global Solar Spectral Irradiance measured at sea level (15).

Wavelength (nm)	Domain	Energy (W/m^2)	%
280-320	UV-B	~ 4-2	~ 6.8
320-400	UV-A	~ 72-50	
400-780	Visible	~ 580-660	~ 55.4
780-1400	IR A	~ 410-430	~ 37.8
1400-3000	IR B		

The **minimal erythema dose** (MED) is the threshold dose required to cause a perceptible reddening of the skin 24 h after exposure. The estimated MED narrow band-UV-B radiation in north Indian patients varied from 500 to 1100 mJ/cm^2 (16). However,

MED values differ among individuals and depend on the endogenous protection provided by melanin and on the **skin type** which is usually categorized according to the Fitzpatrick scale (type-I to IV). Skin type I corresponds to people with white or freckled skin, green or light –blue eyes, red hair and high sensitivity to sun light; skin type IV shows black skin, dark brown eyes and black hair, rarely experiencing sunburn. The repair of UVR skin damages occurs within 24 h only if the irradiation dose is ≤ 60 % of the MED (17, 18). The **sunburn** starts to develop few hours after irradiation, worsening about 18-24 h post-irradiation. When UVR exposure causes irreversible cell damage, cell death follows via apoptotic mechanisms, leading to the appearance of sunburn cells in the epidermis layer (18, 19).

The skin has **natural defenses** against the damaging effects of UVR, such as: (1) The SC that absorbs, reflects and scatters UV rays; (2) skin pigmentation: melanin absorbs and scatters UVR, and is a free radical/ROS scavenger; (3) naturally occurring antioxidants such as lipophilic carotenoids, alpha-tocopherol (or vitamin E), glutathione and ascorbic acid (or vitamin C); (4) antioxidant enzymes such as CAT, SOD and GPx; (5) DNA repair mechanisms and (6) epidermal hyperplasia and hyperkeratosis that increase the physical barrier to UVR (20, 21).

The importance of the skin's natural defenses against the damaging effects of UVR can be observed in several clinical syndromes, such as Xeroderma Pigmentosum and Oculocutaneous Albinism (20).

1.1 Photoaging and Photocarcinogenesis

Aging is a complex process in which several mechanisms operate simultaneously. These mechanisms may include accumulation of genome mutations and toxic metabolites, hormonal deprivation, increased ROS, and cross-linking of macromolecules by glycation (22). '**Photoaging'** or '**extrinsic aging'** is the process by which sunlight or artificial UVR gradually induces clinical and skin histological changes (20). On one hand, UV-A radiation is considered to be the main cause for premature skin aging (23) because it corresponds to the major percentage of total UVR, and has a higher depth of

penetration into the dermis (24). On the other hand, UV-B-induced DNA damage leads to MMP-1 induction, the main enzyme responsible for collagen 1 digestion (25). **UVR-induced cutaneous alterations** may be characterized by dryness, rough texture, irregular pigmentation (hyper or hypomelanosis), yellowish color, telangiectasia, plaque-like thickening, deep creases and wrinkles. These skin changes may vary considerably among skin types (20, 26).

Skin Histological changes (**Table 2**) due to chronic UVR exposure may involve both epidermis and dermis layers, and ultimately, to malignant skin cells transformation (20).

Table 2- UVR-induced Histological Changes in Epidermis and Dermis Layers (adapted from (20, 24, 26))

Epidermis	Dermis
• epidermal hyperplasia or atrophy	
• disappearance of dermal papillae	• elastosis
• thickening of the basement membrane	• presence of deformed collagen fibers
• ↑ and irregular distribution of melanocytes and melanosomes	• ↓collagen
• atypical proliferation of keratinocytes (solar keratosis)	• dilated blood vessels
• ↓ naturally occuring antioxidants (↑ROS)	• ↑ Matrix Metalloproteinases (MMPs)
• thickening of *Stratum Corneum (SC)*	

Solar Elastosis is clinically manifested as yellow discoloration and pebbly surface of the skin, being a prominent characteristic of photoaging. The dermis displays accumulation of elastotic material in the mid and upper layers. In addition, glycosaminoglycans and proteoglycans increase, while the amount of collagen decreases in photodamaged skin. UVR-induced collagen degradation is usually incomplete, contributing to the accumulation of partially degraded collagen fragments in the dermis that may interfere with skin integrity. Besides, the large collagen degradation products inhibit new collagen synthesis. In addition, there is also a cellular accumulation of lipofuscin related to chronological aging that further inhibits proteasome function (24).

UVR may also disrupt the telomere loop, expose the TTAGGG overhang, and promote aging. In fact, intrinsic aging is accompanied by repeated cell divisions that shorten telomeres (24).

Photodamaged skin generally presents an increased number of hyperplastic fibroblasts as well as inflammatory cells. This chronic inflammation in photoaged skin corresponds to **heliodermatitis**. Perivascular veil cells may display dilations (**telangiectases**) in severe photodamaged skin. UVR has also been involved with the **immunosuppression** system which may have implications in cutaneous tumour surveillance (24, 27). Rijken (20) believes that neutrophils may play an important role in the pathophysiology of photoaging.

The **oxidative stress** generated by UVR in keratinocytes and fibroblasts can modify proteins forming carbonyl derivatives which accumulate in the papillary dermis. In addition to their oxidative effects on lipids and proteins, ROS also attack nucleic acids, mainly nuclear and mitochondrial DNA. The 8- hydroxyl-2´-deoxyguanosine (8-OHdG) is a representative DNA base –modified product generated by ROS and is considered as one of the most important biomarkers of carcinogenesis. It induces G-C $\rightarrow$ T-A transversions during DNA replication. The transversions residual presence in DNA of normal and neoplastic tissues results from the imbalance between oxidative attack and DNA repair (28). Upon DNA repair, 8-OHdG is excreted in the urine. According to the European society of pigment research (29), a strong generation of 8-OHdG was related with the proliferation of melanocytes.

Overall, the increase of ROS levels may regulate a cascade of signal transduction, including the involvement of Mitogen-activated protein kinase (MAPK) pathways, and leading to the up-regulation of the activator protein 1 (AP-1) and of the Nuclear Factor (NF-kB), and to the down-regulation of the Transforming Growth Factor (TGF-β). While NF-kB regulation increases interleukin-1 family (IL-1) and Tumour Necrosis Factor (TNF-α) levels, the AP-1 up-regulation activates MMPs. On the other hand, the decrease of TGF-β expression decreases the synthesis of collagen. Ultimately, the combination of these changes lead to the increase of collagen breakdown and elastin production in extracellular matrix and, therefore, to matrix degradation (**Fig.1**) (30).

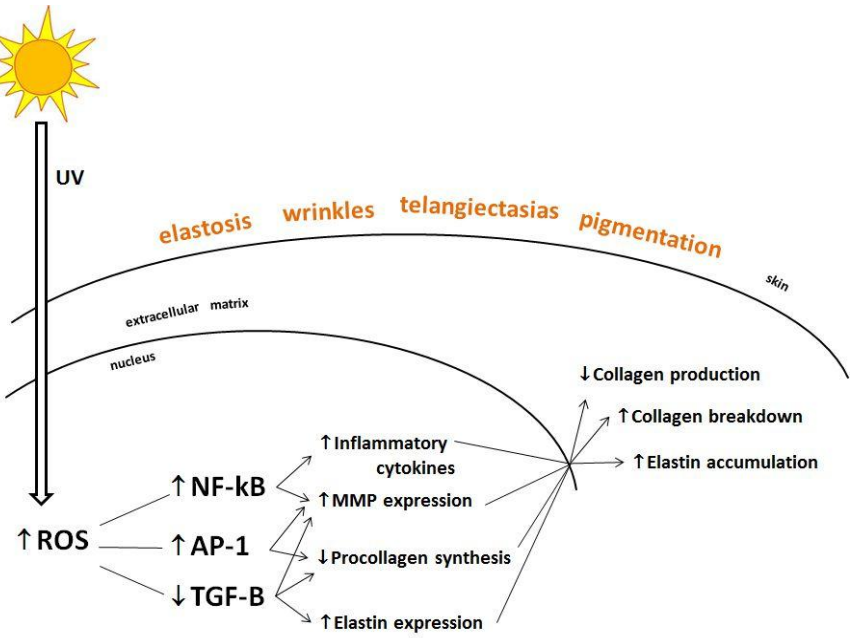


Fig.1- Effects of ROS increase on transcriptional regulation of some important proteins in the skin matrix, involving NF-kB, AP-1 and TGF-β pathways. Adapted from Chen *et al.* (30)

Photocarcinogenesis is a complex multistage phenomenon involving three distinct stages exemplified by initiation, promotion and progression. Each of these stages involves biochemical and molecular alterations in the cell **(Fig.2**).

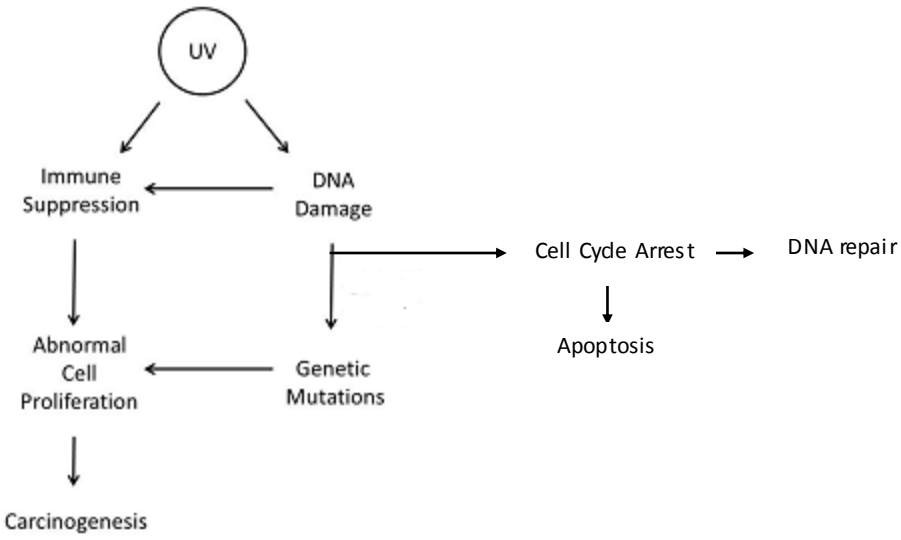


Fig.2- Brief overview of the main cell mechanisms involved in photocarcinogenesis. Adapted from Matsumura & Ananthaswamy (31).

Keratinocytes apoptosis contributes to the regulation of epidermal development and carcinogenesis restrain. The **extrinsic pathway** (via cytoplasm) is stimulated by UV-B or binding of Fas ligand, tumor necrosis factor, or other cytokines to death receptors that results in the activation of a caspase cascade. The **intrinsic pathway** (via mitochondria) is also stimulated by UV-B and involves mitochondrial depolarization and higher membrane permeability, leading to the release of multiple proapoptotic factors including cytochrome c, Smac and the apoptosis inducing factor (AIF) (31). Bid protein links both extrinsic and intrinsic pathways, and the activated Bid fragment will cause the mitochondrial content release. Activation of upstream caspases (caspase-8 or -9) leads to the activation of downstream caspases (caspase-3 or -7) resulting in the cleavage of intracellular substrates, cellular condensation and nuclear fragmentation. Apoptosis inhibitors include caspase inhibitors and the big complex Bcl-2 family proteins, most of which prevent mitochondrial membrane permeability (32).

Caspase-14 is a keratinocyte-specific caspase only found in skin. It was only expressed in HaCaT cells and normal human keratinocytes under culture conditions mimicking terminal differentiation. In addition, caspase-14 could not be induced by death receptors signaling or UV-B irradiation. Although the targets of activated caspase-14 have not been defined yet, it seems to correspond to focal epidermal hyperplasia (32).

In general, the **process of skin cancer development** involves many different cellular mechanisms and events, as synthetically indicated (4, 33-35):

• abnormal stimulation of DNA synthesis and unability to repair DNA damages. A major reason of these abnormal events is the frequent mutation of the suppressor tumour p53 gene, which protects cells from DNA damage through repair mechanisms or apoptosis of damaged cells;

• formation of pyrimidine photodimers in nucleic acids and activation of the ataxia telangiectasia mutated family proteins of keratinocytes;

• deregulation of apoptosis leading to abnormal proliferation of keratinocytes, inflammation, epidermal hyperplasia and immunosuppression (increase of IL-10, IL-12);

• activation of proto-oncogenes (e.g. Ras);

• cell cycle deregulation;

• increase of ROS levels and depletion of antioxidant enzymes activity;

• impairment of signal transduction pathways and induction of ornithine decarboxylase (ODC) activity (an important enzyme linked to the development of skin tumors) and of cyclooxygenase-2 (COX-2, an enzyme involved in inflammation process);

• photoisomerization of *trans* to *cis*-urocanic acid, which acts as an endogenous sunscreen against UV-B.

Exposure to UVR also causes alterations in the morphology and function of antigene-presenting Langerhans cells (36), release of immunosuppressive cytokines (37), and enhanced prostaglandin synthesis (38). In fact, Langerhans cells and dendritic cells of lymphatic system are the major cellular chromophores absorbing UV-B radiation (25).
Excessive UVR leads to calcium increase in keratinocytes, resulting in the activation of the inflammasome (a multiprotein of the innate immune complex) and in the synthesis and release of IL-1, which can activate the synthesis of other proinflammatory cytokines (8, 39). IL-10 appears to be a key mediator of UVR-induced immunosupression (8, 40), while IL-12 was found to accelerate the removal of UVR-induced DNA lesions in keratinocytes by inducing nucleotide-excision repair. These findings strongly suggest that it plays a protective role in photocarcinogenesis (8, 41). In addition, UVR induces the synthesis of granzyme B and perforin in keratinocytes (proteins only present in cytotoxic lymphocytes and natural killer cells), leading keratinocytes cytotoxic to

transformed cells. This cellular transformation strongly suggests that UV-irradiated keratinocytes participate in skin cancer surveillance (24).

Abnormal **DNA methylation** is a hallmark of most cancers. It is a marker of epigenetic events, i.e. susceptible to change with environmental factors, and a fundamental process that modulates gene expression and regulates the chromosomal stability. Hyper or hypomethylation in G-C rich regions can contribute to carcinogenesis by silencing tumour suppressor genes (e.g. p53), upregulating oncongenes at dipyrimidine sites and by decreasing genomic stability. The maximum absorption of DNA purine and pyrimidine bases lies between 230 and 300 nm. Therefore, DNA is a major UV-B-absorbing cellular chromophore in the skin The major types of DNA photoproducts induced by carcinogenic UV-B are: cyclobutane pyrimidine dimmers (CPD) and pyrimidine (6-4) photoproducts (33). The primary response to DNA photodamage is the photolesions repair in surviving cells. Different repair pathways become active, such as: direct repair, base excision repair, mismatch repair, double strand break repair, and nucleotide excision repair. The latter is considered to be the major line of defense against carcinogenesis. Phosphorylated p53-induced transcription of p21 causes G/S cell cycle arrest, allowing the cellular repair pathway to remove DNA lesions before DNA synthesis and mitosis (25). However, if the damage persists into the S-phase, other repair mechanisms might lead to mutagenesis resulting mainly in characteristic C→T substitutions. When such mutations occur in the p53 gene, keratinocytes lose their ability to undergo the apoptosis process following UVR exposure. The degenerative changes in keratinocytes include mitochondrial swelling and rupture, condensation of the cytoplasm and the appearance of pyknotic nuclei. Macrophages bind and phagocytise apoptotic keratinocytes and their number increase significantly in the skin after UV-B exposure (5, 8, 33, 42, 43). Chemopreventive agents may be used to correct aberrant methylation patterns and restore the control of tumour cells growth and/or against UVR side effects (43).

Several strategies are applicable for **photoprotection** and subsequent impairment of molecular and cellular functions. Behavioral changes (avoidance of sun exposure and protective covering) and sunscreens with a high sun protection factor are highly recommended during times of intense exposure. In order to increase the barrier for UVR, the compound should absorb UVR over a broad range of wavelength with high efficacy, and sufficient photostability is also required. Topically applied organic (previously called chemical) and inorganic (previously called physical) **sunscreens** protect by absorbing or reflecting radiation at the skin surface, respectively. Organic sunscreens are usually "not visible" and cosmetically appealing. However, the first-generation of organic sunscreens were quite unstable and reactive. Allergic sensitization could occur due to UVR absorption which could lead to the interaction of the suncreen with cutaneous molecules. Inorganic sunscreens contain 10–100 nm particles (eg. zinc oxide or titanium), and are chemically inert, and thus, not potentially allergenic (24). Nanoencapsulation of organic UV filters is a more recent approach to improve skin retention, photostability and the UV blocking ability of the free molecules (25).

Compounds that interfere with stress-dependent signaling are demanded for the induction of repair systems dealing with UVR-induced damage (11). Furthermore, DNA damage repair enzymes (enzyme T4 endonuclease V; photolyase; oxoguanine glycosylase 1; thymidine nucleotides); antioxidants and dietary lipids; iron quelators (eg. Kojik acid); osmolytes; retinoids (tretinoin); fluorouracil, imiquimod, lipospondin; chemical peels (eg. α-hydroxy acids), dermabrasion, photodynamic, laser and radiofrequency therapies (44), injectables (eg. botulinum toxin and hyaluronic acid), and surgical procedures have also been proposed as strategies for the prevention and/or treatment of photoaging (20, 24). Another approach is related to the induction of melanin production without sun exposure. Although the regulation of the tanning response is complex and only partially elucidated, current facts suggest that UVR-induced DNA damage or its repair is, at least, one of the initial signals that stimulates melanogenesis (24). Gilchrest *et al.* (45) showed that administration of the repair enzyme T4 endonuclease V to irradiated melanoma cells increased both their rate of

repair and UVR-induced pigmentation. These data also suggest that tanning can be stimulated through enhanced DNA repair. Other approaches for increasing skin pigmentation are based on telomere homologue oligonucleotides, Forskolin and α-melanocyte-stimulating hormones analogues (24).

Finally, natural substances extracted from herbs (polyphenols, phenolic acids, flavonoids, carotenoids) can acts as other potential photoprotective resources due to their UVR absorbing and antioxidant properties (46). However, their photoprotective effect is not itself comparable to the use of a sunscreen (7).

2. Skin Cancer

Skin cancer remains one of the most common human cancers and, despite educational efforts, it shows a high incidence which has a tremendous impact on public health and healthcare expenditures (47). Although both the environment and genetic components play a role in the development of skin cancer, UVR is considered to be one of the most efficient skin carcinogenic and mutagenic agents, besides being an effective immunosuppressive agent (48).

The risk of skin cancer is expected to increase as the population ages and larger amounts of UVR reach the surface of the Earth because of depletion of the stratospheric ozone (43, 49). Although keratinocytes are resistant to UVR- induced damage, repeated exposure results in accumulated DNA mutations that can lead to skin malignancies. Therefore, it is desired to develop newer and effective chemopreventive agents and strategies, which can inhibit or slow down the UVR-induced risk of melanoma and non-melanoma skin cancers (**Fig.3**) especially among high-risk human populations (43). Preventive strategies including personal behavioral modifications and public educational initiatives are considered to be both life-changing and life-saving (50).

Fig.3 shows the fate of the different solar UVR components. The specific DNA lesions produced by UV-A and UV-B in this range of wavelengths include the production of various types of DNA photoproducts (33).

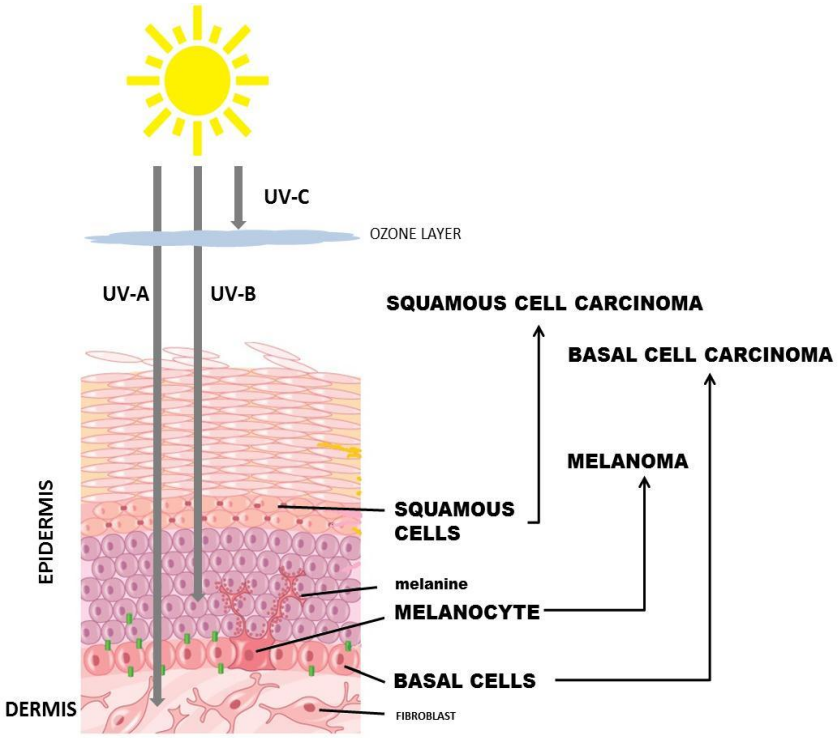


Fig.3- Exposure to UV radiation and development of melanoma and non-melanoma skin cancers. UV-A radiation reaches the dermis and to some extent also the subcutis, whereas UV-B does not pass beyond the epidermal layer.

The importance of epigenetic events is that it represents a mechanism by which gene function is selectively activated or inactivated. Because epigenetic events are susceptible to change, they represent excellent targets to explain how environmental factors, including dietary constituents, supplements, chemopreventive agents may modify cancer risk and tumour behaviour (43, 51).

The formation of skin cancers is a biological process much more complex than narrow scientific aspects that are individually and, sometimes, independently investigated. Recent studies have focused the investigation on the regulation of apoptosis in the skin and its application for understanding skin carcinogenesis (32). Acute UV irradiation causes apoptosis involving p53 and Fas-FasL pathways (FasL, a member of TNF superfamily involved in the elimination of apoptotic cells and prevention of cell transformation). Chronic UV irradiation results in deregulation of apoptosis, leading to the abnormal proliferation of photodamaged keratinocytes, acquisition of p53

mutations, loss of Fas-FasL interaction besides decreased death receptors, Stat3 activation and variation of Survivin, Bcl-2 and Bcl-X_L expression. All of these events contribute to the onset of skin cancer (25, 32). Dysfunctional apoptosis occurring in skin cancer has been studied and gene silencing by RNA interference may become a new approach to reverse apoptosis resistance (52) as it has been successful in tissue cultures and tumor tissues, as well as, in a mouse model (53).

The current **tools for measuring cumulative sun damage** are biopsy histology and skin microtopography. Nevertheless, skin biopsies are too invasive and skin replicas provide only superficial skin architecture data. Reflectance confocal microscopy is a noninvasive imaging tool that allows *in vivo* skin imaging (54). The Raman spectroscopy is well suited for the screening of cancer patients prior to chemotherapy, to detect high-risk patients, who can be successfully treated with topical antioxidants, thus preventing the occurrence of palmar–plantar erythrodysesthesia (PPE), a dermal side effect of chemotherapy (2).

2.1 Non-Melanoma Cancers

The non-melanoma skin cancers (NMSC), **basal cell carcinoma** (BCC) and **squamous cell carcinoma** (SCC) are the most frequent cutaneous malignancies and represent around 80 % and 16 % of all skin cancers, respectively, whereas malignant melanomas represent only about 4 % of all skin cancers (55). Both BCC and SCC are derived from the basal layer of the epidermis. SCC especially affects people over 40 years of age who sunburn easily without tanning, and people who are immunosuppressed (55). BCC doesn't metastasize but can be locally invasive and destructive. On the contrary, SCC is an invasive non-melanoma cancer and can metastasize. Dermoscopy improves the diagnosis of NMSC at different stages of progression (55).

The combination of genetic factors usually with long term sun exposure contribute to the formation of these cancers, being UVR responsible for the induction of DNA damage in keratinocytes (56). However, UVR damaged DNA may be repaired or DNA damaged

cells undergo elimination process via an apoptotic mechanism (57). It is currently considered that deregulated cell proliferation and apoptotic pathways lead to the development of cancer. Therefore, it appears that exploiting the apoptotic potential of cancer cells might lead to new therapies that could be less toxic to normal cells due to their physiologically controlled survival pathway.

The most significant pathogenic event in BCC is considered the mutations in the Hedgehog pathway related genes, especially *PTCH,* a human homologue of the patched gene in *Drosophila melanogaster* (25). In general, BCC have reduced expression of Bax and increased expression of Bcl-2, while Bcl-X_L is overexpressed in SCC. However, a relatively higher apoptotic rate in BCC may account for the slow growth of clinical lesions. In SCC, expression of Survivin, Bcl-2 and Bcl-X_L is associated with metastasis or poor prognosis. Progression of SCC is also associated with constitutive activation of keratinocytes survival signaling pathways (32).

Prevention of SCC is directly achievable through sunscreen protection (58) being dietary factors and nonsteroidal anti-inflammatory drugs potential second-line preventions (59). By targeting different pathways identified as important in the pathogenesis of NMSC, an approach combining multiple agents (with different targets in the cell) or the addition of chemopreventative agents to topical sunscreens may offer the potential for novel and synergistic therapies in preventing and/ or treating NMSC (60). Recently published data showed the failure of topical tretinoin in chemoprevention of NMSC (61). Photodynamic therapy (PDT) with topic 5- aminolaevulinic acid was used in NMSC therapy as a non-invasive therapy with possibility of treatment for multiple lesions in only one session (62). However, the recurrence rate increase after drug-PDT diode laser single session can be observed in a long-term follow-up, and the repetitive sessions is strongly recommended (62). Other potential therapies could include the introduction of p53, bortezomib (proteasome inhibitor) and stat3 decoy (32).

2.2 Melanoma Cancers

Cutaneous malignant melanoma arises through the interplay of both environmental and genetic factors and is highly invasive. This type of skin cancer is capable of metastasizing to distant sites. Melanoma is thought to develop starting with a benign nevus (63). The propensity to develop nevi is genetically determined. For the skin with low propensity, repeated and cumulative exposure to the sun is required for melanoma development. However, in people with high nevus propensity, even short exposure to the sun could lead to melanoma development (64). The role attributed to the intermittent sun exposure in the genesis of most melanoma and BCC in the case of sunbeds has been also discussed (65). Patients with advanced melanoma with dissemination to distant sites and visceral organs have a very poor prognosis. PDT for patients with skin metastases from melanoma only works in 20 −30 % of these patients (66). Melanoma research on the mechanisms by which UVR initiates this cancer are needed to improve prevention strategies and have been investigated in mouse models (67). Eggermont & Robert (68) reviewed the later 40 years of lack of progress on melanoma treatment. However, in the last decade, great advances have been made, both in terms of targeted drugs that kill melanoma cells and in terms of host immune system modulating drugs, which use however still remains under discussion (69, 70).

A new development in 2011 in melanoma adjuvant therapy resulted with interferon IFN-alpha2b on the basis of the ECOG 1697 trial which found no difference in recurrence-free or overall survival (71). In March 2011, pegylated IFN-alpha2b (PegIntron®) was approved by the Food & Drug Administration (FDA) for the treatment of stage III melanoma based on the result of the EORTC 18991 trial, which found that PegIntron® induced a significant increase in recurrence-free survival in patients with stage III melanoma, with lymph-node-positive melanoma (72, 73). Two other adjuvant therapy trials, the EORTC 18071 and DERMA trial are completed. The importance of ulceration and IFN sensitivity in adjuvant trials have led to the EORTC 18081 trial, in course, in patients with ulcerated primary melanomas to prospectively investigate IFN sensitivity (68). A current trial, ECOG 1609, is comparing treatment with Yervoy™ (Ipilimumab) against standard high-dose IFN-2b. Ipilimumab, anti-Cytotoxic T

Lymphocyte Antigen 4 (CTLA-4) monoclonal antibodies treatment induces low response rates but durable responses (74). Vemurafenib (Zelboraf® from Roche) was the first FDA approved treatment for the B-Raf proto-oncogene (BRAF) mutation-positive metastatic melanoma and it has been associated with high response rates in up to 70 % of patients (55). The combination of the BRAF inhibitors with MAPK/ERK kinase protein inhibitors have fewer side effects and induced higher response rates. However, resistance to the drugs was developed and relatively short periods of response were achieved (55).

In conclusion, new drugs and probable combinations are under investigation and substantial improvement in survival from melanoma is expected from the trials in course.

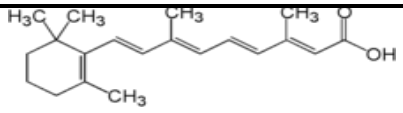
3. Photodamage Skin Care Agents

3.1 Tretinoin

3.1.1 Physicochemical Properties

Tretinoin, also known as all-*trans*-retinoic acid (ATRA), is a naturally occurring derivative of vitamin A (retinol). The 13-*cis* isomer is called isotretinoin. Tretinoin belongs to the first generation of non-aromatic retinoids. The physico-chemical properties of tretinoin are represented in **Table 3**. There are two sources of dietary vitamin A. Active forms, retinaldehyde and retinol, are obtained from animal products. Precursors or provitamins, which are converted to active forms by the body, are obtained from fruits and vegetables containing yellow, orange and dark green pigments, known as carotenoids (75).

Table 3- Physicochemical properties of Tretinoin (75-78).

Molecule	**Tretinoin**
Molecular Structure	
IUPAC Name	(2E,4Z,6E,8E)-3,7-Dimethyl-9-(2,6,6-trimethylcyclohex-1-en-1-yl)nona-2,4,6,8-tetraenoic acid
MF	$C_{20}H_{28}O_2$
MW (g/mol)	300,44
Physical state	yellow to light-orange crystalline powder
Solubility	Insoluble in water (20 °C); Soluble in chloroform; slightly soluble in ethanol
Log P	~ 4-5
Melting Point (°C)	180 – 181
Stability	Stable only under ordinary conditions. Sensitive to air / light and heat, especially in solution

3.1.2 Bioavailability and Metabolism

The human cells convert precursors to retinol, and then the de-esterified alcohol can be converted to metabolically active retinal (the aldehyde of retinol) and retinoic acid (the oxidized form of retinol) formed by carboxylation of retinal. The steps of metabolism, especially the inactivation of the retinoids, are not yet fully understood (77).

When applied on skin, all forms of *trans*-retinoic acid isomerize partly on the epidermis in 9-*cis* and 13-*cis*-retinoic acid, among other metabolites. Approximately 80 % of retinoic acid remains on the skin surface, and it penetrates depending on the type of vehicle formulation (79).

3.1.3 Safety

The collateral effects of topical administration of tretinoin include redness, desquamation, dryness, and burning tingle (80). The transdermal penetration and systemic bioavailability of topical retinoids are not yet completely clarified. Despite its short half-life, some authors defend that overexposure to tretinoin may cause teratogenesis and embryotoxicity (81, 82). However, there is not a consensual opinion about the use of topical retinoids during the pregnancy (83).

3.1.4 Biological Activity and Pharmacological Action

The retinoic acid receptors are **α, β and γ -RAR** and retinoid X receptor (**RXR**), and the cytosolic skin binding proteins are the **CRABP** (cellular retinoic acid binding proteins). This connection promotes various action mechanisms, such as normalization of proliferation and differentiation of the epidermis, decrease of prostaglandins, leukotrienes and cytokeratins release and inhibition of neutrophil chemotaxis (84). An alternative pathway may be via **aquaporin type 3** present in keratinocytes, facilitating the movement of water and glycerol through the skin (79, 85).

Retinoids, including tretinoin, are important regulators of cell proliferation and differentiation, and are mainly used to treat **acne** and **photodamaged skin** and to manage **keratinization disorders** such as ichthyosis and keratosis follicularis.

Retinoids have also been used as **chemopreventive and anticancer agents** because of their pleiotropic regulator function in cell differentiation, growth, proliferation, DNA repair and apoptosis. Although oral retinoids have not been effective for chemoprevention of skin cancer in the general population (86), the use of topical retinoids as chemopreventive agents has yielded variable results, some positive (87) others not (51, 60, 61, 88). However, according to Shimizu *et al*. (89) ATRA can suppress the process of carcinogenesis both *in vitro* and *in vivo*, especially in the treatment of various cancers, including SCC. Tretinoin represents the class of anticancer drugs called differentiating agents and is used in the treatment of acute promyelocytic leukemia (90). Roméro *et al.* (91) have demonstrated *in vitro* that retinoic acids are potent inhibitors of UV-B induced melanogenesis via tyrosinase related proteins (TRP-1 and TRP-2) expressions.

3.1.4.1 Acne

Retinoids are considered the **first line treatment for acne**, being also used in **maintenance therapy**. These drugs cause the desobstruction of the pores, preventing the formation of white spots, which lead to an **anti–inflammatory** and **comedolytic action**. Also, it is usual the topical combination of antibiotics with retinoids to treat comedogenesis, bacterial growth and inflammation. This combination also increases the effectiveness and tolerance to the treatment. On the other way, benzoyl peroxide can be used in combination with topical retinoids to reduce the dose of antibiotic. It is important to use daily solar protection during the treatment due to the skin sensitization to the solar exposure (92, 93).

The currently commercial available formulations include 0.025-0.1 % of tretinoin topical creams or gels. However, new sustaining release systems are emerging, in which the

active substance is vehiculated in microsponges or polymers in order to remain at the SC, the outermost layer of the skin, thus promoting comedolisis and modulation of the keratinocytes proliferation (94). Recently we have developed a new formulation of tretinoin-loaded ultradeformable vesicles, which revealed to be a promising delivery system for tretinoin dermal delivery without promoting skin irritation that is usually observed in currently available commercial formulations (95).

3.1.4.2 Photoaging

Tretinoin is also used on fine wrinkling, mottled hyperpigmentation, roughness associated with photodamaged skin (90). The described (long term) effects of topical retinoids on the skin are: (1) reduction and redistribution of epidermal melanin, (2) improved ultrastructural characteristics of the epidermis, (3) increased anchoring fibrils, (4) increased deposition of papillary dermal collagen, and (5) increased vascularity in the papillary dermis (21, 70).

UV irradiation results in a functional deficiency of vitamin A as a consequence of the lower expression of the two predominant retinoid receptors in human skin, RAR-γ and RXR-α. The same happens in UV-B irradiated cultured keratinocytes and melanocytes. Contrarily to keratinocytes, irradiated melanocytes return to normal values 2 to 3 days after irradiation (27, 96, 97). Pretreatment of human skin with tretinoin blocks dermal matrix degradation following sun exposure inhibiting the induction of the AP-1 transcription factor and AP-1 regulated matrix-degrading MMPs. Tretinoin does not interfere with UVR-induced upregulation of tissue inhibitors of metalloproteinases (TIMP) thus favoring collagen preservation (24, 98). In fact, Singh *et al*. (26) demonstrated by photomicrography the restoration of procollagen-1 in the papillary dermis after treatment with 0.1 % tretinoin during 40 weeks.

Clinically modest but highly statistically signficant improvements in global appearance of photoaged skin treated with tretinoin were shown in several double-blind vehicle-controlled trials involving more than 700 subjects. These data led to the first U.S. FDA

product (Renova®) approval for the indication of improving photoaged skin (24, 99). The beneficial effects are dose-dependent and increase with duration of therapy for at least 10–12 months (24, 99).

Kossard *et al*. (100) reported that 0.05 % tretinoin cream appeared to be effective in reversing epidermal atrophy and diminishing fine wrinkling, mottled hyperpigmentation and skin roughness in a randomized double-blind vehicle controlled trial. However, the solar elastosis was not treated at least during this period (6 months).

Fisher *et al*. (98) demonstrated that a single topical application of 0.1 % retinoic acid for 4 days promoted the proliferation of keratinocytes (increasing the number of cells and therefore the thickness of the epidermis), compression of the barrier and opening spaces between keratinocytes.

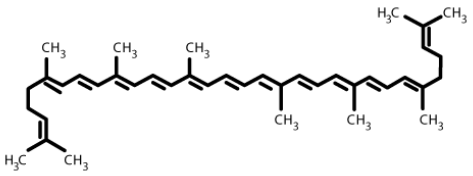
3.2 Lycopene

3.2.1 Physicochemical Properties

Lycopene is a naturally occurring carotenoid found in a number of fruits and vegetables such as the tomato, watermelon, pink grapefruit, guava, apricots, papaya and rosehip (101). Lycopene is an acyclic hydrocarbon and its chemical structure has many conjugated carbon double bonds in the all-*trans* form, which account for lycopene´s stability and also for its attractive colour. These double bonds can be easily attacked by electrophilic reagents, resulting in an extreme reactivity. This reactivity of lycopene is the basis for its anti-oxidant activity (102).

Synthetic lycopene is extremely expensive (103), and the resulting compound is equivalent to natural lycopene, including the isomer content (104-106). While in the plant matrix or in solid form, lycopene is relatively stable, but after extraction from the matrix and dissolution in a non-polar organic solvent, lycopene is quite unstable (107). Therefore, the stability of lycopene must be a consideration during experiments, especially in *in vitro* studies. A summary of the main physicochemical properties of lycopene is represented in **Table 4**.

Table 4- Physicochemical properties of Lycopene (105, 106, 108, 109).

Molecule	**Lycopene**
Molecular Structure	
IUPAC Name	(6E,8E,10Z,12Z,14E,16E,18E,20Z,22Z,24E,26E)-2,6,10,14,19,23,27,31-octamethyldotriaconta-2,6,8,10,12,14,16,18,20,22,24,26,30-tridecaene
MF	$C_{40}H_{56}$
MW (g/mol)	536.87264
Physical state	Orange Powder
Solubility	Insoluble in water, ethanol and methanol Soluble in chloroform, hexane, benzene, carbon disulfide, acetone, petroleum ether and oil
Log P	15
Melting Point (°C)	172-175
Abs.	444, 470 and 502 nm
Stability	Sensitive to light, oxygen, high temperature, acids, catalyst, metal ions. Store at -70°C. Combustible. Incompatible with strong oxidizing agents.

3.2.2 Bioavailability and Metabolism

Although lycopene represents as much as 50 % of carotenoids found in human serum, it cannot be synthesized in human organism (9). Lycopene has **high bioavailability** and, in most cases, its bioavailability from dietary sources is increased by **coingestion of dietary lipids and thermal processing** (for example, in tomato paste) due to ***cis*-isomerization**

of the molecule because of the heat chemical reaction during processing. In addition, the physical disruption of the cell structure in processed tomato products compared to fresh tomatoes partially explains the difference in the bioavailability of lycopene. Although about 90 % of the lycopene in dietary sources is found in the linear, all-*trans* conformation, human tissues contain mainly *cis*-isomers. Several research groups have suggested that *cis*-isomers of lycopene are better absorbed than the all-*trans* form because of the shorter length of the *cis*-isomer, the greater solubility of *cis*-isomers in mixed micelles, and/or as a result of the lower tendency of *cis*-isomers to aggregate (9, 47, 104, 106, 110, 111).

The **lycopene distribution in skin tissue** is about 0.2-0.6 nmol/g wet weight, with higher concentration in the upper skin layers because of the transportation of carotenoids to the skin surface via eccrine sweat glands and/or sebaceous glands and migration of epidermal keratinocytes in which carotenoids have been suggested to be loaded. Through Raman spectroscopy studies, significant differences regarding the distribution and level of carotenoids were observed within different skin areas (2, 7, 18).

Lycopene is cleaved *in vitro* to **acycloretinal, acycloretinoic acid and apolycopenals** (112) in a nonenzymatic manner. Kim *et al*. (113) suggested the susceptibility of carbonyl group of carotenoids, with a long chain of conjugated double bonds, to cleave by autooxidation, radical-mediated oxidation and by singlet oxygen. These reactions may also occur in *in vivo* conditions if the tissues are exposed to oxidative stress (114). Recently it was demonstrated that oxidation/cleavage products formed by chemical transformation impact the proliferation of certain cancer cells. In this process, the dialdehydes seem to be the most active metabolites (3). These studies strongly suggest that oxidation products as well as intact carotenoids have biological effects on human health, whether beneficial or harmful (112).

3.2.3 Safety

Regarding the lycopene safety, **no adverse effects** from lycopene ingestion and topical administration have been described up to moment. Both pure crystalline lycopene and formulated lycopene (in stable conditions) are not genotoxic as demonstrated by a comprehensive battery of tests (106).

3.2.4 Biological Activity

In humans, carotenoids function primarily as dietary sources of provitamin A. When converted to vitamin A, these molecules play an important role mainly in vision and skin. However, lycopene lacks the b-ionone ring structure required to form vitamin A and has no provitamin A activity. The first reported *in vivo* activities of lycopene were the protection against bacterial infection, when lycopene was injected intraperitoneally to mice (115). Furthermore, protection against radiation and development of certain types of ascites tumours was also described (116, 117).

Several studies (114, 118) have demonstrated potential benefits of lycopene related to its biological functions, as it is described above for antioxidant, gap-junction communication, retinoid activity, cell proliferation and apoptosis properties and the consequent implications for prevention of photocarcinogenesis. Some of these biological functions may be mediated by lycopene metabolites with more or less activity or with an entirely independent function (114).

Epidemiological studies indicate that lycopene may be helpful in cardiovascular disease (119), diabetes (120) and cancer prevention (oral, esophageal, pancreatic, rectal, colon, cervical, breast cancer) (121) and, particularly, in prostate cancer prevention, which is reported in many clinical trials (47, 122). Being fat soluble, lycopene appears to be particularly effective in tissues with high lipid content, such as prostate. The skin, also a lipid-rich organ is likely to benefit from lycopene biological activity. However, the studies regarding the role of lycopene against some of these diseases are not completely conclusive and are still ongoing.

Although some published Phase I and II studies have established the safety of lycopene supplementation, these studies do not clearly address the potential efficacy of lycopene as a chemopreventive agent. It has been proposed that the most important studies that are expected to be reported during the next several years will be Phase II clinical trials that are placebo-controlled, randomized and double blind. Well designed and adequately powered clinical studies of lycopene efficacy are still needed (116).

3.2.4.1 Antioxidant Properties

The pharmacological properties of lycopene are mainly due to its antioxidant activity. In fact, antioxidant properties of many carotenoids have been long believed to play critical roles in their anticarcinogenic actions. Using tomatoes or tomato products, numerous studies have demonstrated decreased DNA damage (28), decreased susceptibility to oxidative stress in lymphocytes (123), and decreased low density lipoprotein (LDL) oxidation or lipid peroxidation (124). **Oxidative stress** is one of the major factors of chronic diseases and cancer. *In vitro, ex vivo*, and *in vivo* studies have been carried out to demonstrate the effects of lycopene against oxidative stress (105). However, data regarding the antioxidant effects of lycopene alone in biological systems are limited (114). The investigation into the interaction of free radicals with antioxidants has been strongly stimulated, as it has become possible to measure the carotenoids in human skin *in vivo*, online and non-invasively by resonance Raman spectroscopy (125). In the past, these investigations were only possible by means of chemical analysis of biopsies, i.e. invasive measurements (2, 126).

As mentioned above, lycopene is a **powerful antioxidant both *in vitro* and *in vivo* against the oxidation of proteins, lipids and DNA.** Many factors, including molecular and physical structure, location within the cells, ability to interact with other antioxidants, concentration and the partial pressure of oxygen were described to be key factors regarding the reactivity of lycopene in biological systems (127).

Lycopene exerts its maximal antioxidant activity cellular membranes level and naturally interacts with lipid components. This property is related to its highly lipophilic nature

(128). Tumour initiation could be reversed through lycopene membranes protection from lipid peroxidation. It was demonstrated that lycopene is more efficient than β – carotene in preventing NO_2 induced oxidation of lipid membranes and subsequent cell death (3, 129).

Lycopene has been identified as one of the **most potent scavengers of singlet species of oxygen free radicals** – the highest among the carotenoids (twice as potent as β-carotene and, approximately, 100 times more powerful than vitamin E). This effect is probably due to the greater number of conjugated double bonds in the lycopene structure (8, 114). At low oxygen tension, it can also scavenge peroxyl radicals, inhibiting the process of lipid peroxidation (18).

It is predicted that three possible mechanisms are involved in the **action for lycopene towards ROS**: radical addition, electron transfer to the radical and hydrogen abstraction (**Fig.4**). Lycopene is the best antioxidant based on electron transfer reactions (130).

1. Radical addition: Lycopene + $R^{\bullet}$ → R-Lycopene$^{\bullet}$

2. Electron transfer: Lycopene + $R^{\bullet}$ → Lycopene$^{\bullet+}$ + R^{-}

3. Hydrogen abstraction: Lycopene + $R^{\bullet}$ → Lycopene$^{\bullet}$ + RHL

Fig.4- Possible reactions of lycopene with radical species ($R^{\bullet}$) (102, 105).

Regarding the **interaction of lycopene with other antioxidants**, it is already known that lycopene in combination with other antioxidants such as vitamins E and C, polyphenols and other carotenoids have wide potential for human health (**Table 5**) (127, 131). Bast *et al*. (132) suggested that lycopene might enhance the cellular antioxidant defense system by regenerating the non-enzymatic antioxidants vitamins E and C from their radicals. β-carotene not only quenches oxy radicals but could also enhance the radical-protective properties of both vitamins E and C, as well (**Fig.5).** In addition, the superior protection of these mixtures may be related to the specific location of different antioxidants in cell membranes and, also to different absorption wavelengths regarding the skin photodamage context. Recent formulations of antioxidant mixtures in the

development of nutritional products have been in favor for their health benefits (105, 133).

Fig.5. Squeme of the mechanism proposed by Black & Lambert (134) by which β-carotene participates in quenching oxy radicals and interacts to enhance the antioxidant properties of vitamin E: vitamin E (TOH) firstly intercepts an oxy radical and the tocopherol radical cation ($TOH^{\cdot+}$) formed is repaired by β-carotene (CAR), which in turn forms the carotenoid radical cation ($CAR^{\cdot+}$) latter repaired by ascorbic acid.

Table 5- Examples of some lycopene interactions with other antioxidants (adapted from Kong *et al*. (105)).

Examples	References
The combinations of lycopene with vitamins C and E and β-carotene have exhibited higher scavenging activity on 2,2-DPPH radical than their individual antioxidant activity.	(135)
Lycopene combined with other antioxidants gave a better inhibiting effect towards diene hydroperoxides produced from linoleic methyl ester with 2,2'-azobis (2,4-dimethylvaleronitrile) (AMVN) induced oxidation.	(108)
Lycopene helps in repairing the vitamin E or α-tocopherol radical and the products from this reaction radical cation will be repaired by vitamin C. Some researchers suggested that vitamin E could regenerate the intact lycopene.	(102) (136)
A study done using multilamellar liposomes showed that lycopene and lutein was the best combination toward AMVN-induced oxidation. Lycopene is the strongest reducing agent and able to reduce the radical cations of lutein and zeaxanthin, but not β-carotene.	(6, 137, 138)

There are additional reports that have analyzed the antioxidant or chemopreventive effects of lycopene in combination with cellular antioxidants, such as the hormone melatonin. Moselhy & Al mslmani (139) investigated the chemopreventive potential of lycopene and melatonin on 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced mammary tumours in female rats. These authors reported that melatonin enhanced the

ability of lycopene to decrease the levels of both malondialdehyde (MDA), a marker of lipid peroxidation, and NO in the serum and breast tissues of DMBA-treated rats. Similarly, the co-administration of melatonin significantly increased the activity of the antioxidant enzymes (SOD, CAT and GPx) in this tissue. It was estimated that while lycopene, administrated alone, protected 66.5 % of the DMBA-injected rats from carcinogenesis, the co-administration with melatonin protected 80 % of the animals (3, 139).

Antioxidant Response Elements/Electrophile Response Elements (ARE/EpRE) are *cis* - regulatory sequences at the promoter region of detoxifying enzymes. These elements allow the coordinated induction of these enzymes (3). The major ARE transcription factor Nrf2 (nuclear factor) is a primary factor involved in induction of antioxidants and detoxifying enzymes, and is essential for the induction of several phase II cytoprotective enzymes, including glutathione S-transferases, NAD(P)H:quinone oxidoreductase which play a role in the detoxification of carcinogens. Carotenoids and their metabolites share with all other classes of phase II inducers a common chemical property, the ability to react with sulfhydryl groups (8, 140). Carotenoids activate the ARE transcription system by disrupting the cytosolic interactions between the major ARE-activating transcription Factor (Nrf2) and its inhibitor Kelch-like ECH-associated protein 1 (Keap1) (3, 141). Such effects on gene expression may be related to the protective or adverse properties of carotenoids (9).

Evidence in recent years indicates that beneficial effect of lycopene may be due in part to the protection of the cells from ROS through induction of phase II detoxification enzymes (114, 142-144).

Studies with β –carotene demonstrated that this carotenoid can act as **pro-oxidants** at high concentrations and as antioxidants at low concentrations. However, no conclusive evidence exists for a similar behaviour of lycopene (3). In addition, high lycopene concentrations in cell media may not be associated with its high intracellular concentrations because lycopene uptake by cells is relatively low, which would favour an antioxidant role for lycopene *in vivo* (21, 113). Eichler *et al*. (145) reported that the

antioxidant and pro-oxidant behaviours of lycopene depend on the cellular level of this compound. Optimum protection of skin fibroblasts from UVR-induced formation of TBARS was observed at 0.15 nmol/mg protein and the pro-oxidant effect above 0.15 nmol/ protein. Likewise, only low doses of β -carotene and lycopene were able to prevent cellular DNA damage (146). However, according to Young & Lowe (127), clear evidence for a pro-oxidant activity of carotenoids at physiological relevant pO_2 is still missing.

Although many *in vitro* and *in vivo* studies provided evidence for the antioxidant properties of lycopene, some doubt has recently emerged to whether the health benefits of lycopene really arise from its antioxidant activity. Moreover, most of the *in vivo* studies have been performed with lycopene extracts, so the contribution of other components remains unclear (3, 147).

3.2.4.2 Induction of Gap-Junctional Communication

Gap-junctional communication (GJC) allows small molecular signaling between cell-to-cell through channels that are formed by gap junction proteins such as connexin 43 (Cx-43). GJC has been implicated in the control of cell growth via adaptive responses, i.e., differentiation, proliferation, and apoptosis. It has already been evidenced that a loss of GJC is a hallmark of carcinogenesis (114, 148, 149). Lycopene has been shown to have a chemopreventive activity by **inducing gap junctional communication** (GJC) (3). Stahl *et al*. (150) demonstrated that in human fetal skin fibroblasts, lycopene significantly enhanced GJC after 1 and 3 days of treatment. In the same study, only high concentrations of acycloretinoic acid had a similar effect, supporting that this metabolite may not account for the GJC-inducible activity of lycopene (3).

Cx-43 is the connexin most often induced by retinoids and carotenoids. Among carotenoids, lycopene and its oxidative and enzymatic cleavage metabolites modulate GJC in a differential and dose-dependent manner. The oxidative metabolite lycopene-5,6-epoxide was shown to increase Cx-43 expression in human keratinocytes (114, 151). Livny *et al*. (152) found that the expression of Cx-43 in KB-1 human oral tumour cells

was up-regulated mainly after treatment with lycopene, and to a much lesser extent with β-carotene, thus inhibiting proliferation and enhancing GJC. However, only one *in vivo* study found a positive association between GJC or Cx-43 expression with lycopene consumption (147, 153).

It has been hypothesized that lycopene derivatives may have **hormone – like actions** at low concentrations by acting as ligands for a nuclear receptor, similar to retinoic acid derived from carotene (154). Carotenoids were found to inhibit in a reversible manner the progression of carcinogen initiated fibroblasts to transformed ones, and this achievement was correlated to an increased GJC induced by these compounds. Non-tumour cells are contact-inhibited and have functional GJC, but most tumour cells have dysfunctional homologous or heterologous GJC (9).

3.2.4.3 Retinoid-like Activity

Retinoids are the most important oxidative products of provitamin A carotenoids. Retinoids have been implicated in the management of several human diseases, including chronic diseases, by gene expression regulation at specific target sites (155). The growth inhibitory effects of retinoids in cancer cells have been studied (156). However, it is unclear if **non-provitamin A carotenoids,** such as lycopene (**Fig.6**), and their metabolites may function in a similar way.

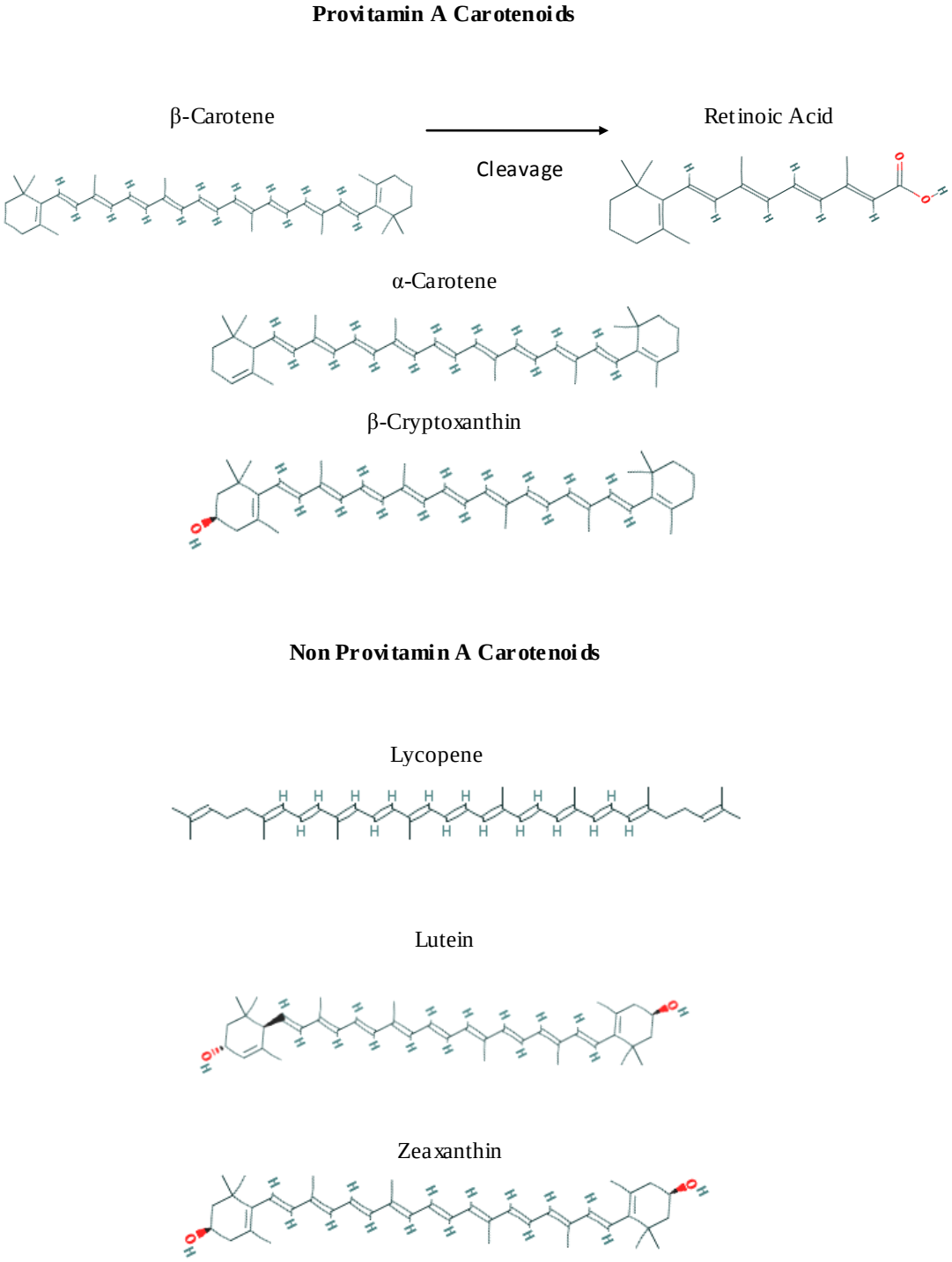


Fig.6- Provitamin A and Non provitamin A Carotenoids structure (chemical structures from PubChem (109); figure adapted from Mein *et al*. (114)).

Recently, it has been suggested (114) that the anti-proliferative effects of the cleavage product apo-10'-lycopenoic acid may be mediated through induced expression of the

RAR-β receptor (and retinoid signaling). The latter has been hypothesized to be a tumour suppressor gene playing an important role in the retinoids growth inhibitory effects mediation, in several cancer cell lines.

3.2.4.4 Cell Proliferation and Apoptosis Regulation

In vivo studies (one with humans and six with animal models) have suggested that lycopene induces apoptosis of cancer cells (147).

The growth inhibitory effect of lycopene was firstly demonstrated by Levy *et al*. (157), who showed that lycopene is a stronger cell growth inhibitor than β-carotene in endometrial, mammary, and lung human cancer cells. In contrast to cancer cells, human fibroblasts were less sensitive to lycopene. In addition, **the growth inhibitory effect of lycopene may also be attributed to induction of apoptosis**. Palozza *et al*. (158) reported that lycopene (0.5-2 mM) inhibited the growth of immortalized RAT-1 fibroblast cells exposed to cigarette smoke by arresting cell cycle progression and inducing apoptosis. However, further investigation is needed into the dose-response effects of lycopene, especially concerning to smoke exposure and/or alcohol ingestion which can affect lycopene metabolism. In addition, a deeper understanding of the metabolism of apo-10´-lycopenoids on carcinogenesis is also needed (114).

Lycopene also prevents carcinogenesis by affecting several cellular processes, such as cell cycle progression and signal transduction pathways modulation (**Fig. 6**) (3, 13).

Carotenoids are able to induce apoptosis in several tumour cells, acting as **potent antiblastic agents**. Their ability to modify the expression of transcription factors and/or proteins involved in the apoptotic process depends on several factors (159). The transcription modulation may be due to direct interaction of the carotenoid molecules (or their derivatives) with transcription factors, such as ligand-activated nuclear receptors, or to indirect change of transcriptional activity, via variations in redox status of the cell.

The signaling pathways targeted by lycopene in different cancer cell lines were summarized by Kelkel *et al*. (3), and represented in **Fig.7**. Briefly, lycopene inhibits insulin-like growth factor (IGF-1R) and the related Ras signaling cascade. This inhibition involves the downstream signaling via the MAPK (ERK, JNK, p38) and PI3K/Akt pathways. Consequently, transcription factors (NF- κB, Sp1 and AP-1) cannot be activated, thus modulating target gene expression. Many of the target genes of these transcription factors are implicated in cell cycle regulation and proliferation (genes of cyclin and CDK families). Cyclins and CDK proteins influence the expression of retinoblastoma (Rb) expression. Unlike normal cells, these cascades are deregulated in tumor cells, allowing cells to continue cell cycle phase transition.

Likewise the inflammatory cascade is repressed. It was demonstrated that lycopene effectively reduced inflammation by inhibiting the release of TNF-α and stimulating IL-10 production. Moreover, lycopene, as other carotenoids, was shown to stimulate gap junctional communication, which is known to play an important role in cell growth control and carcinogenesis (3). This effect supports lycopene antiproliferative effects are also modulated by increasing gap junctional communication (**Fig.7**).

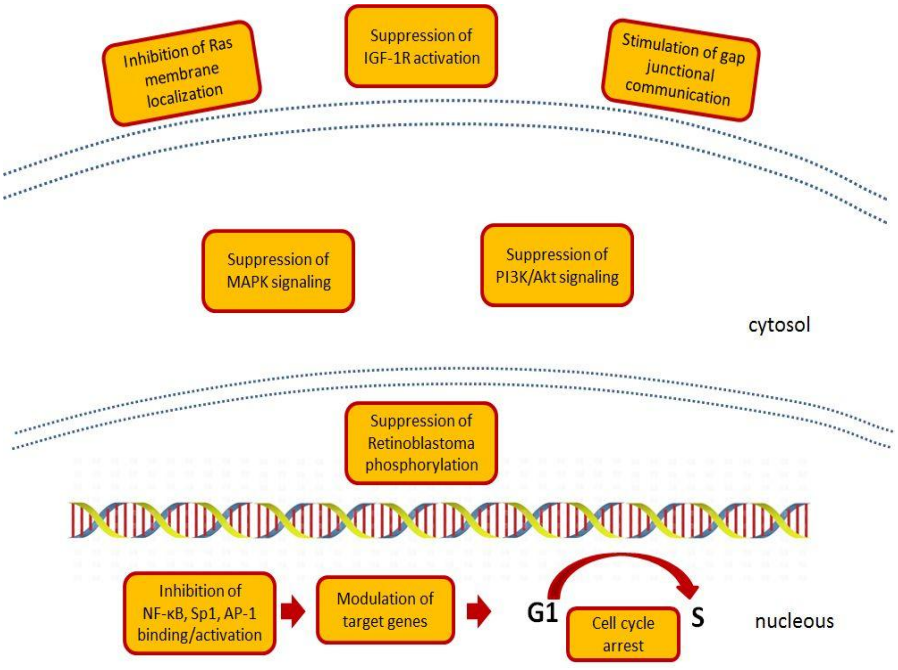


Fig.7- Scheme of the signaling pathways targeted by lycopene in different cancer cell lines and lycopene's points of action (adapted (3)).

We have obtained recent experimental results regarding the effects of UV-B irradiation on human keratinocytes (HaCaT cells) previously exposed to lycopene. According to these results, it seems that lycopene is playing a corrective role in photodamaged cells (unpublished data). However, numerous gaps still exist in understanding how carotenoids modulate the apoptosis process. Many of the results on the pro-apoptotic effects of carotenoids have been demonstrated only *in vitro*. Thus, improved knowledge of the role of carotenoids in apoptosis *in vitro* and *in vivo* will help in understanding their potential role in health and disease (159)**.**

3.2.4.5 Effect on Photocarcinogenesis

Antioxidants can prevent cancer development by protecting DNA from oxidative damage (3). The photoprotection effect of carotenoids (including lycopene) has been studied in several *in vitro/ in vivo* studies, epidemiological studies and clinical trials. Current data remains however controversial, as some studies report a positive effect whereas others report opposite effects.

In Vitro/ In Vivo Studies

A **combination of natural antioxidants** has been proposed to be important for photoprotection according to *in vitro* and *in vivo* data (160). Kowalczyk *et al.* (160) found that several phytochemicals, including lycopene, protect against free radicals' and cytochrome P450 superfamily´s (CYPs) activity *in vitro*. In *in vivo* studies, they also found that lycopene markedly decreased the epidermal thickness (antitumour promotion effect) compared with 7,12-dimethylbenz[a]anthracene-treated group. It also reduced percentages of mice with mutation in 61 codon of Ha-ras (anti-initiation effect). Based on the differential effects observed for the selected phytochemicals on events and processes critical for the growth inhibition of keratinocytes, these authors highlighted the need of phytochemicals synergistic combinations to neutralize detrimental effects of carcinogenesis and tumour promotion. Offord *et al*. (154) studied the photoprotective potential of dietary antioxidants tested in human dermal fibroblasts exposed to UV-A radiation. The carotenoids were prepared in nanoparticle formulations

combined with vitamin C and/or vitamin E. Lycopene and β-carotene alone showed no protection capacity, however, after the addition of vitamin E, their stability and cellular uptake were improved. In addition, the increase of collagenase metalloproteinase 1 (MMP-1) mRNA expression was suppressed. Shixian *et al.* (131) suggested that the synergistic effects observed between **lycopene and vitamin E** may be related to: a) their differential membrane localization; b) their physicochemical properties that allow that one antioxidant may regenerate the second antioxidant; c) their differential interference with signal transduction pathways leading to the synergistic inhibition of cell proliferation. In addition, Afaq *et al*. (161) have shown *in vitro* that anthocyanidins, such as delphinidin, present in pomegranate and also in tomatoes fruits, may act as other photochemoprotective agents.

Butnariu *et al.* (162) studied the **photoprotective potential of lycopene alone** against **UV-A** damage in ***in vivo*** conditions. The effect on the skin's protective mechanisms against UV-A radiation was evaluated by means of experimental acute inflammation on rat paw edema. Lycopene has been involved in the synthesis of prostaglandins and phospholipids components of cell membrane increasing skin protection mechanisms (162). Recently, we have also demonstrated that lycopene induced a therapeutic response by inhibiting mice ear edema (163). The results obtained with lycopene were statistically similar to those obtained with bethametasome (positive control). However, topical application of lycopene reduced inflammatory infiltrate in higher extension than bethametasone, which may support the beneficial potential of lycopene.

Regarding **UV-B** effects, Fazekas *et al.* (47) demonstrated that topical application of lycopene inhibited UV-B induced ODC and myeloperoxidase activities. Additionally, it reduced significantly skin thickness in a dose dependent manner. Topical lycopene was able to prevent caspase-3 cleavage of and to reverse the UV-B-induced normal proliferating cell nuclear antigen (PCNA) inhibition. These authors suggested that lycopene may act as a protective and preventive agent against acute UV-B induced photodamage by: a) inhibiting the epidermal ODC; b) reducing inflammatory responses; c) maintaining normal cell proliferation, and possibly c) preventing DNA damage.

Lycopene ability to inhibit the formation of DNA damage may explain the absence of caspase-3 expression and, thus, by inhibiting caspase-3 lycopene prevents the occurrence of UV-B- induced apoptosis.

Wu *et al.* (164) found that lycopene inhibited **PDGF-BB** (platelet-derived growth factor-BB)-induced signaling and cell migration in human cultured skin fibroblasts through direct binding to PDGF-BB. PDGF-BB facilitates the growth, invasion and metastasis of melanoma. Inhibition of PDGF-BB effects can arrest melanoma progression. *In vitro* studies with human cell lines of foreskin fibroblast and metastatic melanoma shown that lycopene can inhibit PDGF-BB induced fibroblasts migration, attenuate PDGF-BB induced phosphorylation, and reduce PDGF-BB induced signaling (165). In addition, lycopene was shown to bind to PDGFBB also in human plasma (116). Melanoma-induced fibroblast migration was compromised by trapping of PDGF by lycopene (164) suggesting that it may interfere with tumour-stroma interactions. This finding also suggests that lycopene may act as stromal cells and tumour cells inhibitor, contributing to its anti-tumour activity (164).

However, **other data contradict** the putative inhibitory effects of lycopene on UVR damage, including tumor proliferation. For example, Burgess *et al.* (166) concluded that lycopene within physiological ranges did not affect cell proliferation in several human cell lines, including skin carcinoma and non cancerous skin. In addition, Borawska *et al.* (167) suggested that genistein and epigallocatechin-3-gallate but not lycopene could help maintaining or improving skin health through enhancing viability of skin fibroblasts. According to Yeh *et al*. (168) lycopene enhances UV-A induced DNA damage and expression of heme oxygenase-1 in cultured mouse embryo fibroblasts because carotenoids are unstable under light exposure and may have prooxidative effects. Apo-6´-lycopene,2-methyl-hepte-6-one is one of the oxidative products formed during irradiation of lycopene. These *in vitro* studies were also supported by experiments on humans, which demonstrated that lycopene is more sensitive to UVR than other carotenoids (110). Curiously, Nagao (112) found that oxidized metabolites of lycopene,

but not lycopene itself, can inhibit cell growth and stimulate apoptosis in human promyelocytic leukemia cells (HL-60).

These conflicting data supports that further studies on the pathways targeted by lycopene alone are needed, and raise the question of putative synergic effects of lycopene and other compounds.

Epidemiological and Clinical Studies

Few studies have examined the relation of carotenoid exposure and skin cancer in humans. In most of these studies, neither β-carotene nor lycopene appear to be protective for melanomas or non-melanomas skin cancers (51). However, these cohort studies reflect a small number of cases, limiting their power to detect an effect. In contrast, several clinical trials were conducted, but none has found an effect of β-carotene supplementation on either skin cancer or skin cancer incidence (169). According to Black & Lambert (134), the **diet** was the main variable in epidemiological studies that showed photoprotective effects of β-carotene on carcinogenesis and those that showed no effects or even exacerbation. The studies in which a photoprotective effect was observed employed commercial, closed-formula rations whereas the other studies with opposite results employed semi defined diet. In fact, carotenoids or phytochemicals present in closed-formula diets might act to determine the UVR-carcinogenic response in the presence of β-carotene.

According to Heinrich *et al.* (170) a supplementation with a daily **dose** of 24 mg of carotenoid mix comprising the three main dietary carotenoids, β-carotene, lutein and lycopene provided photoprotection against UVR-induced erythema and this effect was comparable to that of β-carotene alone. Protection was correlated with an increase in the carotenoid levels in skin and serum. Long treatment period was required to achieve photoprotection. It should be pointed out that the protective properties of carotenoids are probably at least two-fold, consisting of their potent antioxidant activity and their ability to induce cellular protective responses. Wright *et al.* (171) also reported a

positive association between lycopene intake and a decreased risk of non-melanoma skin cancer. In addition, it was recently demonstrated that lycopene content in the skin was inversely proportional to skin roughness, supporting that lycopene may also be able to reduce skin aging (172).

The **source of lycopene** (natural form vs. synthetic) may also influence its photoprotective properties. For example, Aust *et al.* (173) investigated the effects of synthetic lycopene (~ 10 mg/day) in comparison with a tomato extract and a drink containing solubilized lycopene. The protective effect was more pronounced in the tomato extract (38 %) and lycopene drink (48 %) groups. However, phytofluene and phytoene present in tomato extract and lycopene drink may have also contributed to observed protective effects, since both of these carotenoids exhibit absorption maxima at wavelengths of UVR (173). This study also points to synergic effects of different phytocompounds present in the tomato extract.

Supporting some *in vitro* studies already mentioned, studies on humans also demonstrated that lycopene is able to protect the skin from the effects of UV-B rays, especially when used in association with vitamin C and vitamin E (14). These protective effects have been attributed to redox reactions of carotenoids that may influence UVR carcinogenesis (14).

Another important topic is the **period of lycopene supplementation**, i.e., prior or after the UVR exposure. Rizwan *et al.* (174) examined whether pre- and post-supplementation with tomato paste rich in lycopene could protect human skin against UVR - induced effects partially mediated by oxidative stress, i.e. erythema, matrix changes and mitochondrial DNA damage. At the end of the study, these authors concluded that lycopene provided protection against acute and potentially longer-term aspects of photodamage. Moreover, studies looking at induced sunburn before and after supplementation with lycopene show a significant reduction in skin photodamage when an increased intake of lycopene was performed (175).

Conclusions

Human skin is constantly exposed to the UVR which may induce a number of pathobiological cellular changes, including carcinogenesis. The development of novel preventive and therapeutic strategies depends on our understanding of the molecular mechanism of UVR damage. In this review, the mechanisms of action and potentialities of one provitamin A (tretinoin) and one non-provitamin A (lycopen) active compounds were presented and discussed.

Tretinoin can be used for photoaging treatment by different mechanisms, such as: reduction and redistribution of epidermal melanin, improved ultrastructural characteristics of the epidermis, increased anchoring fibrils, increased deposition of papillary dermal collagen, and increased vascularity in the papillary dermis. Moreover retinoids, such as tretinoin, are considered the first line treatment for acne, being also a maintenance therapy due to their anti–inflammatory and comedolytic actions.

Lycopene has been proposed to play a critical role on anticarcinogenic action at different levels without exerting side-effects, as it was described for antioxidant, gap-junction communication, cell proliferation and apoptosis properties. It is a powerful antioxidant both *in vitro* and *in vivo* at low concentration against the oxidation of proteins, lipids and DNA. However, several gaps remain on the clarification of the role of lycopene, and other carotenoids, as modulators of apoptosis. Thus, improved knowledge of the role of carotenoids and their oxidation products in apoptosis *in vitro* and *in vivo* is still needed. Even many studies regarded the influence of lycopene (and other carotenoids) on cancer risk reduction, this relationship mostly suggested by case-control studies, should be confirmed by additional evidence from prospective studies. For comparison, the tested conditions should ideally be standardized (e.g. lycopene source, period of application, dose). This evidence becomes even more necessary when contradictory results are obtained.

The skin protection against sunburn by these agents is not comparable to the use of a sunscreen, a suitable diet combining different natural antioxidants may contribute to basal protection, and thus increase the defense against UVR-mediated damage to skin.

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Abbreviations

AMVN: 2,2'-azobis (2,4-dimethylvaleronitrile)
AP-1: Activator Protein-1
ARE: Antioxidant Response Elements
ATRA: All-*Trans*-Retinoic Acid
BCC: Basal Cell Carcinoma
Bcl-2: B-cell lymphoma 2
Bid: BH3 interacting-domain death agonist
BRAF: B-Raf proto-oncogene
CAT: Catalase
COX-2: Cyclooxygenase-2
CPD: Cyclobutane Pyrimidine Dimmers
CRABP: Cytosolic Skin Binding Proteins
CTLA-4: Cytotoxic T Lymphocyte Antigen 4
Cx-43: Connexin 43
CYP: cytochrome P450 superfamily
DMBA: Dimethylbenz(*a*)anthracene
EpRE: Electrophile Response Elements
FDA: Food & Drug Administration
GJC: Gap Junction Communication
GPx: Glutathione Peroxidise
HO-1: Hemeoxygenase-1
IFN: Interferon
IGF: Insulin-like Growth Factors
IL: Interleukin
Keap1: Inhibitor Kelch-like ECH-associated protein 1
LDL: Low Density Lipoprotein
MAPKs: Mitogen – Activated Protein Kinases
MDA: Malondialdehyde
MED: Minimal Erythema Dose
MEK: Mitogen-activated Extracellular signal-regulated Kinase
MMPs: Metalloproteinases
MMP-1: Collagenase
MMP-3: Stromelysin-1
MMP-8: Neutrophil Collagenase
MMP-9: Gelantinase
NF: Nuclear Factor
NMSC: Non-melanoma skin cancers
ODC: Ornithine Decarboxylase
8-OHdG : 8- Hydroxyl-2´-Deoxyguanosine
PCNA: Proliferating Cell Nuclear Antigen
PDGF-BB: Platelet-Derived Growth Factor-BB
PDT: Photodynamic therapy
PPE: Palmar–Plantar Erythrodysesthesia
RAR; RXR: Retinoic Acid Receptors
RARE: Retinoic Acid Response Elements
ROS: Reactive Oxygen Species
SCC : Squamous Cell Carcinoma
SOD: Superoxide Dismutase
TGF: Transforming Growth Factor
TNF: Tumour Necrosis Factor
TRP: Tyrosinase related proteins
UVR: Ultraviolet Radiation

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Chapter 1.2

Topical Delivery of Antioxidants

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Topical Delivery of Antioxidants

Abstract

Reactive oxygen species (ROS) and free radicals have been implicated in a number of diseases and disorders, and the skin, for its localization, is exposed to a large number of environmental threats. Free radical scavengers and antioxidants have thus been proposed as protective or therapeutic agents against ROS-mediated injuries. Oral treatment with several antioxidants has been reported to provide skin protection against deleterious effects of ultraviolet radiation. Topical delivery of antioxidants has increasingly gained interest and development, especially by offering better targeting to the upper skin layer. However, the topical delivery of antioxidants for dermal action is a challenging research field since the molecules are, in general, susceptible to degradation. The search for a new delivery system that, simultaneously, preserves the antioxidant stability and enhances its deposition on the skin, opened a new chapter in drug delivery design. Nanocarriers have been successful in enhancing the clinical efficiency of several drugs. More recent approaches in modulating through the skin delivery led to the development of specialized nanoparticulated systems.

The first part of this article presents a review of the potential of antioxidants as pharmacological agents in ROS related diseases, with a special focus on oxidative stress implicated skin pathologies: ROS formation and natural protection against ROS toxicity, ROS-mediated skin damage and skin protection by antioxidants. On the second part of this work, we present reported formulation strategies for dermal delivery of antioxidants focusing the nanoparticulated systems developed in recent years.

Keywords: Antioxidants, Topical Delivery, Drug Delivery Systems

Introduction

The human body suffers constant attacks from ROS, condition that can lead to a state of disease when the available supply of the body's antioxidants is insufficient to handle and neutralize ROS of different types. The result is massive cell damage, cellular mutations, tissue breakdown and immune compromise.

ROS have been implicated in a number of pathologies (1). The over expression of antioxidant enzymes has been found in a number of diseases including infection, inflammation and cancer, either at systemic or at the skin levels (2,3,4).

Skin, besides being the largest organ in human body, is also a major target of oxidative stress (OS). The knowledge of the deleterious effects of ROS led to the utilization of antioxidant therapy for systemic and for cutaneous diseases and disorders, both as prophylactic and therapeutic agents. The antioxidants global market has always been important because many of their constituents are present in traditional herbal medicine. More recently, epidemiological studies presented nutritional compounds involved in the prevention of several health problems.

Topical antioxidant therapies could benefit significantly from the development of appropriate drug delivery systems in either of the following cases: to obviate the rapid absorption and degradation of antioxidants or to allow for the deep skin penetration of the referred antioxidants, and to potentiate antioxidant intracellular effectiveness.

In the present paper, an overview on the formation of ROS in biological systems and the cellular systems of antioxidant defense will be presented. The role of ROS in systemic and in skin diseases will also be reviewed. The use of antioxidants for therapeutic purposes will be discussed. A special focus on new drug delivery systems for topical application is considered due to the potential therapeutic advantages of the delivery of antioxidants to the upper and deep skin.

1. ROS formation and natural protection against ROS toxicity

ROS are highly reactive molecules generated by the biochemical redox reactions that occur as part of normal cell metabolism, and by exposure to environmental factors such as ultraviolet (UV) light, cigarette smoke, environmental pollutants and gamma radiation. The use of some toxic compounds can result in the production of ROS, which include anticancer drugs, anaesthetics, analgesics, etc. However, ROS are also involved in many cellular functions, being a normal part of being alive. For example, ROS are generated when mitochondria oxidizes glucose. The release of free radicals and oxidants from the cells is a physiological process essential for the defense from infection: white blood cells use ROS to attack and destroy bacteria, viruses, and virus-infected cells. The detoxifying actions of the liver also require ROS. **Fig.1** summarizes the main causes of ROS formation, either by exogenous or endogenous factors (5).

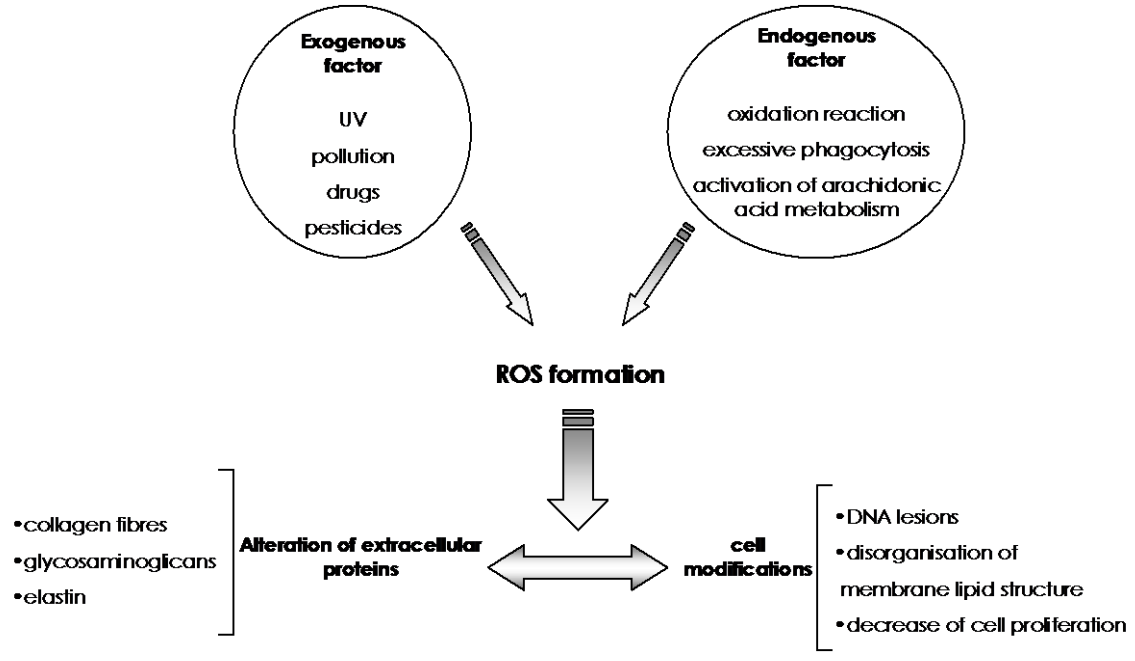

Fig.1- ROS formation and effects (adapted (5)).

The **main ROS**, which occur in the human body, include: superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), nitric oxide radical ($NO^{\cdot}$) and peroxyl radical ($ROO^{\cdot}$). Once formed, these species attack cell structures within the body. Primary targets of ROS attack are the polyunsaturated fatty acids in the membrane lipids, causing lipid peroxidation, which may lead to disorganization of cell structure and function. Furthermore, decomposition of peroxidized lipids yields a wide variety of end-products, including malondialdehyde (MDA).

ROS have special target structures in the cells: the cell membranes, mitochondria, DNA, enzymes and lipids. In the case of cell membranes, ROS compromise the delivery of nutrients and the removal of waste. In the case of the mitochondria, ROS compromises cellular energy production. Oxidative damage to DNA impairs cell reproduction and leads to cancer and autoimmune disease. Oxidized low-density lipoproteins cause arterial hardening and clogging that leads to heart attacks and strokes.

Therefore, ROS have been well studied and characterized (6). They are naturally controlled by the chemical or enzymatic detoxificant systems protecting the organism from its effects. However, the capacity of these systems is not always as efficient as desirable. Their protection decreases in many biological disorders and with aging.

Free radicals are unstable because they have unpaired electrons in their molecular structure. This causes them to react almost instantly with any substance in their proximity. The hydroxyl radical is the most reactive radical. Hydrogen peroxide and peroxynitrite are degraded with $^{\cdot}OH$ formation. $^{\cdot}OH$ reacts preferably with membrane phospholipids.

There are three free radical lipid peroxidation initiation mechanisms:

1-Non-enzymatic process (irradiation, chemical toxicants);

2-Semi-enzymatic process, with $O_2^{\cdot-}$ radicals mediated by enzymes generation. After $^{\cdot}OH$ formation the oxidation is processed in the non-enzymatic way;

3-Enzymatic process, mediated by cycloxygenases or lipoxygenases.

OS is a condition of pro-oxidant/antioxidant disequilibrium: the generation of potentially harmful ROS exceeds the quenching tissue's antioxidant defense mechanisms. Many research works have been performed in order to understand, evaluate their consequences and to treat the damages caused by free radicals (7,8). However, when their production is higher than antioxidant protection, the OS phenomenon occurs, and the protective tools become self-damaging mechanisms: DNA and protein damage, lipid peroxidation, inactivation of anti-peroxidases, depletion of anti-oxidant defenses, activation of NF-kB and cytokines transcription. In sum, the mechanism of ROS formation is dangerous when out of control, making the production of radical species a cause of injury and disease.

In healthy individuals, the **antioxidant system** defends tissues against ROS attack. The main antioxidants are vitamins A, E and C, β-carotene, glutathione, bioflavonoids, selenium, zinc, CoQ10 (ubiquinone), and various phyto-chemicals from herbs and foods. Biochemical antioxidants not only scavenge free radicals, but inhibit their generation from the body, as well. They include lipoic acid, enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, and other repair enzymes. Melatonin, a hormone produced by the pineal gland, is also a potent antioxidant. Cholesterol, produced by the liver, is another major antioxidant that the body uses to repair damaged blood vessels. Vitamin C is water-soluble and the most effective antioxidant within the plasma; it also regenerates vitamin E. Vitamin E is liposoluble and the most effective antioxidant within the cell wall.

The body has the ability to make some of its own antioxidants, three of which are SOD, CAT, and glutathione peroxidase. However, the body is not able to produce enough antioxidants on its own to neutralize all of the ROS. The rest of these antioxidants are originated from food. α-lipoic acid, mixed carotenoids, Coenzyme Q10, cruciferous, N-acetyl-N cysteine, lutein, and a host of bioflavonoids are examples of antioxidants obtained from food.

The role of enzymes like SOD could not be separated from the relationship with other scavengers. SOD has a specific scavenger activity effective for superoxide ion. However,

other low molecular components are useful to complete cell defense from free radicals attack. Copper bound at active site of SOD is claimed to play a role in main processes associated with inflammation: oxygen dismutation, prostaglandin synthesis and collagen cross-linking.

Antioxidants work synergistically with each other. Glutathione peroxidase works in parallel with SOD and CAT and they could also work against different types of free radicals.

For antioxidative action, antioxidants need adequate amounts of the so-called antioxidant minerals copper, zinc, manganese, and selenium, and adequate amounts of folic acid and vitamins B1, B2, B6, and B12, which are enzymes cofactors. These nutrients limit the antioxidant's work.

It is thus possible to describe a coordinated system composed by enzymes and proteins and also by low molecular weight compounds, both hydrophilic and hydrophobic. Different antioxidants play specific roles in different tissues and different organs. Antioxidant enzymes are present in the skin (9). SOD activity in the skin is 5-10 times lower than in other tissues and does not significantly differ from dermis to epidermis. The SOD activity appears increased in some skin diseases but unaffected in others. CAT activity in the epidermis varies in depth being smaller in the deep strata. Specific activity is lower than for the liver but comparable to the brain and the heart. CAT activity is significantly decreased in photo-OS. Glutathione peroxidase activity is more than twice as high in epidermis, compared with dermis, and vary in response to ROS induced by tumors.

The scheme presented in **Table 1** summarizes the various compounds involved in the protection against ROS toxicity and the targets of their action.

Table 1- Different antioxidants and their specific roles.

	Antioxidant	Role
Enzymes	Cu,Zn-SOD Mn-SOD Extracellular SOD	$O_2^{\cdot-}$ quenching
	Catalase	H_2O_2 quenching
	Glutathione peroxidase Glutathione transferase	H_2O_2 and lipoperoxidases degradation
Proteins	Ferritins Transferrins Lactoferrin	Fe ions chelation
	Ceruloplasmine	Cu ions chelation, Fe ions oxidation, $O_2^{\cdot-}$ quenching
	Albumin	Cu ions chelation, $^{\cdot}OH$, $LOO^{\cdot}$, HOCl quenching
Low molecular weight compounds	Vitamin E Ubiquinol N-acetylcysteine	$^{\cdot}OH$, $LOO^{\cdot}$, HOCl, etc. quenching
	Carotenoids	$^{\cdot}OH$, $LOO^{\cdot}$, HOCl, $^1O_2$ etc. quenching
	Vitamin C Glutathione	$^{\cdot}OH$, $O_2^{\cdot-}$ quenching
	Carnosine	various ROS quenching
	Taurine	hypochlorite neutralization
	Uric acid Bilirubin	lipid peroxidation prevention

Antioxidant enzymes act as antioxidants *in vivo*. This is the case of SOD and CAT: their substrates are superoxide radical and hydrogen peroxide, respectively, to prevent accumulation of hydroxyl radical. A dismutation reaction involves one- or two-electron transfers, where the electrons are accepted from $O_2^{\cdot-}$ or H_2O_2. On the other hand, lipid peroxyl radicals result from $^{\cdot}OH$ reaction with polyunsaturated fatty acids of membrane phospholipids and can be inactivated by bioantioxidants such α-tocopherol and reduced form ubiquinon Q10, non-enzymatically.

Other enzymes (10) participate in the regeneration of ascorbic acid involved in the reduction of radicals of antioxidants, such as α-tocopherol radical. Also glutathione–dependent peroxidases using lipohydroperoxides prevent the production of alkoxylradical, contributing for the lipid peroxidation regulation. Proteins are responsible for chelating the transient metals and preventing electron donation. Low

molecular weight compounds complete the depletion of the ROS not scavenged by antioxidant enzymes.

2. ROS-mediated skin damage

Human skin is responsible for protecting internal organs from the toxic external environment and, consequently, the major target candidate of OS. The skin protects from heat, cold, physical injuries and also provides the first defense against invasion by bacteria, viruses, and other toxic elements. The skin is also an excretory organ, removing toxins from the body via perspiration.

The skin essential biomolecules can be severely damaged during photosensitization reactions. In these photodynamic reactions the activation of molecular oxygen is implicated, either by formation of ROS or singlet oxygen. The OS can induce not only cellular death, but also DNA mutations (photogenotoxicity), which in the case of an inefficient DNA repair system can induce oncogenesis. Peroxidation of lipids may occur (photo-peroxidation), the reaction with proteins may induce cell death, immunosuppression or uncontrolled intracellular signalling (11). When associated to photogenotoxicity, it can induce irreversible metabolism and cause cellular transformation.

There are many ROS implicated diseases that can affect the skin (12). **Table 2** shows a possible classification of skin diseases associated with ROS.

The skin protein collagen is particularly susceptible to ROS damage, causing the collagen protein molecules to break down and then link back up again in a different way, in a cross-linking process. Collagen cross-linking causes the normally mobile collagen to become stiff and less mobile. Sunlight also causes the messenger molecules present in skin cells to become active and create inflammatory products. Fisher *et al*. (13) have shown that multiple small exposures to UV irradiation lead to sustained elevations of enzymes that degrade skin collagen and contribute to photoaging. Skin aging accelerating factors produce an imbalance in the turnover of macromolecules in the

dermis. Topical application of antioxidants is able to decrease the modification rate of skin elasticity and thickness (14).

Table 2- Skin diseases associated with ROS (12).

Physicochemical oxidative stress	UV Heat Chemicals
Neutrophilic oxidative stress	Cutaneous vasculitis Behçet's Disease Acne and Rosacea Psoriasis
Other oxidative stress	Infections Ischemia-Reperfusion Injury Autoimmune Diseases Aging Skin inflammatory disorders

Some factors can accelerate skin aging: sun exposure, active or passive cigarette smoke, environmental toxins, poor diet, excess alcohol consumption, stress, harsh soaps or detergent-based moisturizers, sleep deprivation. Skin cancer typically occurs in skin that is photo-aged.

2.1. Photo-oxidative stress in the skin barrier: photo-induced skin lesions

In the last years, with the changes in the ozone layer in the upper atmosphere, it is clear that the effects of UV radiation from the sun are much more dangerous than originally thought.

The exposure of normal skin to UV contributes to well-known acute responses such as erythema and pigmentation, and also to chronic harmful effects such as photoaging and photocarcinogenesis. It is admitted that cutaneous photodynamic reactions resulting from the interaction of UV/visible radiation with endogenous cell photosensitizers generate ROS. The ROS are also involved in phototoxic reactions induced by the

interaction of UV/visible light with drugs or other industrial or environmental chemicals, accumulated in the skin after topical or systemic administration.

Elias and co-workers have provided a large body of evidence that the *stratum corneum* (SC)-the outermost skin layer - lipid composition is essential for maintaining the barrier integrity and suggested that perturbations of the SC lipid or protein architecture may be important for a number of dermatoses (e.g. atopic dermatitis), rather than merely the end result of processes initiated in underlying layers (15,16). The oxidative modification of lipids affects preferably unsaturated fatty acids and leads to their degradation. ROS could alter the lipid composition of the SC and thus potentially affect the barrier function.

In addition to oxidative injury within the SC, the increased formation of stable lipid peroxidation end products (MDA) in the SC could trigger inflammatory responses in adjacent skin layers. A well-known example of such a mechanism, with UV radiation-induced formation of a compound in the SC and subsequent inflammatory response in adjacent skin layers, is urocanic acid, in a process believed to take part in UV radiation-induced immunosuppression (17).

2.2. ROS-mediated skin cancer

The contribution of ROS to skin carcinogenesis has been known for a long time, especially following UV irradiation (18). ROS induce DNA damage and immunosuppression contributes to the skin cancer appearance.

Fig.2 represents the involvement of ROS in skin cancer induction. The DNA damage plays a central role expressed by immune system suppression (with an increase of Langerhans cell depletion, an increase of cytokine release and a decrease of antigen presentation) and by mutations in growth regulatory genes contributing to abnormal cellular physiology (19). The latter, contributes, on one hand, to an increase of cellular derived ROS, and consequently to maintain a cycle of DNA damage and, on the other hand, to potentiate skin neoplasia.

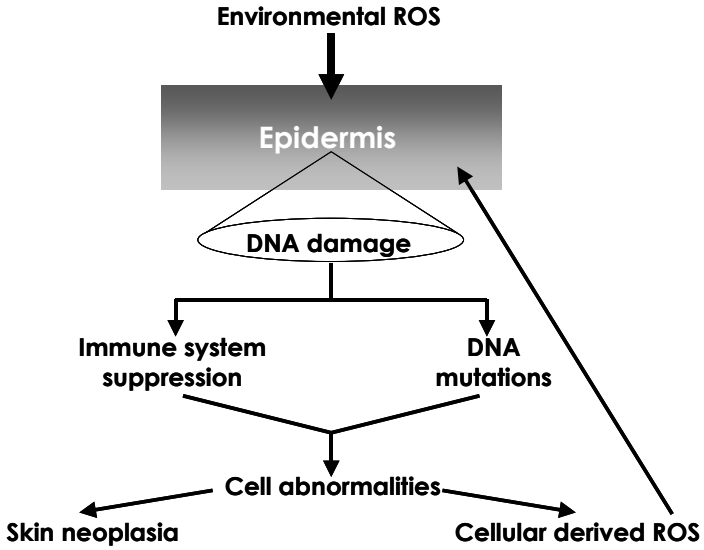

Fig.2- Mechanism of ROS-mediated skin cancer (adapted (19)).

Young skin is also exposed to ROS potentially damaging changes but there is sufficient cellular energy (ATP) for DNA repair and cell renewal. Enzymes that provide antioxidant activity, such as SOD and CAT, are readily available. With aging, the energy for cell repair and renewal diminishes and the antioxidant enzymes are less available. Exposure of skin to solar UV radiation induces OS and suppression of cell-mediated immune responses. Reduction in UV-induced infiltration of CD11b+ cells may be a strategy to reduce solar UV light-induced ROS-mediated skin disorders, such as photoaging and photocarcinogenesis (20).

2.3. Skin inflammatory disorders

Inflammatory skin disorders representing a large proportion and the redox-modulated pathway in inflammatory skin diseases have been reviewed (21).

Free radicals make the membrane more permeable, allowing the cells to dehydrate. Enzymes break down the lipid bilayer and cause inflammation. Chronic inflammation is an underlying cause of common degenerative diseases. Along with causing permanent gene mutations, free radicals activate signal transduction pathways that are related to growth, differentiation, senescence, and connective tissue degradation.

SC of **atopic dermatitis** patients has a less pronounced oxidative state as a consequence of an increase in cutaneous antioxidant defenses due to the chronic inflammation (22). Occupational exposure to metal working fluids causes allergic and irritant contact dermatitis. OS can enhance dermal inflammation caused by this occupational exposure[23]. Studies demonstrated an enhancement in mast cell accumulation caused by topical exposure to metal working fluids (24). In childhood atopic dermatitis pathophysiology, results have shown an impaired homeostasis of oxygen/nitrogen radicals and increased OS, suggesting its suppression as a potentially useful strategy for treatment of this chronic inflammatory disease (25,26).

Generalized severe OS of the skin is expressed as a consequence of contact dermatitis in a restricted area (27).

A systemic unbalance of the antioxidants has been shown (28) and a common profile in patients with different forms of physical **urticaria** has been detected. These alterations may lead to an increased percentage of peroxidable compounds in skin and to the intracellular generation of ROS.

Significantly elevated heme-oxygenase-1 mRNA levels were found in acute inflammatory illness of children, suggesting that monocyte heme oxygenase-1 production could serve as an anti-inflammatory agent to control excessive cell or tissue injury in the presence of OS (29).

Rosacea is a chronic inflammatory disease that affects predominantly the face. Authors found that in mild involvement stage of rosacea patients SOD activity was stimulated to protect the skin against ROS so that the MDA levels were maintained. In a more severe state of the disease, the MDA levels were increased, supporting the “antioxidant system defect hypothesis” in rosacea patients (30).

ROS are also found to be involved in allergic skin diseases. The CAT activity of human skin with **allergic contact dermatitis** decreased both in young and old skin, suggesting that cutaneous CAT may play a role in the pathophysiology of this disease (31).

Alterations in the oxidation products, antioxidant markers, antioxidant capacity and lipid patterns were found in plasma of patients affected by **Pappilon-Lefèvre Syndrome** (PLS) (32). PLS has been grouped with the palmoplantar ectodermal dysplasias. An imbalance in both oxidation product/antioxidant concentrations and essential fatty acid pattern was found.

Alopecia areata is an autoimmune inflammatory disease. Some authors found an affected oxidative status in the disease, suggesting that lipid peroxidation and antioxidant enzymes may be involved in its pathogenesis (33).

Numerous rodent models used in cutaneous inflammation research are based on the induction of acute or subacute vascular type of cutaneous inflammatory responses after the application of chemical agents such as croton oil, arachidonic acid and dithranol (34). Arachidonic acid-induced mouse ear inhibition test (AA-MEIT) is described as the most commonly used *in vivo* test for assessing the anti-inflammatory effects of drugs in the first stage trials. Since neuthrophil influx is a component of the inflammatory reaction, high levels of myeloperoxidase are expected as a reflex of neutrophil accumulation (35).

Studies of dithranol-induced skin irritation in the mouse ear model reveal that the formation of free radicals is essential for dithranol inflammation (36). Significant reductions of Cu,Zn-SOD (37), and of gluthatione S- transferases levels were induced by dithranol.

An acute model of skin inflammation induced by ROS, generated by glucose oxidase as a producer of H_2O_2, has been described to test the anti-inflammatory action of enzymes. The inflammatory response was inhibited predominantly by CAT and to a lower extent by SOD, both attached to polyethylene glycol (38).

3. Skin protection by antioxidants

From a pharmacological point of view, ROS have been seen as a basis for drug design for a long time. However, the study of the skin ROS pharmacology is still in the beginning (39). Skin is easily reached for topical applications, which makes the topical route a preferential strategy for such therapy.

As ROS mediate pathological and toxicological processes, several pharmacological strategies exist and can be classified as different groups (40):

1- Natural or synthetic antioxidants (gluthatione - GSH, flavonoids, butylhydroxytoluene, butylhydoxyanisole, N-acyl dehydroalanines);

2- Enzymes and enzyme mimetics (SOD, CAT, GSH peroxidase, Ebselen®, copper diisopropyl salicylate);

3- Vitamins – A, E, C;

4- Scavengers of O_2^- - derived radicals – mannitol, glucose, dimethylsulfoxide;

5- Chelators - Desferal®;

6- Precursors of intracellular antioxidants – aminothiols, N-acetylcysteine, selenium.

In terms of mechanistic basis for strategies applicable to control ROS, such strategies can be classified as:

1- Enzymatic redox reaction
2- Non-enzymatic redox reactions
3- Radical scavenging
4- Precursors of intracellular antioxidants
5- Metal ion chelation
6- Energy quenching

The use of antioxidant enzymes, as an adjuvant therapy, able to intercept ROS formed and able to increase the natural antioxidant cells defenses (detoxifying enzymes (41), vitamins E and C, carotenoids, whose presence has individual variability) is probably the most challenging strategy. The presence of exogenous antioxidant enzymes could prevent the formation of locally produced ROS (42). SODs and CAT are metalloproteins that use efficient dismutation reactions in their mechanisms to detoxify ROS (8). A large number of studies have shown the advantages of antioxidant enzymes in the treatment

of numerous pathologies. Cuzzocrea *et al.* (8) comprehensively reviewed the use of antioxidants for therapeutic purpose. In treatment of various fibrotic skin diseases, modulation of skin SOD activity to inhibit some OS damages has been described, with clinically varying success (43).

The use of SOD and CAT as therapeutic agents to attenuate ROS induced injury responses, other than skin related, has been reported (44,45). Limitations of enzymes use are their large size limiting cell permeability, short circulating half-life, immunogenicity and cost. For example, the clinical use of SODs has been limited due to the lack of cellular membrane permeability, short plasma half-lives, solution instability, bell-shaped dose response curve and high susceptibility for proteolytic degradation. Enzymes, which can exhibit their pharmacological activity in plasma or on tissue surface, benefit from prolongation of plasma half-life. Liposomes have been extensively studied as carriers for antioxidant enzymes, especially for SOD.

Novel synthetic catalytic scavengers were developed to inactivate toxic oxygen radicals and other reactive oxygen species, including oxygen radicals and hydrogen peroxide, to reduce damage caused in various autoimmune, cardiovascular, neurological, inflammatory and infectious diseases, and studied in animal models (46).

Antioxidant therapy strategies include drug administration via the skin for local therapeutic effect, both on diseased or healthy skin (topical delivery), and for systemic effect (transdermal delivery). Furthermore, many attempts have thus been made to overcome the skin permeability barrier for large molecules. Early studies have shown that skin fluidizers can facilitate permeation of small drugs across the intact skin but it remained practically impossible to deliver a significant amount of large drugs, such as proteins, through the skin without major organ disturbance (47).

Although the skin selectively excludes the passage of proteins, optimized mixed lipid colloidal vesicles, Transfersomes®, are able to mediate the transport, even of macromolecules, through the skin and can ensure targeted drug delivery deep below the application site, as demonstrated in previous studies (48). This is very important since numerous attempts have been made to find acceptable alternatives to an

injection and the use of antioxidant enzymes is limited by the outermost layer of the skin.

3.1. Use of natural or synthetic antioxidants

This dichotomy has relative importance in the food industry, in the addition of antioxidants to food. The so- called natural antioxidants are synthesized most often by plants and also by various microorganisms, fungi and animals. Antioxidants can be also obtained by the synthesis or biosynthesis in the industry. These are called synthetic antioxidants. The former type exists for own producers benefit. Synthetic antioxidants production follows well-defined industrial procedures. They are tested for safety in human health and can be produced in large scale. Other group of antioxidants are identical to the natural antioxidants found in food, but synthesized in the industry and called nature-identical antioxidants. They combine the advantages of synthetic and natural antioxidants: they are pure substances, relatively cheap, easily available, and of reproducible properties, including the antioxidant activity (49).

3.2. Use of low molecular weight antioxidants

Photochemopreventive effects of selected botanical antioxidants have been extensively reviewed (50,51).

Silymarin, a flavonoid antioxidant, possess high protective effect against tumour promotion involving inhibition of promoter–induced oedema, hyperplasia proliferation index and oxidant state (52). Prevention of UV-B-induced immunosuppression and ROS by silymarin may be associated with the prevention of photocarcinogenesis in mice (53).

Studies demonstrated that **activin**, a grape seed-derived proanthocyanidin extract, reduced the inflammatory response and the ROS developed in systemic sclerosis (54).

Soybean isoflavone genistein inhibits UV-B-induced skin tumourigenesis in mice by selective inhibition of oxidative DNA damage (55). Paradoxical effect has been found after oral vitamin C supplementation: the supplementation had no effect on UV

radiation-induced erythermal response with no reduction in skin content of total glutathione and protein thiols (56). A reason for this finding might be the fact that skin bioavailable vitamin C is replacing other reductants of skin cells.

Vitis vinifera (grapes) was proposed as a chemopreventive agent against skin ROS and carcinogenesis (57).

Novel chemopreventive agents as **FA15** derived from natural sources and a hydrophobic derivative of ferulic acid, produce suppression of inflammation and skin tumour (58).

The role of antioxidants on cutaneous photodamage has been recently reviewed (59). The combination of topical **vitamin C and E** produced appreciable photoprotection in pigs (60). Studies suggest that α-tocopherol (vitamin E) protection against UV-B-induced lipid peroxidation is a consequence of both its ability to scavenge peroxyl radicals and its UV-B absorbance resulting in a sunscreen effect (61). Both mechanisms would provide photoprotection in adjacent epidermal layers. α-tocopherol could also regulate the SC lipid homeostasis and thus maintain the physical integrity of the penetration barrier.

Vitamin E is probably the most studied low molecular weight antioxidant. Topical administration of α-tocopherol protects cutaneous tissues against UV-induced oxidative damage *in vivo*, in murine skin and suggests that the underlying mechanism of such effect is related to the up-regulation of a network of enzymatic and non-enzymatic antioxidants (62). High doses of vitamin E significantly lower the urine porporphyrin excretion in studied patients affected by porphyria cutanea tarda (63). Topical application of α-tocopherol was also demonstrated to reduce UV radiation-induced immunosuppression and to prevent UV-B induced carcinogenesis in hairless mice (64). The associated mechanism appears to be via preventing epidermal lipid peroxidation (65).

The study of the mechanisms of UV radiation induced damages has led to the development of skin models for short-term studies. This is the case of *ex vivo* pig skin model used to test the protective effect of topical application of antioxidants (66).

The mechanism for immunosuppression induced by UV-B has been studied (67) and it was demonstrated that chronically applied vitamin E can effectively reduce cancer formation and immunosuppression induced by UV irradiation (64). Topically applied vitamin E scavenges UV radiation-generated lipid peroxides and ROS. This may inhibit loss of membrane integrity protection from local immunosuppression (65).

Identification of common dietary substances with photocarcinogenesis protective effects has been made. Dietary administration of **butylated hydroxytoluene** inhibits UV radiation induction of carcinogenesis (68). Dietary photoprotection by **ω-3 polyunsaturated fatty acids** against photocarcinogenesis in animals has been described (69). In healthy humans, there is evidence of protection by dietary **EPA** (eicosapentanoic acid, a ω-3 polyunsaturated fatty acid) against a range of early genotoxic markers (70).

Lycopene lacks provitamine A activity, but it is a powerful antioxidant both *in vitro* and *in vivo* against the oxidation of proteins, lipids and DNA. Lycopene has been identified as one of the most potent scavengers of singlet species of oxygen free radicals – the highest among the carotenoids (twice as potent as β-carotenoid perhaps because of the greater number of conjugated double bonds in the lycopene structure: 11 vs 9) and, approximately, 100 times more powerful than Vitamin E (71-74).

3.3. Use of high molecular weight antioxidants

Among many others, antioxidant enzymes are effective cellular catalysts responsible for controlling a great number of reactions in the cells. These special active proteins have been employed in many studies as antioxidant therapeutic agents with restricted success related to its *in vivo* behavior. However, successful antioxidant enzyme therapy has been reported for treatment of experimental arthritis (75) and for the treatment of cutaneous lesions (76) when enzymes are delivered by means of colloidal carriers.

Because ROS are responsible for the biological effects of the photo-OS, it may be thought that the use of antioxidants, including the **enzymes SOD and CAT**, may be a promising strategy for preventing or alleviating these effects. UV-B exposure increases

intra- and extracellular H_2O_2 production (77). However, it is important to improve the skin penetration of these macromolecules, enhancing their ability to repair and/or prevent skin damages induced by absorption of visible and/or UV-A light by drugs or other chemicals (called photosensitizers) accumulated in sunlight exposed areas of the skin. This objective can be achieved with an appropriate carrier mediate regio-specific delivery of molecules and enhance enzyme skin penetration into skin and deep below the application site. This would be a relevant goal if we consider the large number of drugs (antibiotics, neuroleptics and anti-arrhythmics) that can induce exacerbated cutaneous reactions in skin areas exposed to solar radiation, mainly radiation UV-A/visible, due to photo-OS induced by these photosensibilizer molecules. This photo-toxicity can be so severe that hospitalization may be needed.

Topical application of a SOD containing gel, on the human normal skin surface, was able to inhibit the acute inflammatory response induced by a locally applied solution of 8-methoxypsoralen followed by UV-A irradiation (78). In parallel, after the application of a hydrogel containing a fluorescently labeled SOD on the human skin surface, the labeled SOD was localized in the upper epidermis and in the epidermal cell layer surrounding the lumina of the hair follicles (42). These results are in accordance with results of other authors, which evidences that the delivery of proteins into the skin has been achieved predominantly by means of accumulation of liposomes in the hair follicles (79).

Studies demonstrated the radiation damage modification in various systems by means of antioxidant enzymes and have suggested the protective role of Cu,Zn-SOD against UV-B-induced injury of the human keratinocyte cell line HaCaT (80).

Topical application of SOD was proposed and investigated for the treatment of skin and mucous membrane lesions (81), with differing and even contradictory results. A lipid peroxidation study in burned patients suggested that polyethylene glycol-conjugated SOD can prevent the conjugated dienes formation by suppressing oxygen radical production (82). Using an acute localized gamma irradiation pig model, it was shown that a successful treatment of fibrosis, involving skin and skeletal muscle, with liposomal SOD might be possible (83). In most studies dealing with the topical application of SOD,

however, the skin was not intact and enzyme delivery through the skin was by diffusion, permeation or using shunts, such as hair follicles and glands.

In the case of thermal injury to the skin, skin SOD activity decreased immediately after experimental burns and topical administration of SOD (84).

Topically applied SOD encapsulated in liposomes reduced the size of post-burn wounds and the formation of oedema in rabbits, compared to pure and intralesionally injected SOD (76).

Using an *in vivo* skin inflammation model, induced by ROS generated by glucose oxidase as a producer of H_2O_2, the inflammatory response was inhibited predominantly by CAT and in lower extent by SOD, both attached to polyethylene glycol (38).

4. Pro-oxidant activity

Almost all antioxidants have pro-oxidant effects *in vitro* in high concentrations or under special conditions because they are by nature quite unstable molecules (85,86). Also many products contain ester derivatives of the antioxidants or non-natural isomer forms that cannot be absorbed and/ or metabolized by the skin (86).

Several of these antioxidants may auto-oxidize readily to free radicals like hydroxyl radical and semiquinone radical. Such species are known to be toxic and are reported to bind irreversibly to various cell constituents by the formation of covalent binding with sulfhydryl groups and/or other essential groups producing secondary free radicals. The formation of these secondary free radicals is responsible for the pro-oxidant activity of these agents (85). The capacity of carotenoids to act as pro-oxidants has been noted in model systems by an increased formation of thiobarbituric acid-reactive substances (TBARS) as a marker of lipid peroxidation (87). Carotenoids act as pro-oxidants at high concentrations and as antioxidants at low concentrations. High carotenoid concentrations in cell media may not be associated with high intracellular

concentrations because lycopene uptake by cells is relatively low, which would favor an antioxidant role for lycopene *in vivo* (88).

A study in C3H cells (71) demonstrated that lycopene possesses prooxidant activity when exposed to UV-A and the oxidative derivatives of lycopene, like Apo-6´-lycopene and 2-methyl-hepte-6-one, rather than itself may be responsible for the increased OS, enhancing the UV-A-induced lipid peroxidation and the subsequent activation of the heme oxygenase-1 cascade.

Lycopene in combination with vitamin E, glabridin, rosmarinic acid or carnosic acid synergistically inhibits the oxidation of LDL, and it has been suggested that the synergistic effects may be attributed to the interaction of these antioxidants with lycopene-derived radicals and/ or with oxidative products of lycopene. Indeed it has been envisioned that a combination of natural antioxidants, including lycopene, may be required for effective photoprotection. These results suggested that it may be premature to use lycopene as a component of cosmetics and further studies are needed to ascertain the safety of using lycopene, and perhaps other carotenoids in cosmetics.

Apart from other mechanisms, pro-oxidative effects have been discussed as a possible reason for an increased risk for lung cancer observed in two intervention studies with β-carotene. In these studies the incidence of lung cancer was about 20% higher than in controls, when β-carotene was supplemented at high doses over an extended period of time to individuals at an elevated risk for this disease. Vitamin E and vitamin C influence the levels of pro-oxidant carotenoid radical cation which could be pro-carcinogenic in nature (85).

Eichler *et al*. (87) reported that the antioxidant and pro-oxidant behaviors of β-carotene, lycopene, and lutein depend on the cellular level of the compounds. Optimum protection was observed at 0.4, 0.05 and 0.3 nmol/ mg protein, respectively and the pro-oxidant effect above 6 and 0.15 nmol/ mg protein for β-carotene and lycopene.

To overcome the vitamin C instability, the more stable esterified derivatives ascorbyl-6-palmitate and magnesium ascorbyl phosphate are often used, but these derivatives are

not well absorbed and are only minimally metabolized by the skin to the active free ascorbic acid form (86). Besides, other authors (85) reported that ascorbic acid-6-palmitate strongly promoted UV-B induced lipid peroxidation and may intensify skin damage following physiological dose of ultraviolet radiation. In order to achieve the benefits to the skin with topical vitamin C, the formulation must contain at least 10 % of L-ascorbic acid, be stable and be at an acid pH, about 3.5 less than the pKa of vitamin C (4.2) (86).

The synthetic isomers of vitamin E are esterified to acetates and succinates for use in commercial vitamins and some topical formulations because of its higher stability. However, the skin has only a limited capacity to cleave the esterified forms of vitamin E to achieve free tocopherol form. Furthermore, the all-rac form of vitamin E has been reported to cause allergic contact dermatitis and erythema multiforme when applied topically. No such adverse reactions have been reported with d-α-tocopherol (85).

High concentrations of α-tocopherol (>> 0.005 mol tocopherol/mol unsaturated fatty acid) accelerated lipid auto-oxidation *in vitro*. By the mechanism of its own recycling, tocopherol depletes other antioxidants, such as ascorbate, thiols and ubiquinols, in this way acting as pro-oxidant. The antioxidant efficiency of ascorbate is strongly dose dependent and ascorbate has the potential to generate pro-oxidant effects in cells and cell free systems (86).

In cells, vitamins C and E interact synergistically to provide antioxidant protection: vitamin C (with its lower redox potential) reduces the oxidized vitamin E to regenerate its activity.

5. Topical delivery systems

The **skin** receives an extraordinary interest as a site of application of drugs both for antioxidant and other molecules. The skin is much more than a body container: it is the biggest (around 7 kg and 18000 cm^2) and elaborate, multilayer functional unit capable of performing multiple and fundamental roles. The skin exhibits a complex structure and a great capacity of regeneration being made up of several layers including SC, viable epidermis and dermis, and appendages that include sweat glands, sebaceous glands, and hair follicles. The SC is the outermost desquamating 'horny' layer of skin, comprising about 15-20 rows of flat, partially desiccated, dead, keratinized epidermal cells. The skin acts as a barrier, avoiding the penetration of foreign bodies (toxins, pathogens, etc) and simultaneously maintaining the body's ingredients, including water. Paradoxically, the cutis, the most exposed layer, still holds many secrets concerning the mechanisms of transcutaneous permeation (89). The hydrophobic parts of SC are one key to the understanding of this process. A substance with molecular weight higher than 300 Da is too large to penetrate the skin. The skin entry is generally described as intercellular or transcellular, but the latter apparently is of little, if any, practical importance. Skin surface differs from the deep skin regions and acts as a semi-permeable barrier. Permeation and penetration through nanopores can be described by equations. The transport of material through a barrier is proportional to a driving-force difference that varies for different permeants. Strictly speaking, permeation relates only to diffusion of molecules through a barrier. Fick's law of diffusion can be generally applied to estimate the transcutaneous material flow. Flow is proportional to the system permeability value, the involved area and the driving-force difference, across the skin barrier. Barrier penetration by a carrier, a macromolecule or molecule aggregates is not sensitive to transbarrier permeant concentration driving-force. Carriers and aggregates can only cross a barrier penetrating the latter. This concept is applicable to the skin barrier. Once applied on the intact skin, low-molecular-weight molecules generate a transcutaneous concentration gradient capable of pushing molecules into the skin. The idea is to simplify the enormous complexity of the skin and to consider diffusion through the skin governed by Fick's law of diffusion. In the case of

penetration of vesicles, an externally created water activity difference through the pore is needed for the motion (90).

Penetration enhancement techniques based on delivery systems have been considerably exploited. However, each drug enhancement strategy may differ and requires optimization.

It is important to restrict the term transdermal delivery to the passage of solutes through the various layers of the skin to reach the systemic circulation and to exert systemic effect. Dermal or topical delivery should be addressed to the local skin delivery that is performed with the aim of targeting the skin and minimizing the systemic absorption. Topical delivery thus permits to extend antioxidant skin deposition and promoting skin antioxidant enrichment.

5.1. Prodrugs and skin penetration modifiers

The chemical approach has contributed to the introduction and commercialization of several permeation enhancers. A chemical enhancer is usually defined as a substance that reversibly alters the skin barrier function in order to permit the drug permeation into the skin faster or better than otherwise in a non-invasiveness approach. Chemical enhancers act mainly at intercellular level. To this moment only few results on the permeation of macromolecules by means of chemical enhancement were obtained. It is the case of the permeation enhancer effect of Azone on *in vitro* percutaneous absorption of heparin (91,92). Furthermore, heparin, a charged macromolecule can serve as a chemical modifier for transdermal ion and molecule transport associated with high-voltage pulsing, suggesting that heparin may stabilize electroporation pathways (93).

Prodrugs can be used as permeation enhancement strategy (94) or, if the prodrug diffuses worst through the skin, a reservoir effect can be achieved with gradual delivery of free drug. This strategy is very interesting for topical antioxidant administration since it could generate a continuous delivery of antioxidants in the skin (95). For topical

delivery of a pro-drug it is important to evaluate *in vitro* degradation and chemical and enzymatic hydrolyses of the prodrug (96).

Soy isoflavones can be used to protect skin from OS induced by UV-B radiation. Using solvents as the vehicle significant amounts of isoflavon were delivered into the skin (97). It is necessary to find an appropriate vehicle to test the lipophilic antioxidants internalization. Solvents, lipoproteins and proteins are able to solubilize the antioxidants and to promote its delivery to cells (98).

5.2. Conventional delivery systems

Over the past few decades there have been many advances in our understanding of the physicochemical properties of both formulation systems and their ingredients. Dosage forms for dermatological drug therapy are intended to produce a desired therapeutic action at specific sites in the epidermal tissue. A drug's ability to penetrate the skin's epidermis, dermis, and subcutaneous fat layers depends on the properties of the drug (physicochemical properties), the carrier base and skin condition. Gels, lotions and creams play an important role as vehicles for the topical treatment of the skin. Both topical and transdermal drug products are intended for external use. However, topical dermatologic products are intended for localized action on one or more layers of the skin (e.g. sunscreens, keratolytic agents, local anaesthetics, antiseptics and anti-inflammatory agents). In modern-day pharmaceutical practice, **semisolid formulations** are the preferred vehicles for dermatological therapy because they remain *in situ* and deliver the drug over extended time periods. In most cases, therefore, the developed formulation will be an ointment, emulsion, or gel. Concerning antioxidant properties, most of the research papers refer gels and emulsions formulations as vehicles. Depending on the lipophilicity of the antioxidant, the chosen vehicle can enhance or reduce its skin deposition being an important factor in the selection of a suitable formulation (99).

The common characteristic of all gels is that they contain continuous structures that provide solid-like properties. Depending on their constituents, gels may be clear or opaque, and be polar, hydroalcoholic, or nonpolar. The simplest gels comprise water thickened with natural gums, semisynthetic materials, synthetic materials or clays. Gel viscosity is generally a function of the amount and molecular weight of the added thickener.

Cassano *et al*. (100) refer a dextran hydrogel linked with ferulic acid for the delivery of vitamin E. Their results showed that ferulate hydrogel was a more effective carrier in protecting vitamin E from photodegradation than hydrogel without antioxidant moieties. Then, the antioxidant hydrogel could be of potential use for cosmetic and pharmaceutical purposes as carrier of vitamin E which can reduce erythema, photoaging, photocarcinogenesis, oedema, and skin hypersensitivity associated with exposure to UV-B radiation (100). Epigallocatechin-3-gallate, a catechin from green tea, has been tested for transdermal delivery and topical application. Catechin gel formulation resulted in prolonged levels in plasma tissues (101). Topical application of resveratrol was tested in a series of hydrogel formulations and solutions, and it was concluded that delivery via a skin route may be a potent way to achieve the therapeutic effects of resveratrol (102).

The most common emulsions used in dermatological therapy are creams, both oil-in-water (O/W), or water-in-oil creams (W/O) emulsions.

To diminish oxidative injury, topically applied antioxidants must reach the susceptible cells. The use of α-lipoic acid and proanthocyanidin significantly enhances collagen synthesis and deposition when used in a standard cosmetic composition (103).

Flavonoids and polyphenols were formulated into an O/W emulsion showing a photoprotective effect when applied into the skin with sunscreens (104).

Zhai *et al* (105) evaluated and proved the antioxidant capacity and preventive effects of a topical emulsion of vitamin E on the response to UV exposure. Other authors (106,107) showed also the protective effect of topical formulations containing antioxidant agents – vitamin C, vitamin E, ferulic acid against UV damage.

Isoflavones (genistein, equol, daidzein, biochanin A, and formononetin) were formulated as 0.5 % individual isoflavone solutions, and the photoprotection effect in a pig skin model was compared to an antioxidant combination solution of 15 % vitamin C, 1 % vitamin E and 0.5 % ferulic acid. The results showed that the protection was less than that provided by the topical antioxidant combination, but nevertheless the isoflavones provided effective photoprotection, and were considered as good candidate ingredients for protection against UV photodamage (108).

Gaspar & Campus (109) evaluated the influence of two different UV-filters combinations, a photostable and a photounstable one, on the photostability as well as on the efficacy of a formulation based on a phosphate-based self-emulsifying wax and on hydroxyethilcellulose, containing vitamin A, C and E derivatives. They concluded that both UV filters combinations did not influence the hydration and anti-aging effects of the formulations containing vitamins, and that the skin irritation was reduced in the presence of these vitamins. In addition, the photostable UV-filters combination had the highest recovery of vitamin A in the photostability studies. Finally, they suggested that the most suitable formulation was the one containing the combinations of vitamins A, C and E with photostable UV-filters.

A commercially available cream containing 0.4 % hydroquinone and 0.15 % retinol with antioxidants was used successfully for topical treatment of melasma (110).

Water-in-oil microemulsions containing quercetin significantly prevented UV-B irradiation-induced skin damages due to the increased penetration into the SC (111). The effectiveness of topical non-ionic emulsion formulations containing quercetin to inhibit the UV-B irradiation-induced skin damages was previously demonstrated by other authors (112). Combined therapy using at least two antioxidants was tested in different microemulsions type (113).

In conclusion, the regular application of skin care products containing antioxidants may be a benefit to efficiently prepare our skin against exogenous oxidative stressors occurring during daily life. Furthermore, sunscreen agents may also benefit from

combination with antioxidants resulting in increased safety and efficacy of such photoprotective products (114).

5.3. Complexation with Cyclodextrins

Cyclodextrins (CyDs) are cyclic water-soluble, non-reducing, macrocycle carbohydrate polymers (115). These starch derivatives are non-toxic ingredients, are not absorbed in the upper gastrointestinal tract, and are completely metabolized by the colon microflora. Besides this, α-CyD, β-CyD and γ-CyD enjoy GRAS status (FDA) for use as an additive in food products, as a flavor carrier and protectant (116,117). Worldwide there are several commercial pharmaceuticals with CyDs based formulations. In topical dosage forms, β-CyD and methylated-β-CyD have more often been used (118-120).

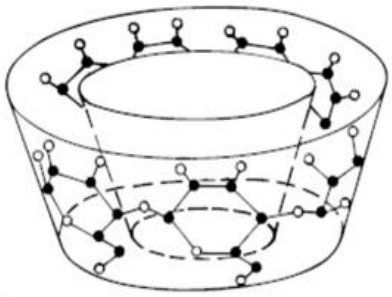

Fig.3- β-Cyclodextrin toroidal structure

CyDs are shown to have a toroidal, hollow, truncated cone structure (**Fig.3**), where the polar sugar hydroxyl groups are oriented to the cone exterior, and consequently the external faces of CyDs are hydrophilic. The result of this amphipathic property is that CyDs can form soluble, reversible inclusion complexes with water-insoluble compounds resulting in compound solubilization (121).

Encapsulation may be a method of protecting molecules as CyDs behave like an empty molecular capsule with the ability to entrap “guest” molecules of appropriate geometry and polarity. The guest molecules (such as antioxidants) are protected from light, heat and oxidation. The entrapped molecules are released from the molecular-inclusion system on contact with water or by exchange with another guest. The method of

encapsulation can also be applied to achieve controlled release in topical formulations. Some of the release mechanisms involves: a change in temperature, moisture or pH; the application of pressure or shear; and the addition of surfactants (122). The method of preparation can affect the effectiveness of the complex (123,124). Accelerated and long-term storage stability of CyD entrapped topical ingredients surpassed that of the traditionally formulated ones (125-128).

Due to their size and hydrophilicity only insignificant amounts of CyDs and drug/CyD complexes are able to penetrate into lipophilic biological barriers, such as intact skin. In general, CyDs enhance topical drug delivery by increasing the drug availability at the barrier surface. At the surface the drug molecules partition from the CyD cavity into the lipophilic barrier. Thus, drug delivery from aqueous CyD solutions is both diffusion controlled and membrane controlled. It appears that CyDs can only enhance topical drug delivery in the presence of water (129).

HP-β-CyD was found to be an effective permeability enhancer for 17-β-oestradiol (130). Methylated CyDs are expected to promote transdermal drug absorption, because they are highly surface active (131).

The entrapment of CyD-drug complexes into liposomes is not a new strategy (132). However, more recently, drug-in-CyD-in-deformable liposomes has been developed for topical delivery (133).

CyDs have been used in formulations for dermal and transdermal delivery (129) and have been used for antioxidant formulation for food industry (134), mainly to promote antioxidant solubilization and chemical stability (135).

Low aqueous solubility of melatonin was significantly increased in a mixture of propylene glycol and HP-β-CyD, motivating enhanced percutaneous absorption of melatonin (136). In the same way the complexation of 4-nerolidylcatechol and HP-β-CyD led to higher drug solubility and stability (137).

Poly(ethylene glycol) citrate (6-armPEG) and its inclusion complex with α-CyD were prepared and used for preparation of vitamin C transdermal patches with stabilization

of vitamin C in topical formulations. The release / diffusion of ascorbic acid across synthetic membrane were considerably enhanced in the presence of 6-armPEG-α-CyD complex (138).

Permeation assays in pig ear skin clearly demonstrated the enhancer effect of β-CyD on 3-O-methylquercetin (3-MQ) permeation. The hydrophilic matrix used controlled 3-MQ release from the β-CyD complex, demonstrating the influence of the inclusion phenomena on 3-MQ diffusion through the gel and partition toward the SC (139).

A topically applied 2 % hydroquinone–CyD formulation significantly reduced the pigmentation of actinic lentigines in Asian subjects by enhanced penetration, bioavailability, and efficacy (140).

Inclusion complex between β-CyD and astaxanthin found to greatly enhance its heat stability under light (141).

Ferulic acid physico-chemical stability was improved by complex formation. Ferulic acid/α-CyD complex showed the most promising properties (high association constant, high degree of photostability, slower drug release) as sunscreen delivery system (142).

Problems associated with sunscreen agents (UV-A and UV-B filters) are their poor solubility and photo degradation which undergoes marked decomposition under sunlight exposure leading to a decrease of its expected UV-protective capacity. It is also desirable to minimize skin penetration of some sunscreens. The inclusion of sunscreen agents into the CyD cavity can suppress their photosensitizing potential and increase their solubility (143).

In spite of a large number of studies dealing with CyD stabilization of antioxidants and topical delivery using CyD containing formulations, antioxidant complexation with CyD appear, thus, as unexploited area with many opportunities for topical delivery.

5.4. Nanoparticulated delivery systems

Novel drug delivery systems have been introduced in the topical delivery of drugs with special incidence on particulate carriers. Particulate type carriers are also known as colloidal carrier systems. Much has been written about the ability of liposomes and other vesicular colloidal carriers to penetrate the SC. The possibility of using such vesicles for transdermal drug delivery was also widely discussed (144-147).

To improve the transdermal drug delivery using lipid-based-aggregates, specialized carriers have been developed, some of which are claimed to cross the skin intact.

Drug association to the carrier can be of three basic different types: encapsulation /inclusion in internal carrier space or carrier core; adsorption to the external surface of the carrier; or covalent association to the membrane or carrier surface.

5.4.1. Liposomes

Liposomes are typically hollow spheres surrounded by a lipid bilayer. Other shapes have also being known and documented (148,149). The hydrophilic parts of lipids, most often of phospholipids, are oriented to the inner and to the outer bilayer surface (**Fig. 4**). Liposomes may be affected by the preparation method as much as this affects the lamellarity and drug distribution, and can be loaded with drugs in vesicle interior or lipid bilayer. Hydrophilic substances are encapsulated in the inner aqueous space and lipophilic compounds are integrated into or on the bilayer (**Fig.4**).

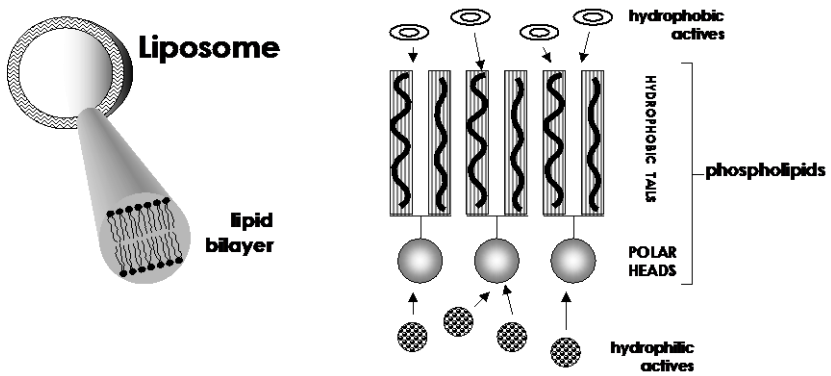

Fig.4- Liposome cartoon. Schematic representation of molecular partition into a bilayer.

Liposome carriers are used in several controlled or targeted release pharmaceuticals. In the cosmetic field, phospholipid liposomes, but also niosomes, i.e. vesicles made of non-ionic surfactants, or sphingosomes, made of sphingolipids, are occasionally used. For transcutaneous delivery, the idea of making vesicles with membranes similar to those present in the SC, led to the preparation of non-phospholipid liposomes, starting with Gray *et al.* in 1973 (150), and many others since then.

Mezei & Gulasekharam (151) were the first to report that liposomes loaded with triamcinolone acetonide facilitate the accumulation within the epidermis and dermis with low systemic levels. Similar results were obtained with local anaesthesia: liposomal tetracaine compares favorably with that mediated by a cream (152).

The years following 89 were rich on topically applied liposome research. Some of the most relevant studies include phospholipids interactions with the skin, lipid vesicle transport through SC, and dermatologically relevant studies with liposomes. Many of these studies revealed interactions between lipid vesicles and the skin via high surface absorption, lipid incorporation or deposition between the intercellular lipid lamella or between the poorly organized lipid regions, and even more likely among themselves, liposomes can also fuse with intercellular lipids (153). In fact, liposomes may affect SC permeability. The basic mechanisms by which this occurs are still uncertain. Two possibilities are proposed: 1) molecules penetrate into the skin associated with intact liposomes; 2) molecules cross the skin, which is affected by the interaction with liposomes or liposome ingredients. In any case, liposomal lipids are incorporate into SC lipid bilayers, and thus facilitate drug transport by changing the lipid layers properties. When this happens, liposomal lipids act as chemical enhancers. Liposomes either act after fusion on the skin via hydration increase or through the action of surfactant or enhancer-like components, such as hydrolyzed fatty acids (**Fig.5**-III)) (145).

Drug deposition is affected by formulation and processing variables involved in liposome preparation. Drug deposition in the skin was studied using a factorial approach to demonstrate the influence of formulation variables. This study explored the topical

delivery of vitamin E acetate (154). Liposomal encapsulation of phycocyanin improved its topical anti-inflammatory activity in cutaneous inflammation models (155). Verbascoside, commonly used in Chinese medicine, was incorporated in large liposomes and resulted in the dermal but not transdermal delivery of the drug (156). Topical application of the antioxidant rh-SOD incorporated in liposomes reduced successfully wound size edema formation in thermally injured tissues comparatively to enzyme solution injected intralesionally or to enzyme gel topically applied (76).

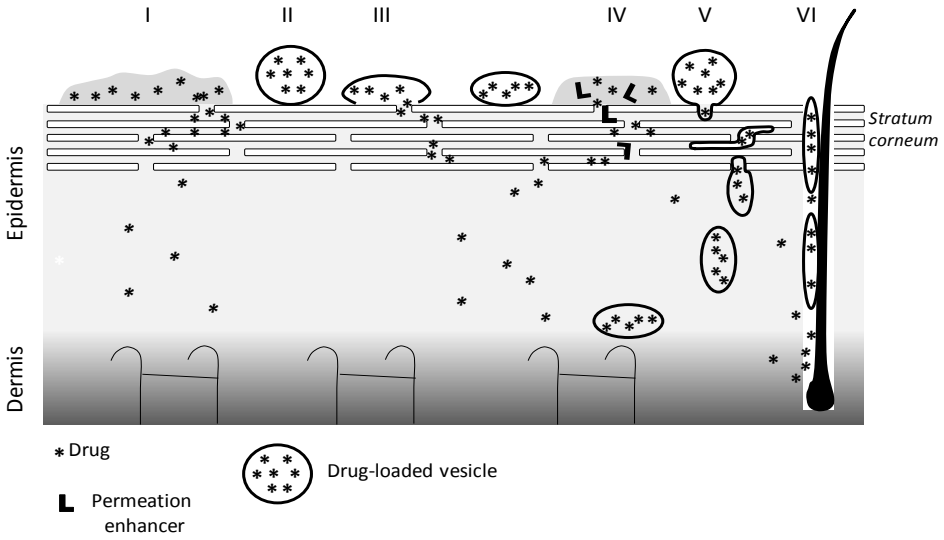

Fig.5- Skin drug delivery: I) drug delivery from a conventional delivery system; II) drug-loaded vesicular carrier with non flexible membrane; III) drug delivery after carrier adsorption and/or fusion with the stratum corneum; IV) drug delivery from a formulation containing a permeation enhancer; V) drug delivery by means of deformable intact vesicle penetration into and through the intact skin; and VI) transappendageal penetration of drug-loaded deformable carriers. (adapted (145))

5.4.2. Deformable vesicles

The hypothesis that liposomes could cross intact SC is very controversial. Liposomes can cross the skin through the pilo-sebaceous units, which are widely open shunts (157) (**Fig.5-**VI)). Carriers with flexible membranes are more deformable than rigid ones, thus penetration of colloidal particles is a function of membrane flexibility. Ideal vesicles for penetration work should pass through pores without suffering extrusion. To ensure vesicle's motion, a strong external driving force must act on the vesicle's components. Naturally occurring transcutaneous hydration gradient is presented as the biophysical mechanistic explanation for spontaneous deformation and passagework (even accepted with some skepticism) (90).

The introduction of special vesicles, which are deformable enough to penetrate even narrow pathways between cells in the skin, started a new chapter in the transdermal delivery field. In recent years, specially designed carriers cross the skin intact and deliver the loaded drugs into the systemic circulation, being at the same time responsible for the percutaneous absorption of the drug within the skin. To differentiate them from the more conventional liposomes are named as deformable vesicles or Transfersomes. With **Transfersomes**, the concept of permeability and penetrability was reviewed. It has now been proven that intact Transfersomes, in contrast to liposomes, penetrate the skin without its disruption (48). Transfersomes® (a trademark of IDEA, AG) composed of highly flexible membranes are the result of combining into a single structure phospholipids (that give the bilayers structure and stability) and an edge-active component (to increase the bilayer flexibility) in order to move spontaneously against water concentration gradient in the skin. These carriers comprise at least phosphatidylcholine and an edge-active molecule acting as the membrane softener. The vesicles have typically 150 ± 50 nm. Many of the techniques used for liposome preparation and characterization can be used for Transfersomes as well. Carrier properties are responsible for membrane flexibility and consequently for vesicle deformability necessary for through-the-skin passagework. This vesicle characteristic is assessed using membrane penetration assays. The transport does not rely chiefly on annexial route but rather is fairly uniform through the entire intact SC.

Simões *et al*. (75) developed and characterized SOD-loaded Transfersomes and have shown for the first time that SOD incorporated into Transfersomes and applied onto a skin area not necessarily close to the inflamed tissue, and is able to promote non-invasive treatment of induced arthritis. Studies demonstrated that this antioxidant enzyme delivered by means of ultradeformable lipid vesicles can serve as a novel region-specific treatment of inflammation after topical application (158).

The driving force for skin penetration by lipid vesicles was firstly argued to be the hydration gradient, or hydration potential difference, across non-occluded SC, and more recently the transepidermal electrical potential was postulated to be the driving force for barrier penetration (144). Additional requirements for successful skin penetration are: 1) lipid vesicles deformability sufficient to allow carriers squeezing through “holes” in the intercellular lipid layers; 2) non-occlusive application which maintains the driving force across the skin. The natural hydration then forces carriers through weak junctions between lipid structures in intercellular space in SC.

The rate of barrier penetration is proportional to the transbarrier water activity difference, which is in turn proportional to the carrier hydrophilicity. Intercolloidal repulsion is essential for carrier penetration. In addition, colloid deformation is needed in order to move particles from high-stress to low-stress sites through pores in a barrier. This transbarrier stress difference can be generated, e.g. when a external pressure produced by a gas stream, is applied on a suspension and this is filtered through narrow pores, or when a vesicle suspension is applied onto the skin, under non-occlusive conditions, and is left to dry out. In this case, a transbarrier hydration gradient is responsible for transbarrier stress difference. The external pressure is then responsible for the vesicle’s motion. **Fig.5** illustrates different possibilities for skin drug delivery. **Fig.5-**V is a schematic representation of the fate of a deformable vesicle suspension on a barrier with narrow pores (145).

Ethosomes are a special kind of unusually deformable lipid vesicles in which the abundant ethanol makes lipid bilayers very fluid (159). This improves delivery of various molecules into deep skin layers such as cannabinoids, testosterone, minoxidil,

propranolol and trihexyphenidil (160). Ethosomes are composed of phospholipid, ethanol and water. The exact process of drug delivery by ethosomes is not completely clear. Ethanol, responsible for the flexibility of the vesicles, evaporates from the formulation once applied on skin surface under non-occlusive conditions. On the other hand, the influence of total ethosomal composition on the bilayer structure of the SC is not yet fully understood.

Regarding the comparison between systems as topical vehicles, the major difference is not technological but in terms of release rate: the diffusional performance through barriers (commonly Franz cells) is, in general, higher for flexible liposomes than for conventional ones (161).

Fang and co-workers developed a new liposomal system which is a mixture of Transfersomes and ethosomes to enhance the skin permeation of cathechins (162).

Other so-called "**elastic vesicles**" were found to be responsible for major morphological changes in the intercellular lipid bilayer structure comparatively to rigid vesicles. The structures are liquid state vesicles made of L-595/ PEG-8-L/sulfosuccinate (50/20/5), and have an average size of 100-120 nm. A two-photon excitation microscopy study has shown that after treatment with "elastic vesicles", thread-like channels were found within the entire SC (163). Fluorescent label incorporated in rigid vesicles was confined to the intercellular spaces of the upper SC.

Deformable vesicles are, thus, alternative and promising drug delivery systems for antioxidant topical delivery, presenting a strategy to overcome the skin barrier. Amongst the various drugs that have been tested, only a few studies present antioxidant skin delivery by means of such carriers.

5.4.3. Non-ionic surfactant vesicles (NSVs)

Niosomes or non-ionic surfactant vesicles, consisting of non-ionic surfactants, are a special example of (non-phospholipid) liposomes. They have vesicular structure, most often stabilized by cholesterol, and have been tested as drug carriers (164). The findings resemble those made with phospholipid vesicles: gel-phase vesicles do not affect transdermal transport and do not cross the skin, whereas fluid phase vesicles can affect molecular diffusion into the skin more strongly. However, no evidence was presented to date that such vesicles could cross SC intact.

NSV can be formulated mainly by lipid layer hydration method, reverse phase evaporation method, or by transmembrane pH gradient uptake process.

NSV alleviate the disadvantages associated with liposomes such as chemical instability, variable purity of phospholipids and high cost, which makes it suitable for industrial manufacture. Besides, NSV are quite stable structures, even in the emulsified form, require no special conditions such as low temperature or inert atmosphere for protection or storage, and provide a large amount of surfactant classes available for the design of this vesicular system (165-167).

NSV appear to have application in topical products containing both hydrophilic and hydrophobic drugs. These vesicles have been applied for cosmetic purposes very successfully and have been experimentally evaluated as carriers of many antiblastic drugs, such as methotrexate, doxorubicin and cisplatin, haemoglobin, indomethacin and the antipsoriatic dithranol. The encapsulation of drugs in NSV can decrease drug toxicity, increase drug absorption and delay removal of drug from circulation due to slow drug release (167). Besides, NSV have also been used to encapsulate lidocaine, estradiol, cyclosporine, erythromycin, α-interferon, plasmid DNA for the human interleukin-1 receptor for topical delivery (168). In fact, topically applied NSV can increase the residence time of drugs in the SC and epidermis, while reducing the systemic absorption of the drug (169).

NSV were used as carriers for tretinoin (TRE), and for selected formulations, niosomes retarded the drug photodegradation (169). Manconi *et al*. (166) evaluated the potential of alkyl polyglucosides (APGs) NSV as topical delivery systems capable of improving the cutaneous delivery of TRE by measuring the influence of drug thermodynamic activity and niosome composition, size, lamellarity and charge on the (trans)dermal delivery of TRE. For this purpose, TRE was incorporated at saturated and unsaturated concentrations in both multilamellar and unilamellar vesicular formulations using two different commercial mixtures of APGs: octyl-decyl polyglucoside and decyl polyglucoside and with a polyoxyethylene lauryl ether (Br30). In recent years, attention has been focused on sugar-based surfactants like APGs for several types of applications, including dermatological ones. Overall, obtained results showed that TRE cutaneous delivery is strongly affected by vesicle composition and thermodynamic activity of the drug. In fact, very hydrophilic surfactants may improve diffusion of TRE through the pig skin. Brij® 30 or Oramix NS 10 (hydrophilic-lipophilic balance (HLB) 9 and 11, respectively) NSV have shown to be able to greatly enhance drug cutaneous retention, especially if compared to the commercial formulation RetinA® and P90 liposomes. On the other hand, small negatively charged niosomal formulations, which are saturated with TRE, have shown to give higher cutaneous drug retention than both liposomes and commercial formulation (RetinA®) (166). Moreover, interactions between skin and vesicles seem to depend on physicochemical properties of the main component of the vesicular bilayer. However, these results are strongly affected by the thermodynamic activity of the drug. Vesicular formulations, which are saturated with TRE have shown to highly promote drug accumulation in the pig skin, while the same does not occur when the vesicular bilayer is not saturated with the drug. Therefore, these results showed an interesting advantage of vesicular systems: once drug saturated vesicular formulations are prepared, they can be diluted to reach the desired drug concentration without losing the saturation of the vesicular bilayer, and therefore, maintaining the same thermodynamic activity of the drug and the same driving force for skin permeation (166).

Another example (167) is the solubilization and stabilization of β-carotene in NSV. The authors found that the maximum entrapment efficiency for β-carotene was reached by the use of Tween® 60, while the lowest was observed in the presence of Tween® 20 probably due to a different HLB. Besides, NSV which contained cholesterol and Tween® 60 in a 1:1 ratio strongly increased their ability in trapping the carotenoid. On the other hand, the addition of cholesterol in a higher ratio decreased the niosomal efficiency in trapping the carotenoid, which could be explained by a possible competitive carotenoid and cholesterol incorporation into NSV. The maximum hydrodynamic diameter of the vesicles was reached by the addition of 0.5 mg/ mL of β-carotene. β-carotene contained in NSV was highly resistant to sunlight, high temperatures and OS induced by different sources of free radicals. They also investigate the behavior of β-carotene in NSV in a highly malignant colon cancer cell line and found that the niosomal formulation strongly inhibited tumour cell growth in a dose-dependent manner. Therefore, NSV provided a convenient, non toxic and inexpensive vehicle for β-carotene in cell culture (167).

Caraf *et al*. (170) showed that NSV could be obtained from polyoxyethylene sorbitan monolaurate-cholesterol in aqueous environment. These authors investigated the delivery of lidocaine HCl and lidocaine base from vesicles through silicone membrane and nude mice skin. The amount of lidocaine permeated through nude mice skin from these NSV was similar to liposomes and only about 2-fold greater than from a micellar system.

NSV were also investigated for their ability to deliver drugs to hair follicles. Niemiec *et al*. (171) investigated the deposition of two polypeptide drugs, interferon-α and cyclosporine into the pilosebaceous units of the hamster ear.

5.4.4. Solid Lipid Nanoparticles (SLN)

Solid lipid nanoparticles (SLN) dispersions introduced in 1991 have been proposed as a new type of colloidal drug carrier system (172-174). The system consists of 0.1-30% (w/w) solid lipid particles in the nanometer range (40-1000 nm), which is dispersed in water, and if necessary, stabilized with preferably 0.5-5 % (w/w) surfactant (173). Generally, they are made of solid hydrophobic core having a monolayer of surfactant coating. The solid core contains the drug dissolved or dispersed in the solid high melting fat matrix. In general, solid lipids can be triglycerides (e.g. tristearin), glycerides (e.g. glyceryl behenate; glycerol tripalmitate), fatty acids (e.g. stearic acid), steroids (e.g. cholesterol) and waxes (e.g. cetyl palmitate). All classes of emulsifiers have been used to stabilize the lipid dispersion. It has been found that the combination of emulsifiers might prevent particle agglomeration more efficiently (175,176).

SLN have different advantages including: good tolerability (avoidance of organic solvents), simple and cost effective large scale production, stability (conferred by surfactants or polymers), site-specific targeting, controlled drug release and protection of liable hydrophilic or hydrophobic drugs from degradation (172,176). However, there is also the insufficient drug loading, drug expulsion after polymorphic transition on storage and relative high water content of the dispersions. To overcome some of the limitations of SLN, **nanostructured lipid carriers (NLC)** were introduced. NLC are composed of solid lipids and a certain amount of liquid lipids, showing an improved drug loading and increased stability on storage thereby reducing drug expulsion. Furthermore, it is possible to control the drug release from the matrix. NLC have been explored for dermal delivery in cosmetics and dermatological preparations (173,175). **Lipid Drug Conjugate (LDC)** nanoparticles were introduced to overcome the limitation of some drug types incorporated in the solid lipid matrix. Lipophilic drugs are usually incorporated in SLN but due to partitioning effects during production, only high potent hydrophilic drugs effective in low concentrations are incorporated in SLN. LDC enables the incorporation of both hydrophilic (e.g. doxorubicin and tobramycin) and lipophilic (e.g. progesterone and cyclosporine A) drugs (175).

Most SLN dispersions are produced by **high pressure homogenization** (HPH). Hot homogenization is the most frequently applied technique, even in temperature sensitive compounds because the exposure time is relatively short. Other production procedures are based on the use of organic solvents (HPH/ solvent evaporation/ diffusion), dilution of microemulsions, w/o/w double emulsion method, membrane contractor technique, phase inversion or also by high speed stirring and/or ultrasonication technique.

Although many different drugs have been incorporated in SLN, there are only few studies about drug release. SLN have been studied and developed for parenteral, dermal, ocular, oral, pulmonary and rectal routes of administration. The results with dermal application are encouraging and probably, this will be the main application of SLN. The dermal administration route further reduces the risks of cytotoxicity or systemic toxicity. The excellent tolerability of the lipid nanoparticles is supported by many available cosmetic dermal products already being introduced to the market fulfilling the regulatory requirements towards tolerability and nanotoxicity. Thus, it seems justified to call the lipid nanoparticles a “nanosave” carrier (173).

In most cases, the incorporation of the SLN dispersion in an ointment or gel is necessary in order to achieve a topical formulation. For dermal application, burst release as well as sustained release (already reported for SLN suspensions) are of interest. Burst release can be useful to improve the penetration of a drug. Sustained release becomes important with active ingredients that are irritating at high concentrations or to supply the skin over a prolonged period of time with a drug.

Hsu *et al*. (177) have reported the preparation of CoQ10 nanoparticles engineered from microemulsion precursors. It was also demonstrated that CoQ10 was more stable within polymeric nanoparticles over dispersion and oil-based formulation when exposed to UV and high temperatures (178). The release profile of Q10-loaded NLC and nanoemulsion was compared (179) and the Q10-loaded NLC exhibited a biphasic release pattern.

Liu *et al*. (180) showed that isotretinoin-SLN formulation with skin targeting may be a promising carrier for topical delivery of isotretinoin. The *in vitro* permeation studies

showed avoidance of the systemic uptake of isotretinoin, a high accumulative amount of isotretinoin in skin and a significant skin targeting effect. Castro *et al*. (181) demonstrated by X-ray powder diffraction that the formation of ion pairing is an interesting alternative for retinoic acid encapsulation in SLN. SLN containing all-trans-retinoic acid (ATRA) were developed and evaluated for topical delivery (182). Authors concluded that the photodegradation of ATRA was reduced, the loading capacity of ATRA in SLN was higher than 95 % (w/w) and the amount of ATRA released from SLN at 4 °C and 37 °C were less than 15 % and more than 60 % (w/w) for 96h, respectively. Jenning *et al*. (183) evaluated the potential use of glyceryl behenate SLN loaded with vitamin A (retinol and retinyl palmitate) and incorporated in a hydrogel and O/W cream. Vitamin A concentrations in the porcine skin tissue suggested a certain drug localizing effect. High retinol concentrations were found in the upper skin layers following SLN preparations. Because of a polymorphic transition of the lipid carrier with subsequent drug expulsion following the application to the skin, the drug localizing action appears to be limited for 6-24h. Best results were obtained with retinol SLN incorporated in the O/W cream retarding drug expulsion. The penetration of the occlusion sensitive drug retinyl palmitate was even more influenced by SLN incorporation. SLN of tretinoin were successfully developed with the help of emulsification-solvent diffusion technique, and the capability of an SLN based gel in improving topical delivery of tretinoin has also been evaluated (184). Drug encapsulation in SLN resulted in a significant improvement in its photostability in comparison to methanolic solution, and also prevented its isomerization. Furthermore, the skin irritation studies carried out on rabbits showed that SLN based tretinoin gel is significantly less irritating to skin as compared to marketed tretinoin cream and clearly indicated its potential in improving the skin tolerability. *In vitro* permeation studies through rat skin indicated that an SLN based tretinoin gel has a permeation profile comparable to that of the marketed tretinoin cream (184).

Souto *et al*. (185) prepared and characterized SLN containing the novel anti-aging substance α-lipoic acid. An entrapment efficiency of 90 % was obtained for all developed formulations using Miranol® Ultra C32 as emulsifying agent. DSC analysis

confirmed that these systems are characterized by a solid-like behavior, even with a very low crystallinity index. Recent studies with SLN and NLC of α-lipoic acid demonstrated an antioxidant activity in developed water-soluble formulation for topical administration (186).

It has been found *in vitro* that SLN have UV reflecting properties. The use of physiological components in SLN is a clear advantage over existing UV protective systems (UV blockers or TiO2) with respect to skin penetration and potential of skin toxicity (176). In addition, it was found that a combination of molecular sunscreens and SLN showed a synergistic effect (172). Wissing *et al*. (187) studied the comparison of two different formulations (SLN and conventional O/W emulsion) as carrier systems for the molecular sunscreen oxybenzone. The release rate was strongly dependent upon the formulation and could be decreased by 30–60 % in SLN formulations. In all test models, oxybenzone was released and penetrated into human skin more quickly and to a greater extent from the emulsions. UV absorption properties of 3,4,5-trimethoxybenzochitin-loaded SLN and SLN free system were also compared (188). Comparing SLN to a conventional emulsion, the amount of molecular sunscreen can be reduced by 50 % in the SLN formulation maintaining the protective level of the conventional emulsion.

5.5. Others

More recently, **pharmaceutical foams** appeared as the solution for skin drug deposition of drugs (189). In order to overcome the poor drug release from the particles, a recent study investigated the capability of dynamic foam to break open nanoparticles upon application to the skin and enhance drug delivery efficiency (190). The study was performed with vitamin E acetate and the results shown that dynamic foam can enhance the release of vitamin E acetate from lipid nanoparticles upon dose application. It is still not clear whether a similar strategy is suitable and effective for other agents. Tocols and derivatives are widely used as antioxidants in food industry and in pharmaceutical compositions, and have been also proposed as drug carriers for topical delivery (191).

Conclusions

ROS are produced by various causes, which can produce lipid peroxidation and other cellular injuries. This production, if excessive, could cause autoxidative damage. It has been demonstrated that oxidative mechanisms are involved in the pathogenesis of both systemic and skin disorders and diseases. An increased interest in antioxidant protection against ROS effects is present in dermatology research and, especially, an increasing focus on the therapeutic topical application of antioxidants has emerged in the last years. However, the real potential of topical antioxidant therapy for the treatment of OS-related skin disorders has not been yet fully recognized. The development of an appropriate and efficient antioxidant delivery for the treatment of OS related diseases is an important goal for the pharmaceutical technology and dermatology fields. The comprehension of ROS role in pathologies, such as skin inflammation and skin cancer could contribute for the establishment of therapeutic strategies involving antioxidant therapy. In the particular case, special emphasis was put on the drug delivery systems of antioxidants and radical scavengers for prophylactic or therapeutic action, to solve problems related to antioxidant skin bioavailability. Incorporation of antioxidants in suitable carrier bases or topical delivery systems could provide a comprehensive solution for OS-related skin diseases.

Abbreviations

ROS: reactive oxygen species

OS: oxidative stress

UV: ultraviolet

MDA: malondialdehyde

SC: *stratum corneum*

CyD: cyclodextrin

3-MQ: 3-O-methyl-quercetin

NSV: nonionic surfactant vesicles

APGs: alquyl polyglucosides

SLN: solid lipid nanoparticles

NLC: nanostructured lipid carriers

LDC: lipid drug conjugate

HPH: high pressure homogenization

ATRA: all-*trans*-retinoic acid

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Chapter 2

Dermal Delivery of Tretinoin Carrier Formulations

Chapter 2.1 Novel Tretinoin Formulations: a Drug-in-Cyclodextrin-in-Liposome Approach

Chapter 2.2 *In Vitro* and *In Vivo* Studies of Novel Topical Tretinoin Formulation

Chapter 2.1

Novel Tretinoin Formulations: a Drug-in-Cyclodextrin-in-Liposome Approach

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Novel Tretinoin Formulations: a Drug-in-Cyclodextrin-in-Liposome Approach

Abstract

Purpose: The aims of this experimental work were the incorporation and full characterization of the system Tretinoin-in-dimethyl beta cyclodextrin-in-ultradeformable vesicles (Tretinoin-CyD-UDV) and Tretinoin-in-ultradeformable vesicles (Tretinoin-UDV).

Methods: The Tretinoin-CyD complex was prepared by kneading and the UDV by adding soybean phosphatidylcholine (SPC) to a nonionic surfactant followed by an appropriate volume of sodium phosphate buffer solution to make a 10 % - 20 % lipid suspension. The resulting suspension was brought to the final mean vesicles size, of approximately 150 nm, by sequential filtration.

The physicochemical characterization was based on: the evaluation of mean particle size and polydispersity index (PI) measured by photon correlation spectroscopy (PCS) and atomic force microscopy (AFM) topographic imaging; zeta potential (ζ-potential) and the SPC concentration determined by Laser-Doppler anemometry and an enzymatic-colorimetric test, respectively. The quantification of the incorporated Tretinoin and its chemical stability (during preparation and storage) was assayed by HPLC at 342 nm.

Results: It was possible to obtain the system Tretinoin-CyD-UDV. The mean vesicle size was the most stable parameter during experiments time course. AFM showed that Tretinoin-CyD-UDV samples were very heterogeneous in size, having three distinct subpopulations, while Tretinoin-UDV samples had only one homogeneous size population. The results of the ζ-potential measurements have shown that vesicle surface charge was low, as expected, presenting negative values. The incorporation efficiency was high and no significant differences between Tretinoin-CyD-UDV and Tretinoin-UDV were observed. However, only Tretinoin-UDV with 20 % lipid concentration formulation remained chemically stable during the evaluation period.

Conclusion: According to our results, Tretinoin-UDV with 20 % lipid concentration seems to be a better approach than Tretinoin-CyD-UDV, attending to the higher chemical stability.

Keywords: Tretinoin; Dimethyl-β-cyclodextrin; Ultradeformable Vesicles; Photon Correlation Spectroscopy/ Laser-Doppler Anemometry; Atomic Force Microscopy

Introduction

Tretinoin, a *trans*-isomeric form of retinoic acid, is effective in the topical treatment of different proliferative and inflammatory skin diseases, such as acne *vulgaris*, ichtiosys, psoriasis, epidermotropic T-cell lymphomas or epithelial skin cancer. Although the exact action mechanism of tretinoin is unknown, current evidences suggest that topical tretinoin decreases the cohesiveness of follicular epithelial cells with decreased microcomedones formation in acne *vulgaris*. Moreover, tretinoin stimulates the mitotic activity and the turnover of follicular epithelial cells causing comedones extrusion. Until now, this is the most effective topical comedolytic agent for the treatment of acne (1). The formulations may contain 0.025-0.1 % of drug on topical creams and 0.01-0.025 % on gels. Unfortunately, this drug presents some technical drawbacks such as chemical instability in the presence of oxygen, light and heat and poor water solubility. Additionally, its topical application may cause irritation and peeling of the treated areas (2-5).

Numerous **drug delivery strategies**, like conventional liposomes, ultradeformable vesicles (UDV), niosomes, solid lipid nanoparticles (SLN), cyclodextrins (CyD), microemulsions and hydrogels have been studied to overcome some drug formulation drawbacks (4). Micro/nano encapsulation of drugs, such as tretinoin (3, 6, 7), has been proposed in order to improve dermal tolerability, encourage adherence, and contribute to better long-term therapeutic outcomes, by facilitating a controlled release (8). For example, Mandawgade *et al.* (9) developed SLN to delivery tretinoin, and more recently Castro *et al.* (10) demonstrated that SLN were efficient as a vehicle to reduce retinoic acid skin irritation, keeping its efficacy. A recent report (6) has presented a new designation of vesicles - permeation enhancer containing vesicles (PEV) and the promotion of dermal delivery of tretinoin by means of such carriers. However, information concerning the drug stability of this drug-carrier association still remains to be clarified.

Phospholipid vesicles, colloidal particles composed of concentric bilayers formed from self-assembly of amphiphilic lipids (e.g., soybean phosphatidylcholine), can act as skin

permeation enhancers as well as drug carrier agents. Their composition affects physico-chemical properties such as size, charge and bilayer elasticity. Several types of vesicles have been described in literature (11). Liposomes, in particular, are nontoxic and biodegradable vesicles, easy to scale up for manufacturing and enabling the encapsulation of both water- and lipid-soluble active components. Besides their unique benefits, liposomes show some disadvantages, such as low stability, low encapsulation efficiency, high cost of manufacturing, degradation by hydrolysis or oxidation, sedimentation, aggregation or fusion during storage (12). Conventional liposomes have been generally reported to remain confined to the upper layer of the *stratum corneum* (SC) and to accumulate in the skin appendages, with minimal penetration to deeper tissues, owing to their large minimum size and lack of flexibility (13). This characteristic has limited their application in delivering drugs to deeper layers of the skin; nevertheless, they were proven to protect tretinoin from light (14, 15).

Recent approaches in modulating vesicle compositions have been conducted in order to develop systems capable of carrying low molecular weight drugs and macromolecules to deeper tissues. **Deformable liposomes or ultradeformable vesicles** (UDV), termed as Transfersomes® (a trademark of IDEA AG, Germany), are the first generation of elastic vesicles introduced in the early 1990s. These vesicles are constituted by phospholipids and an edge activator, which is frequently a single chain surfactant that destabilizes the vesicles lipid bilayers, and consequently increases the bilayers deformability (16). While conventional liposomes were reported to have mainly local or rarely transdermal effects, UDV are considered a carrier for dermal and transdermal drug delivery. They have been proven to be superior to conventional gel-state and even liquid-state vesicles in terms of both enhancement of drug permeation and interactions with human skin (17, 18).

The UDV's action mechanism was investigated, and two hypotheses were proposed (19): first (mechanism 1), intact vesicles may enter the SC carrying vesicle-bound drug molecules into the skin, acting as drug carrier systems. The driving force for the vesicles to enter the skin could be the transcutaneous osmotic, hydrostatic and electrical

gradients (20, 21). Accordingly, vesicles applied on the skin surface follow spontaneously the local hydration gradient, moving into the deeper skin strata (**Fig.1**). This highlights another important difference between deformable and conventional liposomes, the high and stress-dependent adaptability of such UDV, which enables them alone to squeeze between the cells in the SC. Second (mechanism 2), vesicles may enter the SC and change the intercellular lipid lamellae, acting as penetration enhancers of free drug molecules into and across the SC (19, 22, 23).

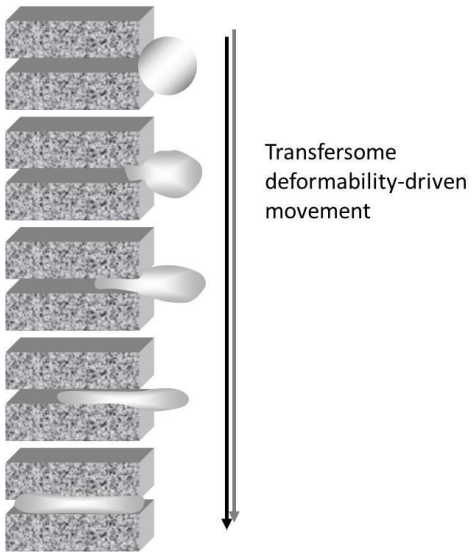

Fig.1 - Deformability-driven movement of Transfersomes through small pores. Adapted from Simões (24).

Accommodation of a poorly water-soluble drug in the lipid bilayer of liposomes is often limited in terms of drug–to-lipid mass ratio. Thus, the entrapment of water-soluble drug/cyclodextrin inclusion complexes in the aqueous phase of liposomes has been proposed in order to avoid such drawbacks, besides the chemical stability (25). Although “drugs-in-cyclodextrin-in-liposome” systems have been previously studied by some authors with other derivatives (β- and hydroxypropyl-β-CD) (25-27), only few studies concern topical formulations (28, 29).

The **aim** of this study was to formulate tretinoin and fully characterize the resulting drug-carrier systems (**Fig.2**): Tretinoin-in-dimethyl beta cyclodextrin-in- ultradeformable vesicles (Tretinoin-CyD-UDV) and Tretinoin-in-ultradeformable vesicles (Tretinoin-UDV) in order to compare their physicochemical parameters during preparation and storage

in use and sterile conditions. In fact, Tretinoin-in-dimethyl-beta-CyD complex has already been studied in previous work (30).

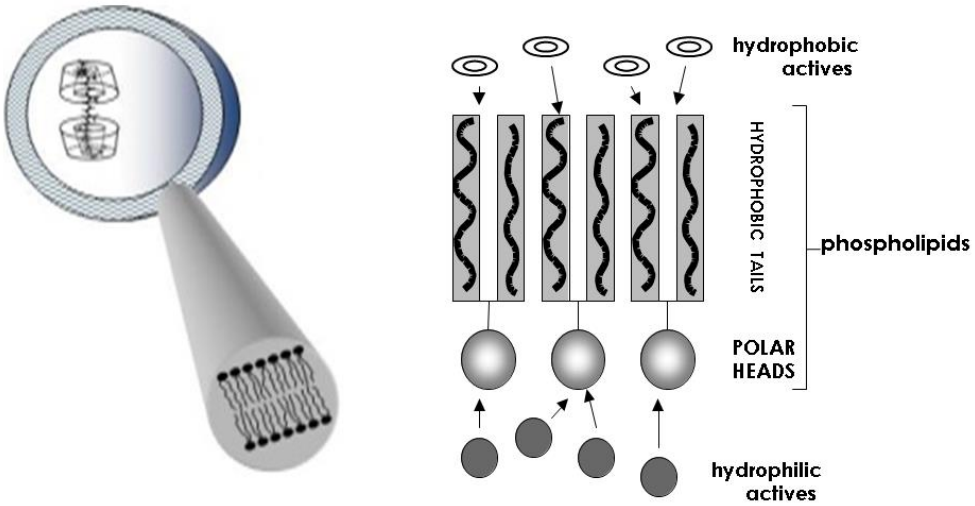

Fig.2- Schematic representation of drug-in-cyclodextrin-in-liposomes (left side) and phospholipid structure and interaction with actives (right side). Adapted from Simões (24).

This experimental work emphasizes the potential of the double vectorization of tretinoin. Theoretically, the entrapment of CyD drug complexes into UDV will increase the advantage of both of them: while the CyD seems to be a solubilizer agent, a chemical protector and sometimes a permeation enhancer (2, 30), one the other hand, the UDV seems to be able to penetrate into the intact skin when administered under non-occlusive conditions (16, 31).

Materials and Methods

Materials

Tretinoin was purchased from Fagron (Terrassa, Spain) and dimethyl-β-cyclodextrin (degree of substitution: 1.8) was a generous donation from Wacker (Stuttgart, Germany). Soybean phosphatidylcholine (S100: 62% Linoleic Acid; 15% Palmitic Acid; 12% Oleic acid and isomers; 5% Linolenic Acid; 3% Stearic Acid) and Tween 80® were purchased from Lipoid AG (Steinhausen, Switzerland) and Sigma-Aldrich (St. Louis, MO, USA), respectively. All other reagents were of analytical grade.

Methods

Preparation of Tretinoin Dimethyl-β-Cyclodextrin Complex (Tretinoin-CyD)

The Tretinoin-CyD complexes were prepared by kneading (mortar and pestle) in 1:4 molar ratio and protected from light and air.

Preparation of Formulations

Three batches of each formulation were prepared according to **Table 1**.

Soybean phosphatidylcholine (SPC) was added to the bilayer softening agent (Tween 80®) and stirred with 50 mM sodium phosphate buffer solution, pH 7.4, to make a 10 – 20 % lipid suspension. Firstly, the crude suspension was filtered to prepare 100 ± 50 nm, assumedly unilamellar vesicles (32). In order to obtain larger vesicles, it was frozen and re-thawed repeatedly (5 cycles). The resulting suspension was brought to the final mean vesicle size, of approximately 150 ± 50 nm, by sequential filtration under nitrogen stream.

The addition of the complex was made in the beginning of the mixture (Tretinoin-CyD-UDV) and, in another similar formulation, only tretinoin was added (Tretinoin-UDV).

At the end of the formulation process, three batches of each one were sterilized by filtration through a 0.22 µm pore filtered and stored at 4 °C.

The formulations were always protected from light and air.

Table 1 – Tretinoin-CyD-UDV and Tretinoin-UDV formulations.

Ingredients	Class	Action	Amount (%)		
			Tretinoin-CyD-UDV	Tretinoin-UDV	Tretinoin-UDV
Tretinoin	Drug	Topical treatment of different proliferative and inflammatory skin diseases	0.1 (Drug:CyD, 1:4 m/m)	0.1	0.05
DM-β-CyD	Hydrophilic water-soluble oligosaccharides	Inclusion-complex formation		_____	_____
SPC	Phospholipid	Vesicles forming component	10	10	20
Tween 80®	Non-ionic surfactant	Vesicles flexibility			
Sodium phosphate buffer solution	Buffering agent (pH 7.4)	Hydrating medium	qs 100	qs 100	qs 100

DM-β-CyD = Dimethyl-β-Cyclodextrin

Characterization of Vesicles Formulations before and after centrifugation

Non-incorporated tretinoin was separated from incorporated one by ultracentrifugation (L8 - 60M ultracentrifuge™ Beckman Instruments Inc., Fullerton, CA, USA). The centrifugation was performed using the following conditions: 180,000 *g* at 15 °C during 2 hours.

Mean Vesicle Size Determination

The mean vesicle size (hydrodynamic diameter) and the mean size distribution in terms of polydispersity index (PI) were measured by photon correlation spectroscopy using Nano-S equipment from Malvern® (Worcestershire, UK). The mean particle diameter

was obtained by averaging three measurements at an angle of 90° in 1 cm diameter cells at 25 °C. All the samples (10 µL) were diluted with 1 mL of filtered distilled water.

Atomic Force Microscopy (AFM) Studies

A NanoWizard II equipment (JPK Instruments®, Berlin, Germany) mounted on the top of an Axiovert 200 inverted microscope® (Carl Zeiss, Jena, Germany) was used for imaging the samples. The AFM head is equipped with a 15-mm z-range linearized piezoelectric scanner and an infrared laser. Samples were diluted to 1/100 (Tretinoin-CyD-UDV) and to 1/50 (Tretinoin-UDV) in Milli-Q water and deposited on freshly cleaved muscovite mica for 20 min. After subsequent washes, the samples were allowed to air dry at room conditions. Imaging of the samples components were performed in air, in tapping mode. Oxidized sharpened silicon tips (ACL tips® from Applied Nanostructures, CA, USA) with a tip radius of 6 nm, resonant frequency of about 190 kHz and spring constant of 45 N/m were used for the imaging. Imaging parameters were adjusted to minimize the force applied on the scanning of the topography of the complexes. Scanning speed was optimized to 0.5 Hz (Tretinoin-UDV) and 2 Hz (Tretinoin-CyD-UDV) and acquisition points were 512 × 512. Imaging data were analyzed with the JPK image processing v.3 (JPK Instruments®). The width and height of the samples were calculated from cross-section plots. All dimensions measurements were performed using the Gwyddion software version 2.19® (Czech Metrology Institute, Brno, Czech Republic). The histograms of the structures width for each sample were constructed choosing the ideal bin size to achieve the best-fitted Gaussian model peak width. The selected binning size was approximately 5 nm. The maximum values of the Gaussian peaks represent the different statistical measures of the width of the samples.

Zeta Potential Determination

Zeta potential values of empty and loaded vesicles were measured by Laser Doppler Anemometry (Malvern Zetasizer 2000®, Worcestershire, UK) after diluting the samples

in sodium phosphate buffer solution pH 7.4 with 10 mM Tween 80® in order to investigate the effect of drug loading on vesicles surface charge.

Pressure Driven Transport

The flow of loaded vesicles was determined with a 1 mL-filtration unit that miniaturizes the commercial available pressure filtration device. The flow of suspension was driven by an external pressure of 0.7 MPa created by a nitrogen stream and measured as a function of time. Track-etched polycarbonate membranes with 30 nm mean pore diameter were used. The suspension was collected into a container on a Sartorius LA620P scale® (Sartorius, Göttingen, Germany). The data were collected with the Wedge software® for Windows (TAL Technologies Inc., Philadelphia, PA, USA).

Incorporation Efficiency (IE) determination

Tretinoin quantification was determined using a validated HPLC method (33, 34). A HPLC system consisting of a 32 Karat Software (Beckman Instruments®, Palo Alto, CA, USA), a Midas Spark 1.1 autoinjector (Spark®, AJ Emmen, The Netherlands) and a Diode-Array 168 detector (Beckman Instruments®) was used for this drug assay. The injector was fitted with an injection loop of 50 µL. Chromatographic separations were performed using a reversed-phase chromatography column (Lichrocart® 250-4, 5 µm C18, 200 mm x 4 mm, Merck Millipore, Darmstadt, Germany). The detection wavelength was 342 nm and the mobile phase was composed of 0.01 % trifluoroacetic acid and acetonitrile (15:85, v/v, %) at a flow rate of 1.0 mL/min.

SPC quantification was made by an enzymatic colorimetric assay (Phospholipids kit, Spinreact®, Sant Esteve de Bas (GI), Spain). Phospholipids are hydrolyzed by phospoliphase D and the liberated choline is subsequently oxidized by choline oxidase to betaine with the simultaneous production of hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide couples oxidatively the 4-aminophenazone (4-AP)

and dichlorophenol to forms a quinonemine dye. The intensity of formed color is proportional to the phospholipids concentration and measured spectrophotometrically at 505 nm (UV-VIS spectrophotometer Shimadzu® UV-160A, Kyoto, Japan).

The IE was then calculated according to the following equation:

Eq. 1. IE (%) = $([drug]_{final} \times [lipid]_{initial}) / ([drug]_{initial} \times [lipid]_{final}) \times 100$

Stability studies

Stability studies were performed in 6 independent batches of each formulation in *use* (3 batches) and *sterile* (3 batches) conditions stored at 4 °C during 90 days. The formulations parameters were determined in each six time points, as described before.

Statistical Analysis

The results are reported as mean ± standard deviation (SD) of at least three replicates for each batch. The results of all these experiments were statistically analyzed by analysis of variance (ANOVA) using SigmaPlot 11.0 software®. The Holm-Sidak Test was used whenever possible (i.e. when the normality and equal variance tests pass) for all pairwise comparisons for stability results. It is more powerful than the Tukey and Bonferroni tests and is recommended as the first line procedure for most multiple comparison testing. The overall significance level is 0.05 and statistical differences were represented by different letters.

Results

Mean Lipid Vesicles Size

The mean vesicles size results obtained for the two formulations tested (Tretinoin-CyD-UDV and Tretinoin-UDV) are represented in **Fig.3** and **Fig.4**.

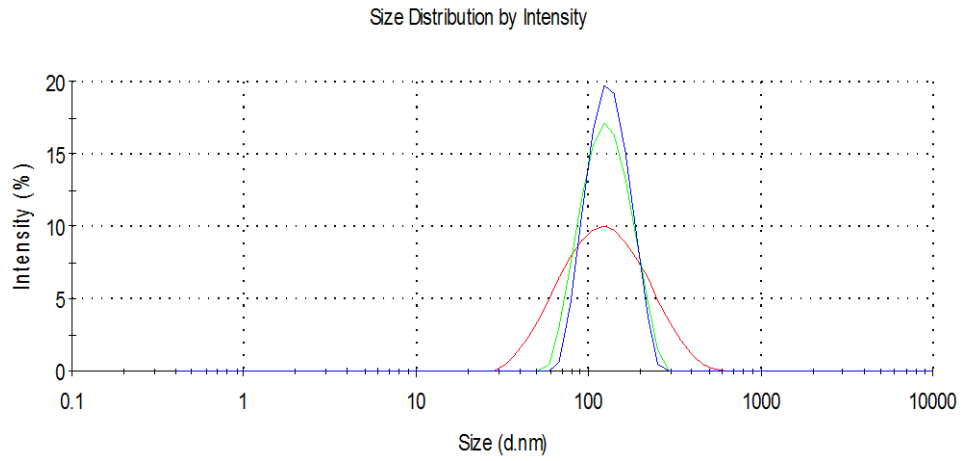

Fig.3 - Mean size distribution by intensity of Tretinoin-CyD-UDV after preparation (n = 3 independent batches).

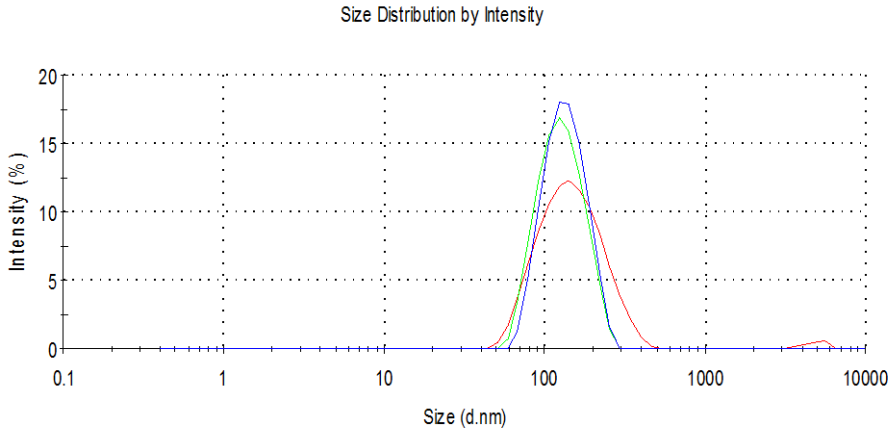

Fig.4 - Mean size distribution by intensity of Tretinoin-UDV after preparation (n = 3 independent batches).

The final mean size distribution obtained for both formulations was approximately 150 ± 50 nm, as expected for midfielders unilamellar UDV. The polydispersity index (PI) was used as a measure of a unimodal mean diameter distribution. Small values of PI (< 0.1) indicate a homogenous population, while higher values (> 0.3) indicate a higher

heterogeneity. All the results met quality criteria and the respective PI was also acceptable (below 0.2). Literature data concerning the influence of CyD presence on liposomal vesicle dimensions are controversial. On one hand, some authors did not find significant differences between the mean size of liposome containing the drug-CyD complexes and the liposomes containing drug alone (27, 35). On the other hand, other authors reported the opposite (36, 37). These contrasting results may depend on the different experimental conditions.

AFM results

Tretinoin-CyD-UDV and Tretinoin-UDV formulations were possible to image by AFM. **Fig.5** shows isolated discoid structures on both types of sample, but with differences in size and height. **Fig.5A** and **5D** are examples of the images analyzed for each Tretinoin-CyD-UDV and Tretinoin-UDV samples, respectively. After conducting width and height measurements with cross sections for each complex imaged, width frequency count histograms (**Fig.5 C** and **5D**, respectively) and a height frequency count histograms (data not shown) were built. The total number of analyzed particles was 110 for Tretinoin-CyD-UDV samples and 193 for Tretinoin-UDV samples. Tretinoin-CyD-UDV has a homogeneous height distribution of 0.954 $\pm$ 0.005 nm, while Tretinoin-UDV samples have two different height groups: 1.05 $\pm$ 0.07 nm (group 1) and 2.37 $\pm$ 0.18 nm (group 2).

The results acquired from the width histograms (**Fig.5C**), after applying the Gaussian model, showed exactly the opposite. Tretinoin-CyD-UDV is a heterogeneous sample, with 3 distinguishable subpopulations with different average cross-section widths: 45.0 ± 0.7 nm (subpopulation 1), 75.9 ± 1.3 nm (subpopulation 2) and 109.4 ± 1.8 nm (subpopulation 3). These subpopulations presented different relative frequencies on the whole sample constitution: 61 %, 24.5 % and 14.5 % for subpopulations 1, 2 and 3, respectively.

For the Tretinoin-UDV formulation, Gaussian fitting of the width histogram showed only one homogeneous population, with an average cross-section width of 38.8 ± 0.3 nm (**Fig.5F**).

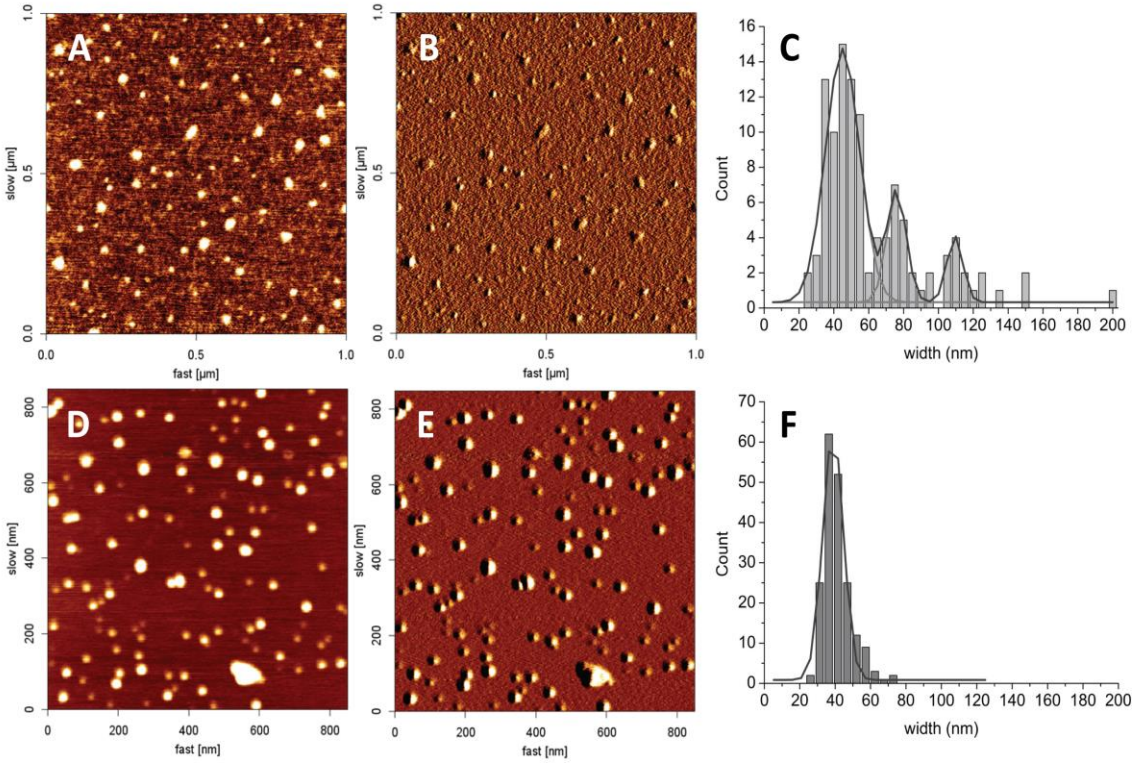

Fig.5- Atomic force microscopy results for Tretinoin-CyD-UDV (A to C) and Tretinoin-UDV (D to F) samples. **A, D** – height images (A - 1 × 1 µm^2, height relative scale of 0.616 nm; D - 0.85 × 0.85 µm^2, height relative scale of 1.855 nm); **B, E** – error images (of the same areas); and **C, F** – width frequency count histograms. Samples imaged on air in tapping mode.

Zeta Potential

The zeta potential characterizes the surface charge of the particles, giving information about repulsive forces between particles or vesicles, and makes a prediction about the stability of colloidal dispersions (38).

The zeta potential was measured for both formulations and also for the respective controls (**Fig.6**).

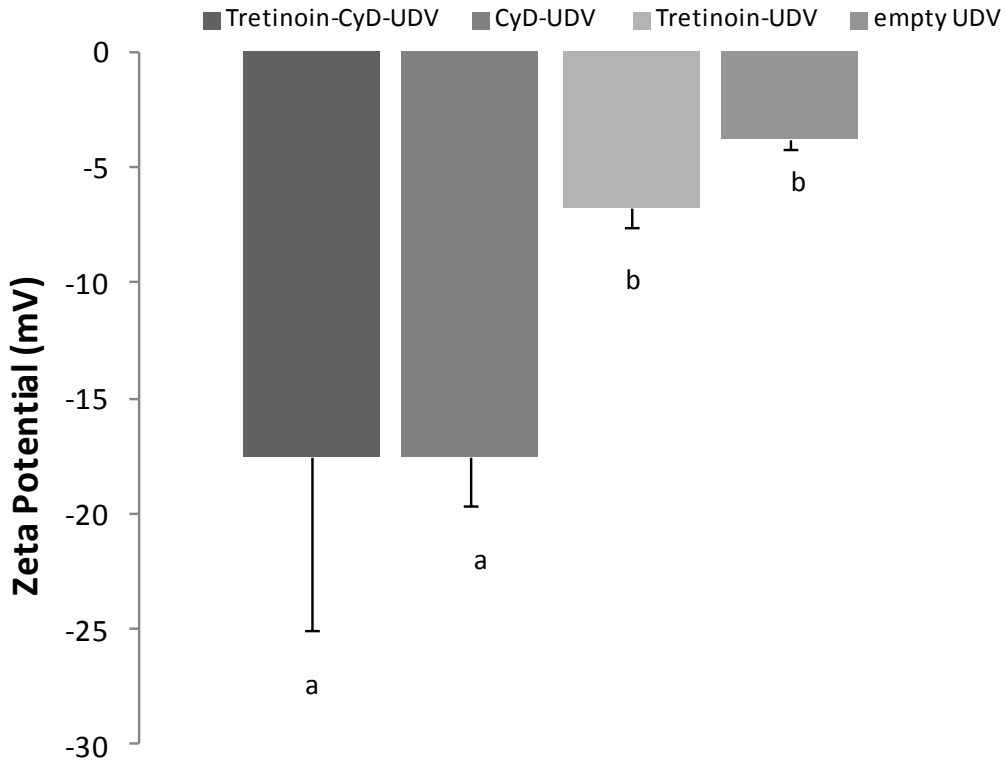

Fig.6- Zeta potential of the two loaded formulations: Tretinoin-CyD-UDV and Tretinoin-UDV before centrifugation and the respective controls (CyD-UDV and empty UDV). (mean ± SD, n =3 independent batches, statistical differences marked by different letters)

The results of the zeta potential measurements revealed that vesicles surface present negative charge values, as expected attending to the use of a nonionic surfactant in the formulation (Tween 80®) and also to the neutral pH of the buffer. There were statistically significant differences between the mean zeta potential values of Tretinoin-CyD-UDV and Tretinoin-UDV besides empty UDV ($p<0.05$). One possible explanation for the drug-induced-vesicle negative zeta potential values may be related to the changes on the vesicles surface structure due to the complex incorporation, a phenomenon also observed by other authors (39). This may result in the change of the phosphatidylcholine head groups orientation at the surface of vesicles, with the choline group plane lying below the phosphate group plane, contributing to the negative surface potentials measured. This measurement was higher for Tretinoin-CyD-UDV formulation probably due to CyD´s interaction.

Pressure Driven Transport Measurements

The flows of both formulations are represented in **Fig.7.**

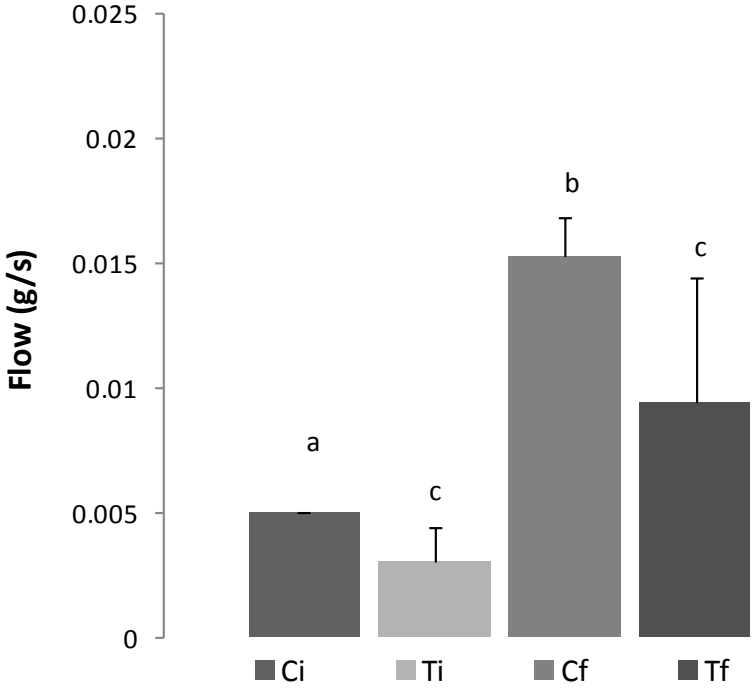

Fig.7 - Flows of the two formulations: Tretinoin-CyD-UDV before centrifugation (Ci), after centrifugation (Cf) and of Tretinoin-UDV before centrifugation (Ti), after centrifugation (Tf). (mean ± SD, n=3 independent batches, statistical differences marked by different letters)

It can be observed in **Fig.7** that after centrifugation and the consequent separation of the non-incorporated drug fraction from the vesicles, they became more flexible. The flow was higher for Tretinoin-CyD-UDV perhaps due to the weak intermolecular interactions (CyD non-covalent interactions). In fact, CyD-UDV (control) had the highest flow (0.022 ± 0.100 g/s) which may justify the flow differences obtained between the two formulations. However, the differences between the median values among the formulations are not large enough to exclude the possibility that the difference is due to random sampling variability. There is not a statistical difference, except for Tretinoin-CyD-UDV before and after centrifugation (Ci *vs* Cf, $p<0.05$).

Incorporation Efficiency

Incorporation efficiency (IE) is the percentage fraction of the total drug incorporated into UDV. This is a crucial parameter in the evaluation of vesicular formulations, as vesicles with maximum IE would improve drug bioavailability. This parameter represents the degree of interaction between the carrier and the drug (32).

The incorporation efficiencies of the tested tretinoin formulations are represented in **Fig.8**.

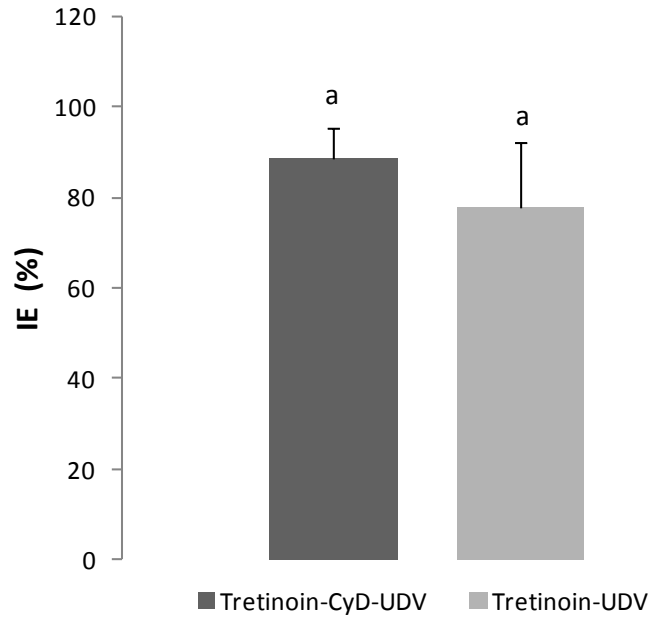

Fig.8 - Incorporation Efficiency (IE) of the two formulations: Tretinoin-CyD-UDV and Tretinoin-UDV. (mean ± SD, n=3)

The IE was high, approximately 90 %, and no significant differences were observed between Tretinoin-CyD-UDV and Tretinoin-UDV ($p = 0.305$).

Stability studies

Stability studies were performed in both formulations in *use* and in *sterile* conditions, during 90 days, regarding the following parameters: vesicles size, drug content, and incorporation efficiency. The results are presented in **Table 2**.

The mean vesicle size was checked at different time points and the results obtained were around 120 nm for both formulations (Tretinoin-CyD-UDV and Tretinoin-UDV), as theoretically expected (32). According to the size results obtained, it is possible to conclude that the vesicle's diameter was a very stable parameter during stability study time course.

In general, there were no significant differences between both formulations (Tretinoin-CyD-UDV and Tretinoin-UDV) in *use* and in *sterile* conditions concerning the incorporation efficiency during study time course, which reflected the good stability of these formulations regarding this parameter. However, considering the chemical stability, the drug assay varied significantly over time for both formulations, especially in *use* conditions ($p<0.05$). In fact, some chromatograms (**Fig.9 and Fig. 10**) of the 60^{th} day showed a great increase of the isomers peaks areas for these formulations, which led us to consider the tretinoin degradation. The drug assay was lower for Tretinoin-CyD-UDV due to a possible lost during the complexation process.

Table 2- Stability study of Tretinoin-UDV (Tret-UDV) and Tretinoin-CyD-UDV (Tret-CyD-UDV) with 10 % lipid concentration, in *use* and *sterile* conditions (mean values of 3 independent batches ± SD).

Formulation	Storage conditions	Time period	Parameters		
			Vesicle size (nm)	Tretinoin (mM)	IE (%)
Tret-UDV	In *use conditions*	0	129 ± 2	4.5 ± 1.8	77.8 ± 14.6
		7	126 ± 3	4.0 ± 1.5	63.9 ± 1.7
		15	128 ± 3	3.6 ± 0.9	93.9 ± 8.0
		30	127 ± 3	3.2 ± 0.9	78.9 ± 15.6
		60	125 ± 1	2.8 ± 1.8	87.0 ± 4.3
		90	129 ± 4	2.8 ± 0.8	62.2 ± 2.7
	Sterile conditions	0	128 ± 2	4.5 ± 1.8	85.2 ± 16.4
		7	124 ± 1	4.3 ± 1.5	76.2 ± 15.0
		15	128 ± 3	4.4 ± 1.4	82.6 ± 8.0
		30	126 ± 1	3.9 ± 1.3	93.4 ± 11.3
		60	129 ± 9	3.5 ± 1.5	87.1 ± 0.1
		90	126 ± 5	2.3 ± 1.1	90.3 ± 16.8
Tret-CyD-UDV	In *use conditions*	0	117 ± 1	3.4 ± 2.4	88.7 ± 6.7
		7	122 ± 4	2.9 ± 2.0	77.7 ± 11.1
		15	120 ± 2	2.9 ± 2.0	66.0 ± 8.4
		30	122 ± 0	2.4 ± 1.7	62.9 ± 9.2
		60	125 ± 3	2.0 ± 1.5	89.9 ± 14.3
		90	126 ± 1	1.8 ± 2.2	91.5 ± 1.4
	Sterile conditions	0	117 ± 1	3.4 ± 2.4	88.7 ± 6.7
		7	119 ± 0	3.1 ± 2.3	81.4 ± 11.2
		15	120 ± 3	3.0 ± 2.2	88.2 ± 3.9
		30	122 ± 2	2.6 ± 1.8	82.2 ± 5.2
		60	124 ± 4	1.3 ± 2.0	80.5 ± 1.5
		90	121 ± 3	0.6 ± 0.6	80.7 ± 16.7

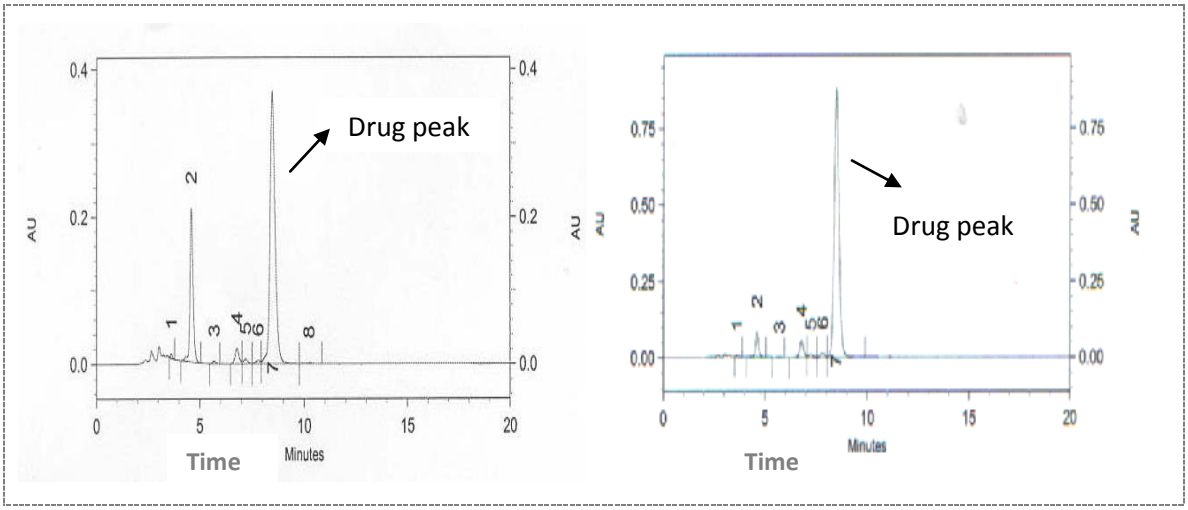

Fig.9 - Chromatogram of Tretinoin-UDV formulation (10 % lipid concentration) in *use* (at left) and in *sterile* (at right) conditions, after 60 days of storage. AU = Arbitrary units. Tretinoin standard presented a retention time of 8.4 min.

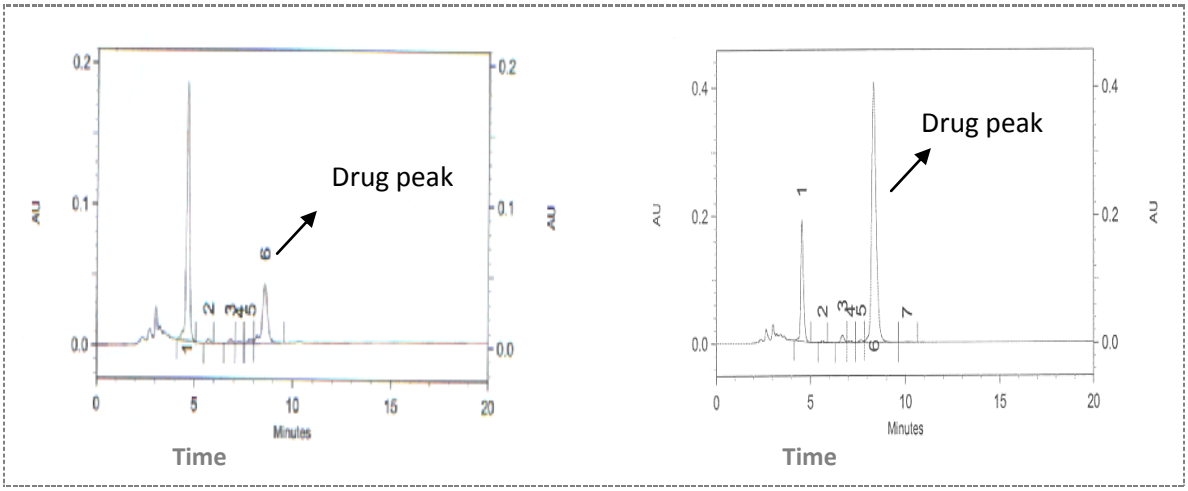

Fig.10- Chromatogram of Tretinoin-CyD-UDV formulation (10 % lipid concentration) in *use* (at left) and in *sterile* (at right) conditions after 60 days of storage. AU = Arbitrary units. Tretinoin standard presented a retention time of 8.4 min.

Taking into consideration the results obtained in this comparative study, an attempt was made to prepare a formulation with higher phospholipid content in order to achieve a Tretinoin-carrier system with suitable physical characteristics and proper drug chemical stabilization. **Fig.11** and **Table 3** present the results obtained with the increase of total lipid concentration from 10 to 20 % in Tretinoin-UDV formulation regarding its stability. In this case, five parameters (vesicles size, zeta potential, drug content and drug loading, incorporation efficiency) were determined in each 6 time points during 90 days.

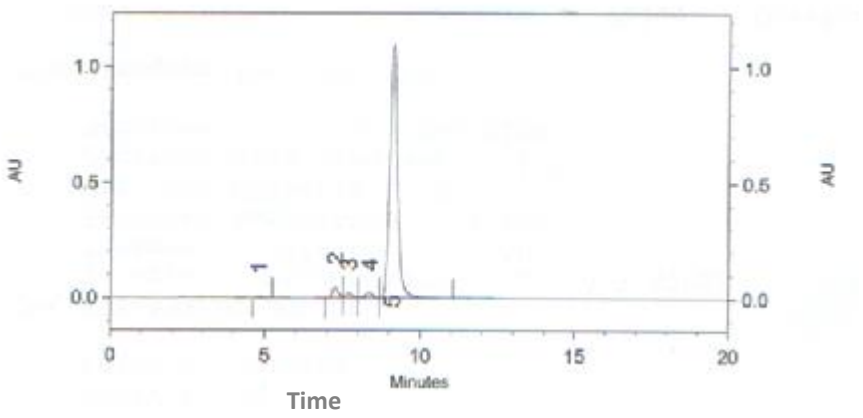

Fig.11 - Chromatogram of Tretinoin-UDV formulation (20 % lipid concentration) in *use* conditions, after 60 days of storage. AU = Arbitrary units. Tretinoin standard presented a retention time of 8.4 min.

In general, this formulation presented around 130 nm of vesicles size and low negative zeta potential. However, although the vesicles size remained stable over time, the same did not happen with zeta potential. In fact, this parameter decreased during 90 days. In terms of colloidal systems, this variation was not important since there was no evidence of particles aggregation according to the size evaluation. Nevertheless, important parameters such as IE and drug loading remained quite stable (approximately higher than 90 % and 13 µg/ mg SPC, respectively).

In summary, the incorporation of tretinoin in UDV with total 20 % lipid concentration (IE> 90 %), resulted in adequate physicochemical parameters of the system and higher stabilization of Tretinoin in the lipophilic environment.

Table 3- Stability study of Tretinoin-UDV (Tret-UDV) with 20 % lipid concentration in *use* and in *sterile* conditions (mean values of 3 independent batches ± SD). ND = Not Determined

Formulation	Storage conditions	Time period	Parameters				
			Vesicle size (nm)	Zeta Potential (mV)	Tretinoin (mM)	IE (%)	Drug Loading (µg/ mg SPC)
Tret-UDV	In use	0	127 ± 2	-6.5 ± 0.3	2.3 ± 0.4	113.0 ± 24.9	13.4 ± 2.4
		7	135 ± 2	-3.4 ± 0.2	ND	ND	ND
		15	129 ± 1	-3.0 ± 0.0	2.0 ± 0.0	68.7 ± 12.3	14.4 ± 6.3
		30	134 ± 1	-1.6 ± 0.4	2.0 ± 0.0	98.0 ± 6.8	13.2 ± 1.5
		60	135 ± 2	-1.3 ± 0.3	2.2 ± 0.2	97.1 ± 25.5	14.1 ± 2.3
		90	132 ± 0	-2.0 ± 0.7	2.0 ± 0.6	ND	13.1 ± 3.3
	Sterile conditions	0	131 ± 10	-5.9 ± 0.6	2.4 ± 0.4	104.0 ± 19.3	14.7 ± 3.0
		7	135 ± 6	-3.9 ± 0.7	ND	ND	ND
		15	132 ± 4	-2.6 ± 0.1	2.1 ± 0.1	84.5 ± 9.0	13.0 ± 1.2
		30	136 ± 6	-1.3 ± 0.3	2.3 ± 0.5	95.9 ± 18.0	15.3 ± 3.1
		60	135 ± 4	-1.5 ± 0.2	2.2 ± 0.3	92.3 ± 14.8	15.4 ± 0.7
		90	135 ± 5	-1.4 ± 0.3	1.6 ± 0.7	ND	14.9 ± 5.4

Discussion

In this research work, the stability of six independent batches of each Tretinoin-CyD-UDV and Tretinoin-UDV formulations prepared in the same conditions were fully analyzed.

Transfersomal or UDV formulations were prepared according to Simões *et al.* (40, 41). Transfersomes preparation method permits the formulations to be handled without temperature, organic solvents or drastic mechanical conditions (40, 42). Taking temperature as example, this is a factor that could have a negative influence on tretinoin stability because of its high susceptibility. In fact, high temperatures used in other methods represent an obstacle to laboratory handling and industrial production of tretinoin formulations.

The preparation method involves a stirring step, after which midfielders unilamellar UDV are obtained (**Fig.12**).

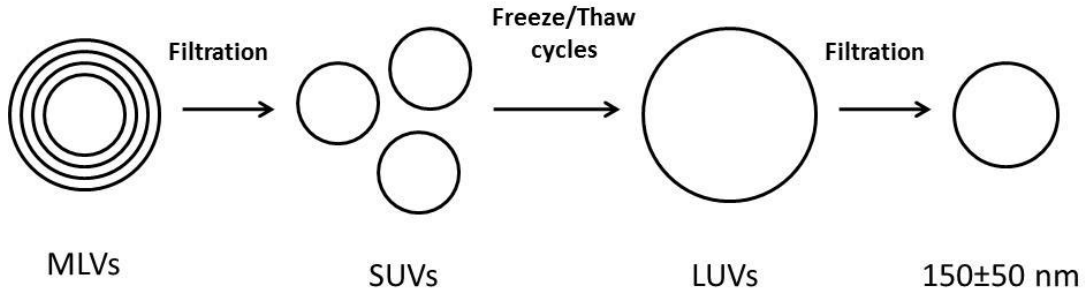

Fig.12 – Scheme of vesicle size and lamelllae variation during midfielders unilamellar vesicles production. MLVs (multilamellar vesicles); SUVs (small unilamellar vesicles); LUV (large unilamellar vesicles).

After stirring, and in order to obtain small size unilamellar vesicles (SUVs), the formulations were filtered under pressure through decreasing pore size membranes. Formulations were then freeze-thawed, so that vesicle fusion occurred and large unilamellar vesicles (LUVs) were formed (32). To get the desired **vesicle size** of unilamellar structures (150 ± 50 nm), a last sequential filtration was made. Although the filtration steps were time consuming, the relatively simple method used proved that it is

possible to produce nanovesicles with uniform characteristics. In fact, the average size of the vesicles did not oscillate much, being quite stable during the 90 days of storage. This is an important indicator of the formulation stability since if aggregation of UDV has occurred, the average size would have increased. It is also important to state that the resulting size is necessary to ensure vesicles deformation, and for further skin penetration (32, 43). Moreover, the size obtained is also dependent on the formulation composition. In fact, the results confirmed an appropriate composition to produce nanovesicles, and the vesicle size was the most stable physical parameter obtained from the stability studies.

The differences between Tretinoin-CyD-UDV and Tretinoin-UDV size could be due to the different molecular interaction that might occur in these two different UDV formulations. Cyclodextrins, particularly the methylated derivatives, are known to remove lipid components from cell membranes and liposomes by forming inclusion complexes with the lipids, especially with cholesterol. It is thus possible that, during or after entrapment of the drug/cyclodextrin complex solutions, lipids enter the cyclodextrin cavity, replacing the drug. This could destabilize the bilayers to some extent, enabling partial or complete leakage of drug content from vesicles and varying the vesicles size (higher PI) (44). Another hypothesis is the different localization of the two Tretinoin forms inside UDV. Tretinoin-CyD might be located in the UDV aqueous lumen (the complex was added at the beginning of the vesicles preparation). On the contrary, the free Tretinoin form will be located within the UDV lipid bilayers.

From subsequent AFM scanning images one can also conclude that the isolated species on both formulations were stable and do not change their structure upon scanning with the AFM tip. The results obtained for Tretinoin-CyD-UDV samples showed 3 different subpopulations, in terms of width, instead of only one. Although it was observed a subpopulation with larger size due to sample aggregation, it had the lowest frequency of occurrence. For Tretinoin-UDV formulation, AFM width results showed only one homogeneous population, but with lower width/diameter value (38.8 ± 0.3 nm). The differences on the diameter of the particles measured by AFM and DLS could be

explained by different factors: (i) sample preparation (dryness and consequent vesicles shrinkage in AFM, in contrast to DLS); (ii) with AFM, after sample deposition on mica, it gains a semi-discoid shape and the tip only could scan over the surface of the attached particles, while on DLS the particle size is determinate by the light intensity of the sample in colloidal suspension; (ii) DLS may have technical limitations for bi- or trimodal samples, as it has been reported that DLS is inappropriate to accurate measure particle mixtures with a large difference at the ratio between the diameters of the particles (45). Hoo *et al.* (46) made a comparison of AFM and DLS methods to characterize nanoparticle size distributions, and concluded that heterogeneous samples in size could not be accurately characterized using conventional DLS due to the intensity difference of the light from the small particles *versus* large particles. Thus, AFM was used here to further observe the size homogeneity since a complex system is being characterized (CyD + Drug + UDV).

Zeta potential is another parameter that can help determining the physical stability of liposomal formulations. Tretinoin formulations used in this study presented a negative charge just after preparation. Soybean phosphatidylcholine (SPC) is a phospholipid often used in topical dosage forms due to the presence of oleic and linoleic acids in its compositions, both permeation enhancers (40, 47). Sinico et *al.* have shown that negatively charged liposomes strongly improved newborn pig skin hydration and tretinoin retention in the skin (48). However, Kitagawa and Kasamaki found that cationic liposomes consisting of double-chained cationic surfactant, phosphatidylcholine increased the delivery of retinoic acid about two-fold (12, 49).

In the tested formulation, Tween 80®, a non-ionic surfactant, acts as an edge activator to provide high deformability to the vesicle (40, 50). In addition, soybean phosphatidylcholine (SPC), is a neutral or zwitterionic phospholipid over a pH range from strongly acid to strongly alkaline (47). Thus, the low vesicles surface charge is an expected result. Interestingly, in spite of a low surface charge (especially, nearly zero) which in theory is prone to vesicle aggregation and fusion (6), such phenomenon did not occurred, as it was demonstrated by the vesicle size evaluation during the stability

studies. The absence of this phenomenon may be explained by steric stabilization, which in this case is obtained from Tween 80®, a bulky surfactant that prevents vesicle fusion.

The **incorporation efficiency** (IE) was high for both Tretinoin-CyD-UDV and Tretinoin-UDV formulations. According to Maestrelli *et al.* (51), multilamellar vesicle liposomes (MLV) containing ketoprofen-CyD complexes exhibited reduced IE in comparison with those containing drug alone, which was explained by the drug location inside the vesicles. In the case of MLV, the volume occupied by the aqueous phase is smaller than that occupied by the lipidic phase. Thus, when the drug is added to the organic phase, it is entrapped in greater amounts in the multi-layer liposomal membrane, when compared to the hydrophilic complex into the aqueous lumen. However, in this work, this difference was not the same as the UDV prepared were midfielder unilamellar vesicles. In addition, the CyD derivative used, dimethyl-β-CyD, was added in the beginning of the suspension formation, as already mentioned. Therefore, the complex entrapment into the aqueous core of these vesicles was probably more favorable attending to the higher water solubility and stability of the complex (30), and to the UDV preparation method. UDV have already been proven to be a versatile system for the incorporation of both hydrophobic and large hydrophilic entities (52).

Regarding **chemical stability**, several attempts have been made to incorporate Tretinoin in pharmaceutical dosage forms in order to achieve an effective and stable drug delivering system (2, 3, 6, 53). Some of these approaches, such as liposomes, niosomes and nanocapsules or complexation with cyclodextrins, have showed some improvement of topical application of Tretinoin, either by actually enhancing the bioavailability of the drug or by slightly improving Tretinoin chemical stability (6, 30, 53-55). However, until now, none of those formulations can do both things successfully enough. In this research work, Tretinoin was entrapped in one of the most promising vesicles in nanotechnology, Transfersomes (56). The aims were to achieve both drug bioavailability and chemical stability. However, it was observed that only Tretinoin-UDV formulation with 20 % lipid concentration assured drug chemical stability when stored protected

from light at 4 °C. Considering that the other UDV formulations with 10 % lipid concentration, even in the presence of CyD, were not sufficient to stabilize this drug, the lipid concentration was found to be the most important formulation requisite in our experimental conditions.

Conclusions

In the present work, we investigated the use of the combined approach of CyD complexation and UDV entrapment in order to obtain adequate physicochemical parameters, including drug loading capacity. Although the enhanced water solubility of the Tretinoin-CyD complex may allowed its entrapment in the internal aqueous lumen of the vesicles instead of drug incorporation into the lipid bilayers, the results from stability studies showed, in general, no significant differences between the two different formulations, including the chemical stability. However, increasing the total lipid concentration from 10 to 20 % allowed getting comparable results in terms of vesicle´s characteristics and reducing Tretinoin isomerization in storage. This last formulation also showed high and stable incorporation efficiencies, which means it could be a good candidate to improve topical bioavailability of this drug. This study also showed that sterilization by filtration did not change vesicle and/or vesicle-drug system characteristics, especially regarding the lipid-concentrated formulation. After these characterization and stability studies, future work will continue with Tretinoin-UDV with 20 % lipid concentration concerning the topical delivery of the drug.

Acknowledgments

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Chapter 2.2

In Vitro and *In Vivo* Studies of Novel Topical Tretinoin Formulation

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In Vitro and *In Vivo* Studies of Novel Topical Tretinoin Formulation

Abstract

Introduction: Ultradeformable vesicles are highly promising tools to enhance the percutaneous transport of different drugs such as tretinoin across the skin barrier**,** alter the drug bioconversion in viable skin, and also to increase the formulation stability at absorption site and reduce the drug induced irritation.

Methods: Topical delivery of tretinoin - loaded ultradeformable vesicles (tretinoin-UDV) was evaluated concerning different studies, such as: the release and permeation profiles (tape stripping); skin penetration (fluorescence analysis); induced electrical changes in skin barrier properties; cytotoxicity (MTT and Trypan Blue assays) and skin irritation in *in vivo* conditions (Draize test). The novel formulation performance was also compared to a commercial tretinoin formulation regarding *in vivo* studies.

Results: Results with synthetic membranes indicated a sustained and controlled drug release, as expected for this formulation. Regarding the permeation study, a dermal delivery was obtained since it was not detected any drug amount in the receptor phase after 24 h. Nile Red-UDV stained intensively mostly in the *stratum corneum*, corroborating the tape stripping results. Tretinoin-UDV decreased skin resistance, suggesting its ability to induce skin barrier disruption. Finally, the formulation vehicle (empty UDV) and tretinoin-UDV were not toxic in *in vitro* and *in vivo* conditions, at least, at 5×10^{-3} mg/mL and 0.5 mg/mL of tretinoin, respectively.

Conclusion: Tretinoin-UDV is a promising delivery system for tretinoin dermal delivery without promoting skin irritation (unlike other commercial formulations), which is quite advantageous for therapeutic purpose.

Keywords: Tretinoin; Ultradeformable Vesicles; Topical Delivery; Skin Barrier Function; Toxicity

Introduction

Tretinoin has long been considered the gold standard, being the most common retinoid used for treatment of skin diseases (1). However, retinoids have the potential to cause troublesome dose-dependent irritation and dryness (2), and are usually associated with poor tolerability, resulting in limited patient compliance (3). In addition, the tretinoin teratogenicity is the most serious side effect. This teratogenic effect is caused by the interference of the exogenous retinoic acid with endogenous retinoic acid signaling, which plays a role in patterning the developing embryo (4). However, the transdermal penetration and systemic bioavailability of topical retinoids are not yet completely clarified. Thus, there is not a consensual opinion about the use of topical retinoids during the pregnancy (5).

In order to exert its action, tretinoin must overcome the main physiological barrier of human body – the **skin**. The skin consists of 250 µm thick inner skin region (dermis) and of 500 µm thick outer skin region (epidermis). The barrier function is mainly due to the outermost part of the epidermis, the *stratum corneum (SC)*, which is composed of approximately 90 % corneocytes. These cells are tightly packed in stacks that run perpendicular to the skin surface, and clusters of which are responsible for skin permeation resistance and *reservoir* effect. Quality and crystallinity as well as total quantity of lipids in the SC determine the skin barrier perfection (6, 7). There are several natural pathways through the skin barrier, such as sweat ducts, sebaceous glands, hair follicles, clusters-junctions, inter-cell-clusters and inter-corneocyte paths. Small hydrophilic entities have the propensity to cross normal skin between corneocytes clusters (20-30 nm diameter), molecules heavier than 400-500 Da are nearly completely confined to skin surface (6, 8).

Dermal and transdermal drug delivery is problematic because skin has a low permeation rate. However, skin delivery offers the advantage of enhanced local effect (dermal), elimination of first pass hepatic metabolism and sustained drug release for a prolonged period of time (transdermal) (7).

Considering skin properties and tretinoin physical and chemical characteristics, besides its irritation potential, it becomes clear that it is extremely difficult to formulate this

drug in a way that can, at the same time, overcome the skin barrier without irritation and provide the necessary stability to the drug. In recent years, technological advances have been made, aiming to overcome skin barrier, which can generally be divided into physical and chemical methods (9). **Physical methods** employed for increasing transport across the skin may use some form of mechanical, electrical (e.g. electroporation and iontophoresis), magnetic (e.g. magnetophoresis) or thermal energy to disrupt the skin (transiently), and are much more invasive then chemical methods. **Chemical methods** use permeation enhancers such as alcohols, fatty-acids, amines, surfactants and others. These chemicals may be included in various types of vesicles, helping those vesicles penetrating the skin (7-9).

Numerous **drug delivery strategies** such as liposomes have been studied to overcome some drug technical drawbacks (10). Liposomes were shown as an interesting carrier for tretinoin in skin disease. In a comparative clinical evaluation of liposomal gel of benzoyl peroxide and tretinoin for acne, it was concluded that the liposomal tretinoin gel was shown to have better response in the treatment of comedones, whereas the liposomal benzoyl peroxide gel of this investigation showed a predominant response in the treatment of papules and pustules (11). Hence, concomitant therapy with liposomal tretinoin and liposomal benzoyl peroxide gel is expected to give more effective treatment of acne (11, 12). *In vitro* permeability experiments with [^{3}H]trans retinoic acid showed that its encapsulation into SC lipid liposomes not only prolongs drug release but also promotes drug retention by the viable skin in order to reduce systemic absorption and the side effects associated with topical application (11, 13).

Transfersomes®, also known as ultradeformable vesicles (UDV), result from the combination of phospholipids with surfactant in the right proportion to make them highly deformable, and to have one single bilayer. They were first introduced in the early 90s by Cevc & Blume (14) and, in contrast to liposomes which cannot penetrate the skin (only passing through the most favorable pathway), Transfersomes actually *penetrate* skin, by self-modifying their form and passing through pathways much narrower than their own diameter. Shape variation is only possible because the surfactant moves all the way through the bilayer into the zones of major tension. The

quantity of lipid determines the extension of Transfersomes penetration (8, 15). The surfactant is often a single chain edge activator, such as sodium cholate, Span® 60/65/80 or Tween® 20/60/80, and at the concentrations normally used, there is no toxicity associated (6, 7, 16, 17). The type and quantity of surfactant determines the transfersomal stability and permeability, and thus UDV must be designed and optimized on a case-by-case basis (9, 17).

Applied on the skin surface under non-occlusive conditions, these elastic vesicles are able to squeeze through intercellular regions of the SC under the influence of the transepidermal water-activity gradient, which creates very strong force acting on the skin via vesicles (18). This enforces widening of the weakest intercellular junctions in the barrier and creates 20-30 nm wide transcutaneous channels. The channels allow sufficiently deformed lipid vesicles to cross the skin along the hydration gradient. Spontaneous motion of the highly deformed, hydrophilic entities through the skin barrier is consequently based on barrier penetration rather than on trans-barrier permeation (8). Therefore, it is not the ideal situation to apply the diffusion concept to vesicle transport across the skin (19). Transfersomes can effectively protect the drug against undesired rapid clearance into cutaneous blood vessels and are capable of retaining the drug long enough on, in and below the skin barrier. Furthermore, they can across the SC independently of the drug concentration (20), and may improve the dermal drug delivery while reducing systemic absorption. Therefore, the therapeutic dose could be reduced and, consequently, also the dose dependent side effects (11). The ability of Transfersomes to deliver drugs across the skin is therefore well-documented and widely accepted. Transfersomes have been used as carriers for various compounds, amongst which are proteins and peptides (21) such as insulin (22), corticosteroids (23), nonsteroidal anti-inflammatory drugs such as ketoprofen (20) and anticancer drugs (24).

The **aim** of this research work was to study the topical delivery of tretinoin in ultradeformable vesicles (tretinoin-UDV), a stable formulation already successfully characterized in previous experiments (25). The drug topical delivery was achieved by

combining different experimental techniques: by combining different experimental techniques: *in vitro* release and permeation/penetration studies; electrical resistance measurement; skin cells viability/cytotoxicity and the *in vivo* evaluation of skin irritation.

Materials and Methods

Materials

Tretinoin was purchased from Fagron (Terrassa, Spain). Soybean phosphatidylcholine (S100) was purchased from Lipoid AG (Steinhausen, Switzerland). Tween 80®, Nile Red, Phospate Buffer Saline PBS 7.4, Thiazolyl Blue Tetrazolium Bromide (MTT), Dimethyl Sulfoxide (DMSO) and Trypan Blue were purchased from Sigma-Aldrich (St. Louis, MO, USA), respectively. All other reagents were of analytical grade.

Methods

Preparation of Tretinoin Loaded Ultradeformable Vesicles

Phosphatidylcholine (SPC) was added to the bilayer softening agent (Polysorbate 80) and stirred with 50 mM sodium phosphate buffer solution pH 7.4, to make a 15 and 20 % of lipid suspension. Tretinoin was added (0.05 %) to the lipid suspension since the beginning of preparation, always protecting samples from light and air.

Firstly, the crude suspension was filtered to prepare 100 ± 50 nm, assumedly unilamellar vesicles. In order to obtain larger vesicles, suspension was frozen and re-thawed (5 cycles). The resulting suspension was brought to the final size, of approximately 150 ± 50 nm, by sequential filtration.

Tretinoin Analysis

Tretinoin quantification was determined using a validated HPLC method (26, 27). An HPLC system consisting of a 32 Karat Software (Beckam Instruments®, Palo Alto, CA, USA), a Midas Spark 1.1 autoinjector (Spark®, AJ Emmen, The Netherlands) and a Diode-

Array 168 detector (Beckman Instruments®) was used for this assay. The injector was fitted with an injection loop of 50 µL. Chromatographic separations were performed using a reversed-phase chromatography column (Lichrocart® 250-4, 5 µm C18, 200 mm × 4 mm, Merck Millipore, Darmstadt, Germany). The detection wavelength was 342 nm. The mobile phase used was composed of 0.01 % trifluoroacetic acid and acetonitrile (15:85, v/v, %) at a flow rate of 1.0 mL/min.

Topical Delivery

All topical delivery experiments were performed under light protection.

Regarding the release study, two tretinoin-UDV formulations with 15 and 20 % of lipid concentration were tested. However, considering previous stability data (see results and discussion), only the selected formulation (tretinoin-UDV with 20 % of lipid concentration) was used for the following topical delivery experiments. The *in vivo* skin irritation potential of tretinoin-UDV was also compared to the marketed 0.05 % tretinoin formulation (Ketrel®, Saninter, Lisbon, Portugal).

In Vitro Release Study with Synthetic Membranes

In vitro release profile was determined by using vertical Franz diffusion cells with a diffusion area of 1.0 cm^2. About 0.25 g sample was spread over the donor side of the membrane (Polysulfone, Tuffry® 25 mm, 0.45 µm, Pall Corporation, Port Washington, NY, USA) under non-occlusive conditions. Polysulfone membranes were soaked for thirty minutes in isopropyl myristate, a skin enhancer.

The receptor phase contained a 3.7 mL mixture of 0.01 M saline phosphate buffer (pH = 7.4) and 0.1 % PEG-40 (m/v) in order to solubilize tretinoin in the receptor solution maintained at 32-37 °C under stirring (Julabo U3 Thermostat and Multimagnetic stirrer SBS®, Labexchange, Paris, France). Care was taken to remove any bubbles between the underside of the diffusion membrane and the solution in the receiver compartment.

At pre-determined times (2.0, 3.0, 4.0, 5.0 and 6.0 h), samples (200 µL) were collected and the same volume was replaced with fresh solution. The drug solubility in the receptor phase was 0.0061 mg/mL, thus assuring the maintenance of sink conditions

during the diffusion experiments. The tretinoin amount in the receptor phase was quantified by HPLC, as described above. Data was expressed as cumulative amount of tretinoin permeated through the membrane filter, considering the total amount of drug applied for each formulation.

In Vitro Skin Permeation/Penetration Studies

In vitro skin permeation study was performed according to the method described by OECD Guideline 428 (28) at the same conditions as described before, except the use of fresh pig ear skin. Tretinoin-UDV formulation (200 µL) was spread over the skin (1.77 cm^2) in contact with 7 mL of receptor phase, and 24 h later, the skin samples were rinsed to remove excess formulation and dried with filter paper. After the skin samples had been attached and fixed on a smooth surface, the SC was removed using 10 adhesive tapes (Scotch® 3M, S. Paulo, Brazil). In order to improve the reproducibility of the tape stripping technique, a cylinder (2 kg) on a foam and an acrylic disk (both with an area of 5.73 cm^2) was used resulting in a pressure of 349.3 g/cm^2. This pressure was applied for 10 sec for each tape. All the tapes (including the first one) with the *stratum corneum* (SC) removed and the remaining skin (viable epidermis and dermis, ED) were cut into small pieces used for the extraction process, previously validated. In this extraction process, 5 and 1 mL of tetrahydrofuran (THF) 70% were added to the SC tapes and ED pieces, respectively. Both samples were stirred for 2 min in a vertical mixer, and sonicated for 20 minutes in order to obtain the cell lysis. After filtration (0.45 µm), the supernatant was injected on HPLC to quantify the amount of tretinoin retained in each skin layer.

In Vitro Skin Penetration Study with Nile Red

Nile Red was incorporated in the UDV formulation instead of the drug and then applied to the donor compartment of Franz diffusion cells. Fresh skin sections treated with PBS pH 7.4 were used as control to determine tissue autofluorescence. After 24 h, the surface of the skin was carefully cleaned, and the diffusion area of skin samples was completely frozen using isopentane at ~ -30 °C with liquid nitrogen, embedded in

Cryomatrix™ (Shandon, Thermo Scientific, Runcorn Cheshire, UK) and sectioned using a cryostat microtome (Shandon 5030). The longitudinal skin sections (8 µm) were mounted on positively charged microscope slides (Superfrost® Plus, Shandon). The slides were visualized without any additional staining or treatment using a fluorescence microscope (Olympus BX60®, Tokyo, Japan) equipped with a camera (Olympus DP50®) and filters for Nile red, excited at 530 nm with emission fluorescence at 550 nm (adapted from Lopes *et al.* 2009 (29)).

In Vitro Skin Electrical Resistance

To evaluate the effect of tretinoin-UDV on the skin barrier function, the electrical resistance of the tissue was measured before and after application of this formulation and water (control). Resistance across each membrane was measured by passing a fixed electrical current across the skin sample using a LCR multimeter (Tenma T.M. 72-960®, Taiwan, China) connected to two stainless steel electrodes, using a setting of 100 Hz. Skin samples were mounted in diffusion cells, and the donor and receptor compartments were filled with PBS pH 7.4. The cells were maintained at 32-37 °C under stirring (Julabo U3 Thermostat and Multimagnetic stirrer SBS).

After 20 min of equilibration, the electrodes were inserted in the donor and receptor compartments to measure the baseline skin resistance, taking care not to touch the skin membrane. At 4, 8 and 24 h, the skin samples were rinsed with water and carefully blotted dry. The donor compartment was then refilled with PBS, and electrical resistance was measured after about 20 min of equilibration (adapted from Lopes *et al.* 2009 (29)).

In Vitro Cytotoxicity in Human Keratinocyte Cell Line HaCaT

The cytotoxicity potential of tretinoin-UDV was evaluated in HaCaT cell line, a non-tumorigenic immortalized human keratinocytes cell line obtained from Cell Lines Services - CLS® (Eppelheim, Germany). Thus, the handling of these cells culture was always according to CLS protocol procedures. These cells were grown in Dulbecco's modified Eagle's medium (DMEM), rich in glucose, supplemented with 10 % fetal bovine

serum (FBS), 2 mM L-glutamine, 1% penicillin–streptomycin (10000 U/mL) and 1 % fungizone (250 U/ mL) (Gibco®, Grand Island, NY, USA) at 37 °C in a humidified incubator with 5 % CO_2 atmosphere (adapted (30, 31)).

Tretinoin-UDV formulation was previously sterilized by filtration (0.2 µm) and diluted in culture medium at 5, 10 and 15 µg/mL. The osmolarity (automatic osmometer, Knauer®, Berlin, Germany) of the sample with the highest concentration was also measured. Beside the negative control (non exposed cells), both assay results were also compared to empty UDV formulation and tretinoin (complexed with cyclodextrin) aqueous solutions (32) with the same dilution factors.

Cell metabolic activity 24 h after tretinoin-UDV formulation exposure was assessed as described below by the MTT assay (often used as viability method). The cells were seeded at a density of 7,000 cells/well and the culture medium was added until 200 µL to a cluster plate of 96-well. After incubation, the cell culture was exposed and incubated again for a period of, approximately, 24 h. The MTT assay was performed according to the following conditions (33): after exposure, cells were immediately washed with PBS and incubated with new culture medium (200 µL) containing 50 µL of MTT (1 mg/mL in PBS pH 7.2) during 4 h at 37 °C, 5 % CO_2. After this time, the medium was carefully removed and the same volume of DMSO was added to each well to solubilize the crystals. The plate was gently shaken for 2 h and protected from light. The optical density of reduced MTT was measured at 570 nm by spectroscopy on an automatic microtiter plate reader (Synergy™ HT Multi-Mode from BioTeK® Instruments Inc., Winooski, VT, USA) equipped with a specific software (Gen5, Biotek®). At least, three independent assays with six replicates of each sample were performed, and the cell metabolic activity (MA ≈ viability/proliferation characteristics) was calculated according to **Eq.1**.

Eq.1 $MA\ (\%) = (Abs\ 570\ nm_{sample} - Abs\ 570\ nm_{DMSO}) / (Abs\ 570 nm_{negative\ control} - Abs\ 570\ nm_{DMSO}) \times 100$

MTT is a yellow water-soluble tetrazolium dye that is reduced by mitochondrial reductase of only metabolically active live cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized (in DMSO) and quantified by spectrophotometric means. Regarding the Trypan Blue assay, it relies on the breakdown in membrane integrity determined by the uptake of a vital dye to which the cell is normally impermeable (34).

The cell viability after tretinoin formulation (and the respective controls) exposure was also confirmed by Trypan Blue assay and calculated according to **Eq.2**. The cells were incubated in a cluster plate of 12 wells. After trypsinization according to Boukamp *et al.* (35) and resuspension of the cells, an aliquot was taken and vortexed with 0.4 % trypan blue solution (1:1). After 5 min of incubation at 37 °C, the cells were counted with the hemacytometer.

Eq.2 Cell Viability (%) = [viable cells **/** (viable cells + non viable cells)] × 100

In Vivo Skin Irritation Potential and Histological Assessment

The skin irritation potential of tretinoin-UDV was also compared to the marketed tretinoin formulation (Ketrel®) when applied to back skin of shaved BALB/c female mice (20-25 g). Animals were housed in separate polypropylene cages of suitable size and kept under standardized environmental conditions throughout the study.

Mices were divided into three groups of three animals each and treated daily by topically applying to the back (6 cm^2), for 10 days, 100 µl of: group a) empty UDV; group b) 0.05 % tretinoin-UDV; and group c) 0.05 % Ketrel®. Animals were examined for signs of skin irritation each day before treatment throughout the treatment period and given an overall "irritation score" defined by Draize (36): 0 = no differences, 1 = light erythema, 2 = well defined erythema, 3 = strong erythema, and 4 = very strong erythema with presence of a scar. At the end of tenth day, the animals were sacrificed. Skin biopsies (1 cm^2) were taken from all treated areas, preserved in 10% formalin solution and processed for histopathological studies. Tissue sections were stained by hematoxylin and eosin standard procedure, and finally examined under a light microscope (Olympus, AH-2 Vanox, Tokyo, Japan).

All animal experiments were carried out with the permission of the local animal ethical committee in accordance with the EU Directive (2010/63/UE), Portuguese law (DR 129/92, Portaria 1005/92) and all relevant legislations. The experimental protocol was approved by Direcção Geral de Veterinária (DGV).

Statistical Analysis

The results are reported as mean ± standard deviation (SD) of at least three replicates/ treatment. The results of all these experiments were statistically analyzed by Analysis of Variance (ANOVA) using SigmaPlot 11.0 software®. The differences were considered statistically significant when $p < 0.05$.

Results

In Vitro Release Study with Synthetic Membranes

Lipid composition, size and surface charge of liposomes, and drug solubility can affect stability and pharmacokinetic profiles of liposomes and drug release pattern (11, 37).

The release profiles of tretinoin-UDV formulations (with 15 % and 20 % of lipid concentration) are described in **Fig.1**.

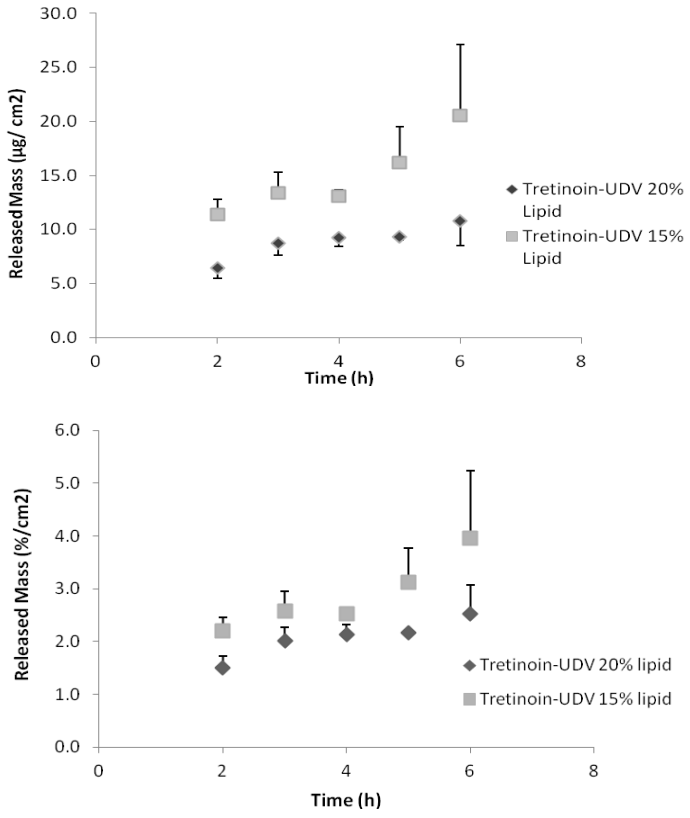


Fig.1- Cumulative Released Mass (µg/cm^2 and %/cm^2) of tretinoin-UDV formulated with 15 and 20 % lipid concentration during 6 h (mean ± SD, n= 3 for each formulation).

The release study of these formulations revealed a sustained and controlled profile, as theoretically expected attending to the formulation carriers (UDV) and to the increasing lipid concentration. Although the release rate was significantly higher for tretinoin-UDV with 15 % of lipid concentration ($p = 0.012$), this formulation was unstable at the 7th month (data not shown) unlike tretinoin-UDV formulation with 20 % of lipid concentration. Thus, this last formulation was chosen for the following topical experiments.

In Vitro Skin Permeation/Penetration Study

The *in vitro* skin permeation study was carried out through the whole skin also under non-occlusive conditions. After 24 h, no drug was detected in the receptor phase. Regarding the tape stripping experiments, 70 % tetrahydrofuran (THF) was selected as the extraction solvent due to its greater reproducibility among the samples and higher recovery rate of tretinoin in SC and ED samples, when compared with mobile phase. In fact, a greater drug retention in SC (~ 10-25 µm of thickness) was obtained than in epidermis and dermis (**Table 1**), as theoretically expected. All measurements also showed low coefficient of variation indicating good reproducibility.

Table 1- Tretinoin quantification (µg/cm^2) in *stratum corneum (SC)* and epidermis + dermis (ED) samples (n = 6).

Tretinoin Assay	SC	ED
Mean (µg/cm^2)	0.372	0.050
Standard Deviation (±)	0.018	0.008
Variation Coefficient (%)	4.926	15.730

In Vitro Skin penetration study with Nile Red-UDV

The skin penetration of Nile Red previously incorporated in the same UDV formulation was visualized by fluorescence microscopy. In general, untreated skin presented some autofluorescence (**Fig.2A and C**), especially in the SC. Nile Red incorporation resulted in a strong fluorescent staining of SC, epidermis and dermis, but especially of the first ones

(**Fig.2B and D**). In this case, the fluorescence seemed fairly homogenously dispersed into the skin.

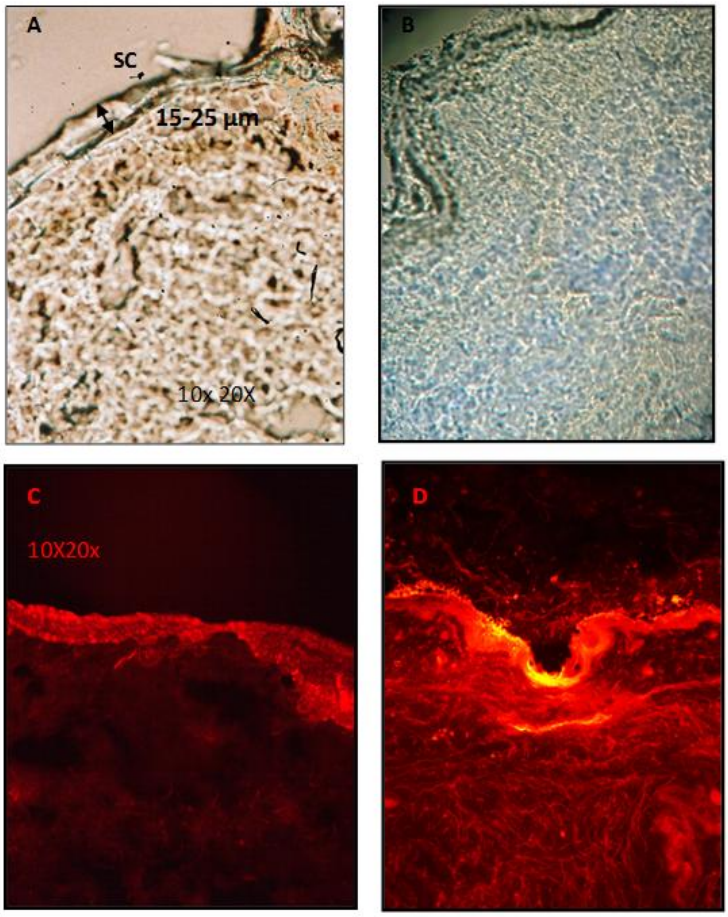


Fig.2- Light microscopy (A and B) and corresponding fluorescence microscopy (C and D) of skin sections untreated (A,C) and treated with Nile Red-UDV formulation (B,D), 24 h after formulation topical application (original magnification 10x20x).

In Vitro Skin Electrical Resistance (ER)

Electrical resistance (ER) is a quick, safe and valuable tool for measuring the integrity of skin membranes. Ensuring that the skin barrier maintains its integrity is an essential factor to the successful performance of such experiments, as specified in test guideline OECD 428 (28).

Compared to water (control) which maintained the skin electrical resistance until 24 h (20.8 ± 3.4 kΩ), tretinoin-UDV decreased the skin electrical resistance to 88.5 % and 59.3 % after 8 and 24 h, respectively. This data suggests the ability of this nanocarrier formulation to induce skin barrier disruption, which was also confirmed by the skin penetration study.

Cytotoxicity Assay

The growth curve for MTT-formazan absorbance was analyzed to ensure that absorbance was proportional to the number of cells (**Fig.3**). Attending to the recommended absorbance range (between 0.75 and 1.25 values), it was chosen a seeding density of ~ 7,000 cells/well.

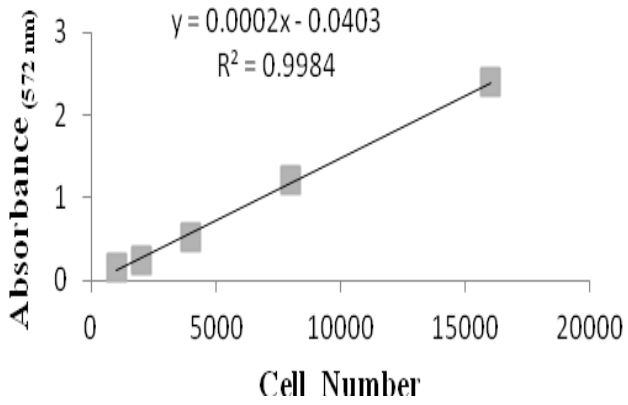
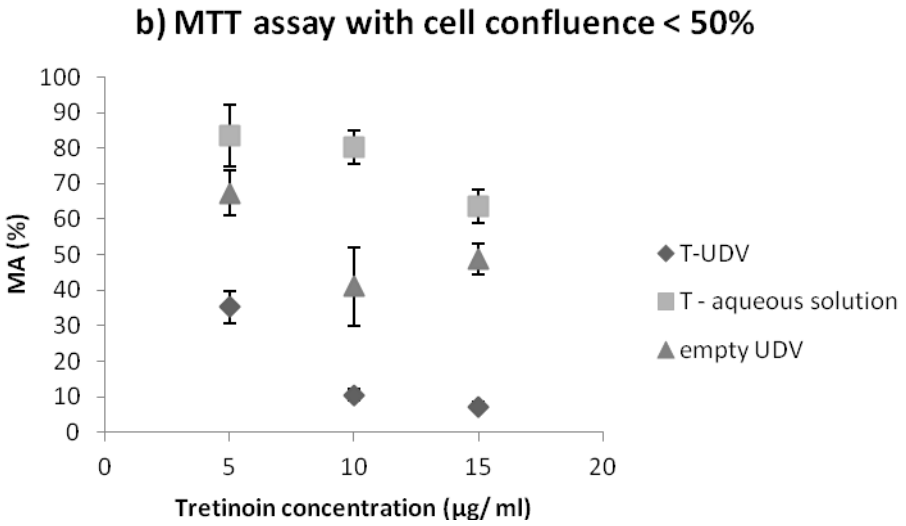
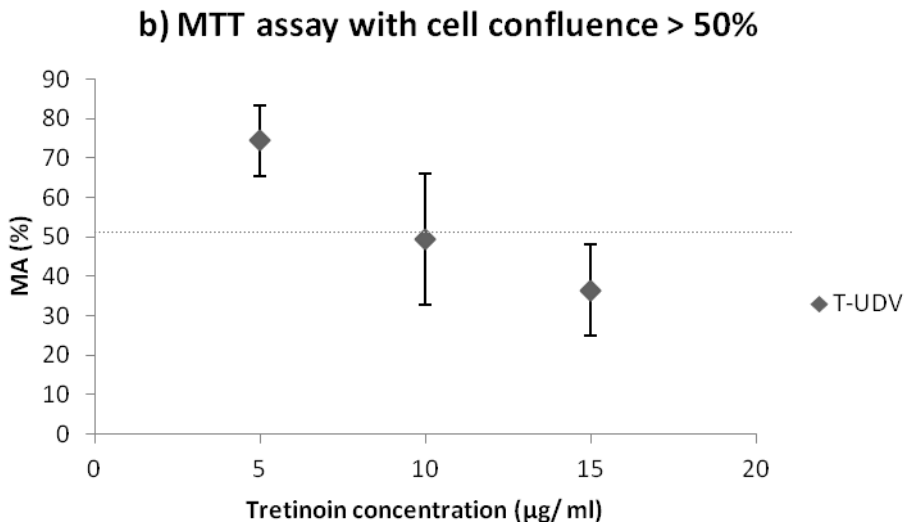


Fig.3- Growth curve of HaCaT cells for MTT-formazan absorbance at 572 nm (mean ± SD).

In preliminary studies it was observed that the formulation toxicity was highly variable and strongly dependent on cell confluence, being much lower when the cell confluence was higher (**Fig.4a and b**). According to this observation and also to usual exposure procedures, the cells were only exposed after achieving more than 50 % of confluence. In addition, all the samples had a mean osmolarity lower than 300 mOsm/ Kg.



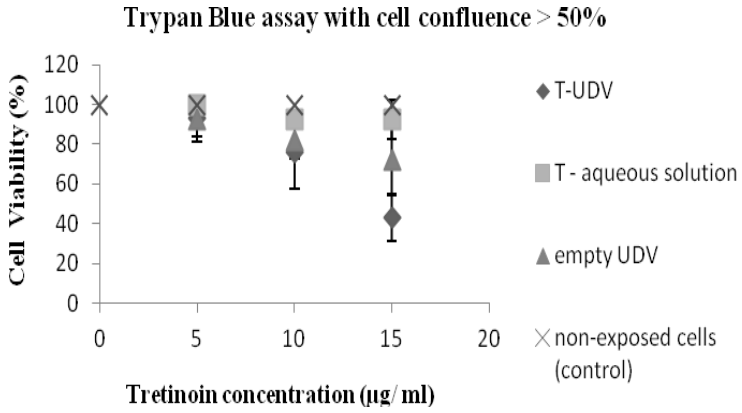
Comparison	Significant (P < 0.05)		Significant (P < 0.05)
Between the vehicles to each dose		**Between the doses to each vehicle**	
T 5 µg/mL vs T_UDV 5 µg/mL	**Yes**	T_UDV 5 µg/mL vs. T_UDV 15 µg/ mL	**Yes**
UDV 5 µg/mL vs T_UDV 5 µg/mL	**Yes**	T_UDV 10 µg/ mL vs. T_UDV 15 µg/ mL	**No**
T 5 µg/mL vs UDV 5 µg/mL	**Yes**	T_UDV 5 µg/mL vs. T_UDV 10 µg/mL	**Yes**
T 10 µg/mL vs T_UDV 10 µg/mL	**Yes**	T_5 µg/mL vs. T_15 µg/ mL	**Yes**
T 10 µg/mL vs UDV 10 µg/mL	**No**	T_10 µg/ mL vs. T_ 15 µg/ mL	**Yes**
UDV 10 µg/mL vs T_UDV 10 µg/mL	**No**	T_5 µg/mL vs. T_10 µg/mL	**No**
T 15 µg/mL vs T_UDV 15 µg/ mL	**Yes**	UDV 5 µg/mL vs. UDV 15 µg/ mL	**Yes**
UDV 15 µg/mL vs T_UDV 15 µg/mL	**Yes**	UDV 10 µg/ mL vs. UDV 15 µg/ mL	**No**
T 15 µg/mL vs UDV 15 µg/mL	**Yes**	UDV 5 µg/mL vs. UDV 10 µg/mL	**Yes**



Comparison Between the Doses	Significant (P < 0.05)
T_UDV 5 µg/mL vs. T_UDV 15 µg/ mL	**Yes**
T_UDV 10 µg/ mL vs. T_UDV 15 µg/ mL	**Yes**
T_UDV 5 µg/mL vs. T_UDV 10 µg/mL	**Yes**

Fig.4- Determination and statistical analysis of MA (mean ± SD) by MTT assay 24 h after exposure to tretinoin-UDV (T-UDV), tretinoin aqueous solutions (T) at 5, 10 and 15 µg/mL and empty UDV (UDV) at the same concentration range under cell confluence: **a)** lower than 50 %; **b)** higher than 50 %. Data expressed as a percentage of control (non exposed cells).

The cell viability assessed by the Trypan Blue assay is expressed in **Fig.5**. In general, a highly decrease of cell viability was found when using both empty UDV (the formulation vehicle) and tretinoin aqueous solutions (T). There are only significant differences of cell viability between the three tretinoin concentrations in T-UDV formulation and there are no significant differences between the empty diluted UDVs, except for the highest "dose" (equivalent to 15 µg/mL of tretinoin formulation).



Comparison with control	Significant (P < 0.05)
T_UDV 5 µg/mL vs Non Exposed Cells	**No**
T_UDV 10 µg/mL vs Non Exposed Cells	**No**
T_UDV 15 µg/mL vs Non Exposed Cells	**Yes**

Comparison	Significant (P < 0.05)		Significant (P < 0.05)
Between the vehicles to each dose		**Between the doses to each vehicle**	
T 5 µg/mL vs T_UDV 5 µg/mL	**No**	T_UDV 5 µg/mL vs. T_UDV 15 µg/ mL	**Yes**
UDV 5 µg/mL vs T_UDV 5 µg/mL	**No**	T_UDV 10 µg/ mL vs. T_UDV 15 µg/ mL	**Yes**
T 5 µg/mL vs UDV 5 µg/mL	**No**	T_UDV 5 µg/mL vs. T_UDV 10 µg/mL	**Yes**
T 10 µg/mL vs T_UDV 10 µg/mL	**No**	T_5 µg/mL vs. T_15 µg/ mL	**No**
T 10 µg/mL vs UDV 10 µg/mL	**No**	T_10 µg/ mL vs. T_ 15 µg/ mL	**No**
UDV 10 µg/mL vs T_UDV 10 µg/mL	**No**	T_5 µg/mL vs. T_10 µg/mL	**No**
T 15 µg/mL vs T_UDV 15 µg/ mL	**Yes**	UDV 5 µg/mL vs. UDV 15 µg/ mL	**Yes**
UDV 15 µg/mL vs T_UDV 15 µg/mL	**Yes**	UDV 10 µg/ mL vs. UDV 15 µg/ mL	**No**
T 15 µg/mL vs UDV 15 µg/mL	**Yes**	UDV 5 µg/mL vs. UDV 10 µg/mL	**No**

Fig.5- Determination and statistical analysis of cell viability (mean ± SD) by Trypan Blue assay 24 h after exposure to tretinoin-UDV (T-UDV), tretinoin aqueous solutions (T) at 5, 10 and 15 µg/mL and empty UDV (UDV) at the same concentration range under cell confluence higher than 50 %. Non exposed cells are represented by (X).

In both cytotoxicity assays (**Fig.4 and 5**), the tretinoin-UDV (T-UDV) formulation was not cytotoxic at the lowest concentration, 5×10^{-3} mg/mL, and the differences between the all three concentrations were statistically very significant ($p \leq 0.001$) in T-UDV formulation under cell confluence higher than 50 %.

Some heterogeneity among replicates was mostly observed in the Trypan Blue assay shown by the SD bars **(Fig.5)**. In addition, this method provided higher cell viability values than the MTT assay due to technical differences between them.

In Vivo Skin Irritation Potential

Data obtained from the skin irritation experiments with mice are represented in **Fig.6,** and as it can be observed, there are significant differences between the commercial (Ketrel®) and tretinoin-UDV formulations ($p < 0.05$). In fact, the UDV formulation had the lowest skin irritation potential.

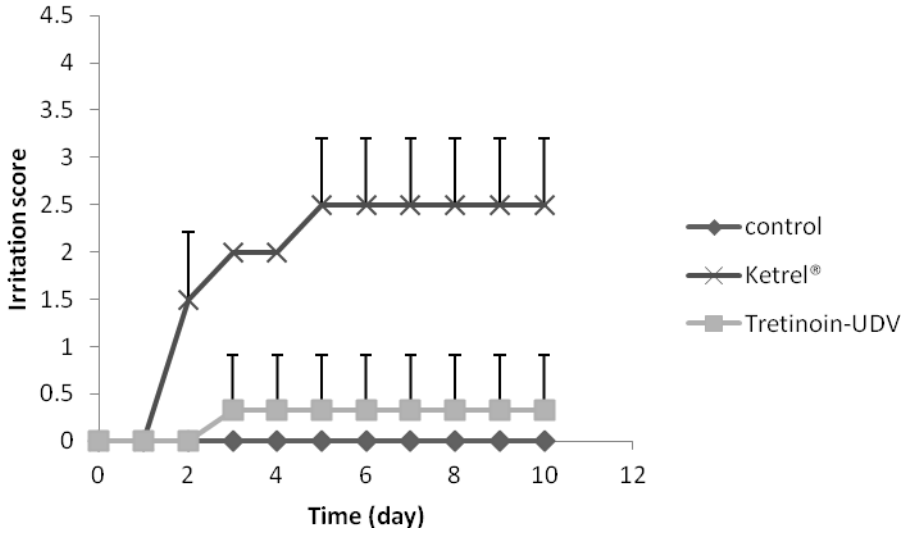


Fig.6- Skin Irritation Potential of tretinoin-UDV and a marketed tretinoin formulation in shaved mice (mean ± SD, n = 3 for each formulation). Irritation score: 0 = no difference between the tretinoin-treated animals and control, 1 = light erythema, 2 = well defined erythema, 3 = strong erythema, and 4 = very strong erythema with presence of a scar.

The epidermal width was augmented after treatment with both tretinoin formulations. Tretinoin–UDV treated skin showed irregular hyperkeratosis and hyperplasia of

sebaceous glands and presence of fibroblast proliferation on superficial dermis (**Fig.7B)**. For Ketrel®- treated skin, hyperplasia of the epidermis and superficial perivascular dermitis were observed (**Fig.7C**).

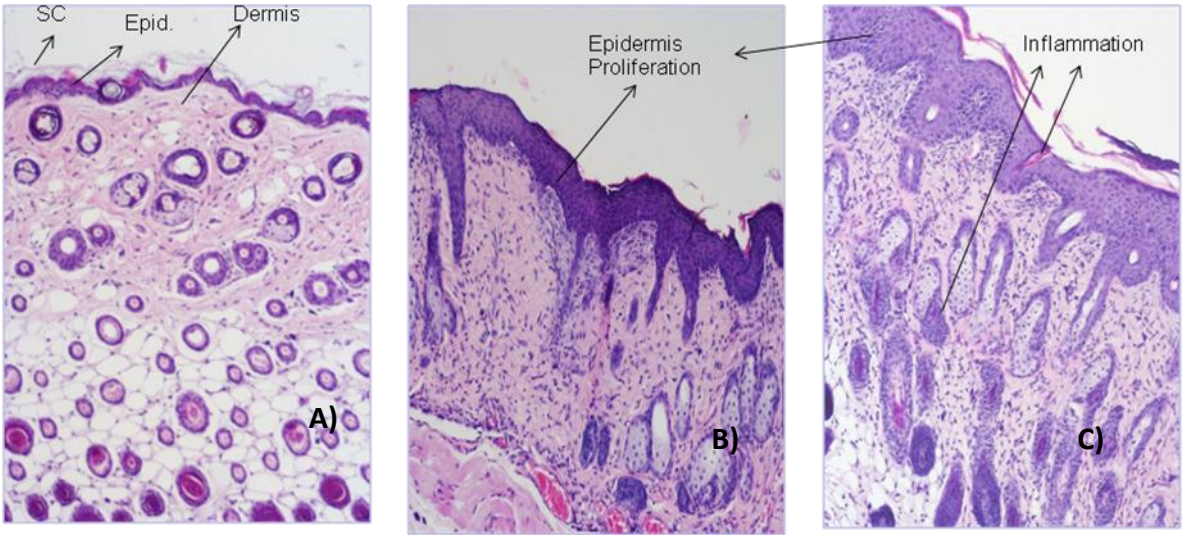


Fig.7- Sections of mouse skin **A**) untreated (serving as the control) or treated with **B**) Tretinoin-UDV or **C**) Ketrel®, stained by hematoxylin/eosin standard procedure and observed by microscopy (original magnification 10x10x).

Discussion

The observed release profiles of tretinoin formulations, and especially, their respective chemical stabilities were the basis of the selection of **tretinoin-UDV formulation with 20 % of lipid concentration** to perform the following studies. In fact, this formulation had also a sustained release **(Fig.1)** and higher chemical stability when compared to other formulation prepared in previous studies: tretinoin hydrogel formulation, in which tretinoin was in free form or complexed with dimethyl-beta-cyclodextrin (38).

The ***in vitro*** **skin permeation study** was carried out through the whole porcine ear skin also under non-occlusive conditions which are more suitable to improve the UDV flux under the transepidermal water-activity gradient (8). On the other hand, occlusive conditions would not only eliminate the osmotic gradient (19), but also improve drug accumulation into the skin layers (39, 40). In addition, porcine ear skin was selected as a

model because of the similarities with human skin regarding biochemical properties, hair follicle density and thickness of SC (41). The drug concentrations in SC and epidermis/dermis are indicative of topical delivery, whereas the concentration in the receptor phase is indicative of transdermal delivery. In fact, no drug amount was detected in the receptor phase after 24 h. Thus, the results showed that tretinoin-UDV formulation has a dermal instead of a transdermal delivery, which will serve the therapeutic purpose since the tretinoin receptors, such as RAR-γ, are mainly located in epidermis layer (42). In addition, tretinoin transdermal delivery is not desirable as long as tretinoin teratogenic effect should be avoided.

According to Manconi *et al.* (10), tretinoin cutaneous delivery is strongly affected by thermodynamic activity of the drug and vesicle composition (charge, structure and size) and, therefore, amphiphile physico-chemical properties. Moreover, interactions between skin and vesicles seem to depend on physico-chemical properties of the main component of the vesicle bilayers. In this case, the vesicles surface charge was low (due to the use of a nonionic surfactant in the formulation (Tween 80®) and the neutral pH), and the vesicles final size was approximately 150 ± 50 nm (25).

In **tape stripping** experiments, the amount of SC removed by a single adhesive tape strip depends on several intrinsic and extrinsic factors. Extrinsic factors that have been reported to affect the amount of SC removed by tape stripping are the type of adhesive tape (43), the force of removal from the skin (44, 45), the duration of pressure onto the skin (46) and topically applied formulations which may interfere with the disorganization level of SC and the skin hydration (47). Even constant extrinsic factors following a strict standard protocol, would not lead to a constant amount of SC in each tape strip, due to the various intrinsic parameters (e.g. corneocytes size). Therefore, all the procedures of the tape stripping experiment were previously validated. In this case, the SC was removed only with 10 adhesive tapes, and the tretinoin UDV formulation was mainly retained in this skin layer (**Table 1**), as theoretically expected from to the formulation composition and lipophilicity of SC layer.

To evaluate whether penetration of the lipophilic drug (tretinoin) from UDV formulation was homogenous throughout the surface of the skin or was limited to certain skin structures, the **skin penetration study and distribution of a fluorescent lipophilic compound** (Nile Red) was performed (29). Nile Red was chosen as a model compound because it is a well-known fluorophore, and presents relatively high absorptivity and good fluorescence quantum yield. Its affinity to the intercellular lipids and lipophilic solvents is also well-known (48), and therefore it will readily stain the hydrophobic UDV membranes. Nile Red incorporation was predominant in SC and epidermis (**Fig.2**), rather than in dermis, which is desirable because retinoic acid receptors are mainly located in epidermis, as already mentioned before (42). These results are quite consistent with others obtained from previous skin penetration studies with another different hydrophilic fluorophore (Fluorescein, FITC) incorporated in the same formulation, and with the lag phase obtained in the releasing profile and the tape stripping results.

Some delivery systems that contain penetration enhancers and/or surfactants can increase the skin penetration of drugs by reversibly decreasing the skin barrier function, and consequently, the **Electrical Resistance** (ER) (29). Rachakonda *et al.* (49) showed a significant agreement between the resistance technique and the standard permeation experiments, thus confirming the efficacy of resistance technique for screening potential chemical penetration enhancers. Lipids of the SC provide the principal transport and electrical resistance in the skin. It has been reported by others (50) that ER is dependent on skin hydration state, ionic strength of the receptor phase, alternating current frequency and current density. An increase in hydration of the SC results in decrease of ER. The most dramatic changes in ER have been observed with different ionic strength medium and alternating current frequency. Thus, the variability in these ER measurements was reduced by certain precautions as recommended by Rachakonda *et al.* (49), such as: 1) using skin samples with the same characteristics (previously stored at -20 °C instead of -70 °C); 2) maintaining the skin hydration; 3) using PBS pH 7.4 as the receptor phase; 4) using a setting of 100 Hz; 5) maintaining the temperature of the receptor compartment at about 32-37 °C. This last condition is

important because at lower temperature, the skin resistance might be higher due to increased rigidity of the lipid bilayers, and at higher temperature, the individual lipid molecules may have more vibrational energy, which makes the lipid bilayers more fluidic and may offer less resistance. Even taken all these precautions for ER measurements, a high variability was obtained, perhaps due to the ionic strength and zeta potential of the UDV formulation. However, tretinoin-UDV decreased the skin electrical resistance especially after 24 h, suggesting its ability to induce skin barrier disruption, also confirmed by the penetration study.

Attending to the importance of developing safe, non-irritating topical delivery systems, the **cytotoxicity potential** of tretinoin-UDV was determined by the MTT and Trypan Blue assays. However, the drug concentration (**Fig.4 and 5**) must be much lower since the cells do not mimic the complex structure of the skin tissue. Thus, the tretinoin-UDV formulation was diluted at 5, 10 and 15 µg/mL. Measurement of cell viability and proliferation is the basis for numerous *in vitro* assays of a cell population's response to external factors.

Both cytotoxicity assays confirmed that the tretinoin-UDV (T-UDV) formulation was not cytotoxic at the lowest concentration. The differences between all the values obtained from the two assays are probably due to the respective assay procedure, such as the trypsinization during the Trypan Blue assay in which most of dead cells are removed, thus leading to higher cell viability values. The tretinoin-UDV formulation cytotoxicity observed at the highest concentration (15 µg/mL) may be due to the combination of two main components: tretinoin and surfactant, since the cell viability slightly decreases with increasing concentrations of these components. The discovered correlation between surfactant and UDV toxicity seems not to be surprising since all other remaining components (e.g. lipids) are considered as non-toxic. The toxic effect of surfactants is mainly explained by different hypothesis related to their chemical nature. The hydrophilic and lipophilic parts of surfactant molecule interact with polar head groups and lipophilic tails of cellular lipid bilayer respectively, resulting in a disruption of the plasma membrane (51, 52). Therefore, long hydrophilic chains could prevent the destabilization of the cell membrane by limiting the penetration of surfactant into the

lipid bilayer, and in contrast, the hydrophobic part would promote the disruption of the membrane. One critical point is still the fact that some of the surfactants contain unbound PEG chains that do not contribute to the surface activity (52). Maupas *et al.* (52) have established the toxicity of different pure surfactants in cultured HaCaT cells: TPGS1500 > Solutol® HS15 > Tween® 20 > Tween® 80 > Cremophor® EL > Simulsol® 4000. Tween® 80 (the surfactant used in UDV formulation) is a PEG sorbitane monooleate and has short PEGylated hydrophilic chains with approximately 20 units (52).

The higher cytotoxicity of tretinoin-UDV formulation relative to empty UDV may be explained by the interaction between this lipophilic drug and the UDV lipids, which could probably alter the surfactant surface activity and, consequently, destabilize the cell membrane, especially when the cells confluence is lower and there are many empty spaces between them. Díaz *et al.* (53) tested the cytotoxic effect induced by retinoic acid (50 and 25 μg/mL) loaded into galactosyl-sphingosine containing liposomes on human hepatoma cell lines, and their results showed that even though liposomes could be a good delivery system for this drug, the study of the interactions between lipids and the drug and lipids and the membranes, as well as the specific mechanisms of internalization (simple endocytosis or receptor-mediated endocytosis), could predict the effect that this agent would have in cells.

Although tretinoin has many therapeutically advantages, the **skin irritation** after its topical application limits significantly the patient compliance. Controlled drug delivery systems such as UDV can be an interesting alternative to protect the skin from direct contact with the drug which is incorporated in the lipid matrix and, consequently, gradually released (54). In addition, these vesicles help to reduce skin irritation by hydration of the epidermis due to contributing vesicular lipids to SC and water content (55). The search for a tretinoin formulation that could be therapeutically active and without skin irritation problems is a matter of interest in dermatotherapy. Here, we have demonstrated that the developed UDV formulation had the lowest skin irritation potential (erythema and scaling) (**Fig.6 and 7**). Castro *et al.* (54) have also obtained similar results with retinoic acid (RA) loaded in solid lipid nanoparticles (SLN), when compared to conventional formulations (gel or cream at 0.01 % and 0.05 %) using rabbit

and rhino mouse models. Moreover, our histological results observed for keratinocytes proliferation and compaction of the skin barrier support other findings in literature (56, 57), and clearly indicate the potential of UDV formulation in improving the skin tolerability to tretinoin.

Conclusions

Nanotecnology is not only used to protect unstable drugs but also to modify the drug permeation and/ or penetration by controlling its release rate and increasing the period of permanence on the skin, besides ensuring a direct contact with SC and skin appendices (58). Based on our results, we propose that tretinoin-UDV formulation has a suitable dermal delivery, with special targeting to SC and epidermis layers. In addition, the formulation vehicle (empty UDV) and tretinoin-UDV were not toxic under *in vitro* and *in vivo* conditions, at least at 5×10^{-3} mg/mL and 0.5 mg/mL, respectively.

In fact, Tretinoin-UDV can be considered as a promising delivery system for tretinoin dermal delivery without promoting skin irritation (unlike other commercial formulation), which is quite advantageous for therapeutic purpose for different skin diseases and/or disorders.

Acknowledgments

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Chapter 3

Dermal Delivery of Lycopene Carrier Formulations

Chapter 3.1 Lycopene from Tomatoes: Vesicular Nanocarrier Formulations for its Dermal Delivery

Chapter 3.2 The Effect of UV-B Irradiation on Human Keratinocytes after Lycopene Exposure

Chapter 3.2

The Effect of UV-B Irradiation on Human Keratinocytes after Lycopene Exposure

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The Effect of UV-B Irradiation on Human Keratinocytes after Lycopene Exposure

Abstract

Introduction: Human skin is constantly exposed to the UV irradiation that may induce a number of pathobiological cellular changes. Lycopene has been reported as the antioxidant most quickly depleted in skin upon exposure to solar radiation, and thus might play a role against UV radiation. Regarding this, our goal was to investigate the effects of pre-exposure to lycopene in UV-B-irradiated skin cells (human keratinocytes HaCaT cells line).

Methods: Thiazolyl Blue Tetrazolium Bromide viability assay was firstly used to set the protocol specific conditions: cells were exposed for 24 h to 10 μM complexed lycopene, and subsequently, cells were irradiated with UV-B radiation (225 mJ/cm^2) and left to recover for another 24 h period. After exposure and irradiation experiments, the following parameters were analyzed: a) Genotoxicity/Clastogenicity by assessing the cell cycle distribution by Flow Cytometry (FCM) and analyzing Gene Expression of Apoptosis Biomarkers by RT-PCR; b) Apoptosis by performing the Annexin assay by FCM; c) Oxidative Stress Damage by reactive oxygen substances (ROS) quantification by FCM.

Results: Complexed lycopene (10 μM) does not affect the profile of apoptotic, necrotic and viable cells, nor shows cytostatic effects despite slightly increasing the ROS content. However, cell populations previously treated with lycopene and then exposed to UV-B radiation show an increase in both dead and viable subpopulations compared to non exposed-UV irradiated cells. This is accompanied by a cell cycle delay at S transition and consequent decrease of cells in G0/G1 phase, which is not observed in irradiated/non exposed cells.

Conclusion: According to data obtained from all experimental assays, complexed lycopene seems to play a corrective or cytotoxic role in irradiated cells depending on the level of photodamage. Thus, our findings may have implications for the management of skin cancer and/or other proliferative skin disorders.

Keywords: UV-B irradiation; Lycopene; HaCaT Cells; Cytotoxicity; Genotoxicity; Oxidative Stress

Introduction

Human skin is constantly exposed to the **UV irradiation** that may induce a number of pathobiological cellular changes. Through lipid peroxidation, protein crosslinking and DNA damage, UV-A and UV-B radiation can cause photoaging and photocarcinogenesis (1-3). Skin is endowed with a variety of enzymatic as well as small molecular antioxidants that can inhibit oxidative damage. However, the antioxidant ability of the skin is often overwhelmed by excessive ROS production (4). In this regard, emphasis in developing novel preventive and therapeutic strategies based on phytocompounds capable of ameliorating the adverse effects of ROS has become an important area of research. Moreover, primary prevention approaches of skin cancer proved to be inadequate in lowering the incidence of this type of cancer, emphasizing the need to develop novel skin cancer chemopreventive agents. Among the vast number of photochemoprotective agents, botanical antioxidants have given promising results (4). Two types of **chemopreventive agents** could be useful for the management of skin cancer. Primarily, the agents that could inhibit the damages caused by UV may prevent the formation of initiated cells (cells with cancerous potential). Secondly, the agents that could eliminate the initiated cells may reduce the risk of skin cancer (5).

Lycopene is a powerful antioxidant both *in vitro* and *in vivo* against the oxidation of proteins, lipids and DNA, and it has been identified as one of the most potent scavengers of singlet species of oxygen free radicals – the highest among the carotenoids (6, 7). At low oxygen tension, it can also scavenge peroxyl radicals, inhibiting the process of lipid peroxidation (8). Lycopene was reported as the most quickly depleted antioxidant in skin upon exposure to solar radiation (9) and might play a role of protection against UV radiation. Recent research has been developed to assess if lycopene has potential for prevention of skin cancer. In fact, lycopene has been shown to inhibit proliferation of several types of cancer cells through different mechanisms in *in vitro* systems (10, 11). Chemopreventive antioxidants are mostly studied for their role as radical scavengers, but this preventive role can be complemented by a corrective activity as selective inducers of apoptosis in transformed cells (12). Moreover, Ribaya-

Marcado *et al.* (9) suggested a role of lycopene in mitigating photooxidative damage in tissues.

HaCaT cells, a transformed nontumorogenic human cell line from keratinocytes epithelial cells were used in this research work (13). Keratinocytes are the predominant cell type in the epidermis, the outermost layer of the skin, constituting 95% of the cells found there (14). Considering that the principal site of action of UV-B is the epidermis layer (15), keratinocytes might be more susceptible to UV-B-induced apoptosis than fibroblasts which are located in dermis layer (16). However, keratinocytes may be more UV-B resistant in terms of their proliferative ability as measured by colony survival assays and have greater ability for UV-DNA repair (16).

To date, most of the studies on the therapeutical potential of lycopene have been performed *in vivo* (17, 18). These studies may be obscured by the complexity of biological system models. *In vitro* conditions may circumvent some of these contingencies, and complement *in vivo* data within the 3Rs perspective (*Refine, Replace, Reduce)*. Despite the lower complexity of *in vitro* systems, the study of cellular photoprotection by antioxidants could be challenging because of the **high chemical instability** (specially to air and light) and strong **lipophilicity** of many antioxidant molecules such as lycopene. According to Zefferino *et al.* (11) *in vitro* experiments may occasionally produce inconsistent results due to lycopene's poor solubility in cell culture media (19). In fact, lycopene is very hydrophobic (log P ≈ 15) and is usually solubilized in organic solvents such as tetrahydrofuran (THF). However, an uncontrolled precipitation process may occur upon addition to aqueous media, besides the high toxicity associated to these solvents. The solubility and uptake of these large crystals in the cells is quite limited and there is almost no protection against chemical degradation (20). Alternative ways of delivering lipid-soluble compounds include micelles, microemulsions, nanoparticles, water-dispersible beadlets, artificial liposomes, enriched bovine serum, or specialized formulations, each of which has an influence on the uptake and stability of hydrophobic compounds (19, 21-24). According to Palozza *et al.,* niosomes provide a convenient, nontoxic and inexpensive vehicle for β-carotene in cell culture (25). Lipid-

based delivery systems also show UV-blocking effects dependent on lipid composition and the particle size (the smaller the particle size, the higher the sunscreen activity). Lipid matrices can act as sunscreen carriers and increase the sun protection factor obtained after topical application of UV absorbers ($BaSO_4$, $SrCO_3$, TiO_2) incorporated within these carriers because they provide a fixation medium for these pigments (26, 27). However, it is difficult to use these hydrophobic systems for cell culture studies.

The main limitations of different **vehicles** used for lycopene cell delivery are summarized on **Table 1**. Each vehicle provides specific advantages but also offers some limitations such as cytotoxicity, poor solubility and crystallization in the cell medium (28).

Table 1- Limitations of different vehicles used for lycopene cell delivery (adapted from Lin *et al.* (28)).

Vehicle	Limitations	References
Tetrahydrofuran (THF)	Rapid oxidation in media, leading to lycopene instability and cytotoxicity	(18, 21, 23, 29-33)
Dimethyl sulfoxide (DMSO)	Reduced solvent capacity for carotenoids (< 0.01 mg/mL for lycopene).	(21, 34-36)
Tween®	Possible oxidation of carotenoids, after solvent drying and filtration	(31)
Micelles	Low carotenoid stabilization and increased cytotoxicity	(21, 28, 37)
Water-dispersible Beadlets	Low toxicity, but also low cellular uptake; depends on chemicals that interfere in assays (e.g. hexane, chloroform)	(29, 38, 39)

In addition, the half-life of free lycopene in solution at 37° C is less than few hours. Thus, until an efficient method of solubilizing lycopene in aqueous buffers and cell culture media is developed, in *vitro* studies on the effects of lycopene on living cells will continue to show considerable variation between laboratories and cell lines, and should be interpreted with caution (40).

Pfitzner *et al*. (19) have demonstrated that methyl-β-CD (M-β-CD) was an improved vehicle for the investigation of carotenoids and other lipophilic compounds in *in vitro* test systems, compared to organic solvents. Carotenoids-M-β-CD complex was superior concerning biological availability, missing cytotoxicity and presenting excellent stability

when compared to other application forms such as organic solvents, mixed micelles, liposomes or beadlets. At least, the solubilization with M-β-CD was easily and reproducibly achievable under routine laboratory conditions.

According to these literature references (19, 28) and pre-formulations studies, we decided to use another similar CD derivative, dimethyl-β-CD (**DM-β-CD**), to solubilize and stabilize lycopene for cell exposure experiments. We propose that lycopene delivered in DM-β-CD complex may be used as a photochemopreventive agent against UV-B irradiation (17). To test this hypothesis, we studied the main effects of UV-B irradiation on human keratinocytes (HaCaT cells line) after complexed lycopene (Lyc-DM-β-CD, or more abbreviated, Lyc-CD) exposure under *in vitro* conditions. For this, several assays related to cytotoxicity, genotoxicity, oxidative stress and apoptosis were performed.

Materials and Methods

Preparation of Lycopene Complex Solution

In order to avoid the use of organic solvents, lycopene (Extrasynthese, Genay, France with a purity ≥ 98%, UV assay) was solubilized by complexation with dimethyl-beta-cyclodextrin (CD, degree of substitution: 1.8) which was a generous gift from Wacker (Stuttgart, Germany). Aqueous solutions of lycopene complexed with CD (1:4 molar ratio) were prepared under aseptic conditions within concentrations of 0, 5, 10, 15 and 20 µM from a concentrated lycopene solution previously stirred with CD during approximately 48 h, and sonicated 30 min (before use) always protected from light and air. Lycopene solutions were always freshly prepared under light and air protection and stored at -20 °C (except the pure lycopene standard, stored at -70 °C). The osmolarity of the sample containing the highest lycopene concentration was determined using an automatic osmometer (Knauer®, Berlin, Germany).

When non specified, all higher grade reagents were from Sigma-Aldrich (St. Louis, MO, USA). All solutions were prepared using ultrapure water obtained in a MILLI-Q System from Millipore® (Billerica, MA, USA).

Human Immortalized Keratinocytes (HaCaT) Cell Line

The HaCaT cell line was obtained from Cell Lines Services® - CLS (Eppelheim, Germany). Handling and culture of these cells were adapted to meet CLS protocol procedures. Cells were aseptically grown in Dulbecco's modified Eagle's medium (DMEM, no HEPES, no Pyruvate), high glucose, supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 1 % penicillin–streptomycin (10,000 U/mL) and 1 % fungizone (250 U/mL) (Gibco®, Life Technologies, Grand Island, NY, USA) at 37 °C in a humidified atmosphere with 5 % CO_2.

HaCaT Cell Growth and Confluence under normal culture conditions

The standard cell growth conditions were established after the analysis of HaCaT growth curves. HaCaT cells were seeded in a 6-well cluster plate (300,000 cells/ well). Cell confluence and morphology was daily observed under microscope. After 3 days, cells were harvested by trypsinization according to CLS procedure. Briefly, the culture was rinsed twice with phosphate-buffered saline solution (PBS Gibco®, without Ca^{2+} neither Mg^{2+}) and PBS containing 0.05 % EDTA to remove desmosomes and incubated at 37 °C about 5-10 min. After this time, the EDTA solution was replaced by a 1:1 mixture of EDTA/trypsin-solution (final concentrations of 0.025 % and 0.05 %, respectively). The trypsinization was achieved after incubating at 37 °C for 15-20 min. At this time, a double volume of complete culture medium (with FBS) was added to stop the detaching process and cells were suspended with stronger shaking, followed by the use of a syringe (21G) because of its mechanical resistance. All cell manipulation and growth and subsequent exposure conditions were performed under strict aseptic procedures.

Cell density was calculated by counting with a hemocytometer (Neubauer® Improved) under a phase contrast microscope Nikon Eclipse 80i® (Coyoacán, Mexico).

Selection of Exposure Conditions:

Selection of UV-irradiation Dose: Ten UV-B lamps (Sankyo Denki G8T5E, Kanagawa, Japan) with a peak emission at 312 nm were used as the UV-B source. The UV-B irradiation was measured with a VLX 312 radiometer equipped with a UV-B sensor (Vilber Lourmat, Marne-la-Vallée Cedex, France). Around 7,000 cells/well were cultured in a 96-well cluster plate with complete medium. Twenty four hours after, the cells were exposed to five different UV-B irradiation doses [based on related literature (41-43)] of ~ 75, 150, 200, 225 and 325 mJ/cm^2. In order to prevent UV quenching, prior to irradiation, the cell culture medium was replaced by the same volume of PBS after two washing steps with PBS. After UV-B irradiation, cells were fed with fresh growth medium and incubated for 24h. Cell metabolic activity was assessed as described below

by the MTT assay (often used as viability method) in order to choose just one UV-B dose. Non irradiated samples were used as negative control.

Selection of Lycopene Complex Dose: Cells were seeded and grown in DMEM, the modified Eagle's medium (α-MEM, without nucleosides, Gibco®, Life Technologies) for 24 h, then DMEM medium was replaced with alpha-MEM (also supplemented with 10 % FBS, 1 % penicillin–streptomycin and 1 % fungizone) containing complexed lycopene solutions (0, 5, 10, 15 and 20 µM). Cells were exposed for 24 h and, after UV-B irradiation, HaCaT irradiated cells were grown under standard culture conditions for a final 24 h period. The metabolic activity of cell culture was analyzed by MTT assay in order to choose just one lycopene dose.

From cell viability results, the exposure and irradiation conditions were determined according to **Fig.1**. Briefly, for further experiments, HaCaT cells were seeded in a 6-well cluster plate (150,000-300,000 cells/2 mL well) and incubated 24 h under the same culture conditions, as mentioned above. After this period, the cells were exposed to complexed lycopene (10 µM) for 24 h in order to achieve its cellular internalization and a higher cell confluence, and after UV-B irradiation (225 mJ/cm^2), cells were allowed to grow under standard culture conditions for another 24 h period in order to give cells time to possibly activate some repair mechanisms.

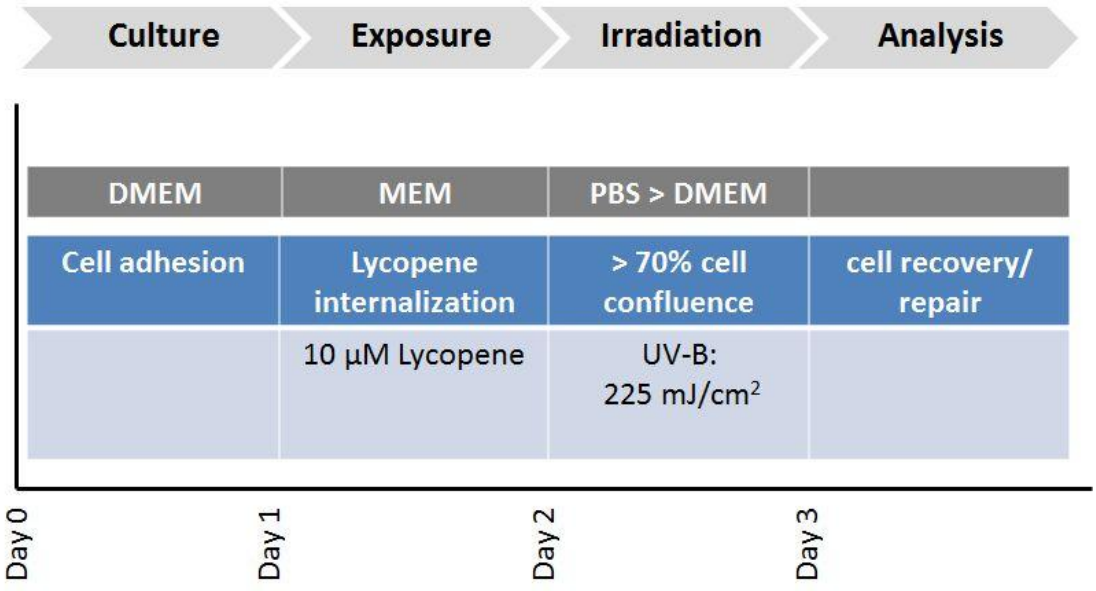


Fig.1- Scheme of culture conditions regarding complexed lycopene exposure and UV-B irradiation.

MTT Assay

Cell metabolic activity was assessed by the Thiazolyl Blue Tetrazolium Bromide (Sigma-Aldrich, St. Louis, MO, USA) assay, which is often used to roughly estimate culture's viability/proliferation characteristic. Cells were seeded at a density of 7,000 cells/ well in a 96-well cluster plate. After complexed lycopene exposure and UV-B irradiation, the MTT assay was performed according to the following conditions: cells were incubated with culture medium containing 50 µL of MTT (1 mg/mL in PBS, pH 7.2) during 4 h at 37 °C, 5 % CO_2. After this time, medium was carefully removed and the 150 µL of dimethyl sulfoxide was added to each well in order to solubilize the formazan crystals. The plate was gently shaken for about 2 h and protected from light. The optical density of reduced MTT was measured at 570 nm by spectroscopy on an automatic microtiter plate reader (Synergy™ HT Multi-Mode from BioTeK® Instruments Inc., Winooski, VT, USA) equipped with specific software (Gen5, Biotek®), and cell metabolic activity (MA ≈ viability/proliferation characteristics) was calculated according to **Eq.1**.

Eq. 1. $\text{Cell MA (\%)} = (\text{Abs 570 nm}_{\text{sample}} - \text{Abs 570 nm}_{\text{DMSO}}) / (\text{Abs 570nm}_{\text{negative control}} - \text{Abs 570 nm}_{\text{DMSO}}) \times 100$

Besides the negative control (non exposed and non irradiated cells), the assays results were also compared to CD aqueous solutions (vehicle control) with the same dilution factors used for complexed lycopene samples.

Analysis of Apoptosis by the Annexin Assay

Apoptosis was measured by flow cytometry using FITC Annexin V Apoptosis Detection Kit from BD Pharmingem™ (Franklin Lakes, NJ, USA). After complexed lycopene exposure and UV-B irradiation as described before, the cells were gently harvested with Accutase® (PAA Laboratories, Pasching, Austria) and washed twice in cold PBS (1 mL). Finally, Fluorescein Isothiocyanate (FITC) and Propidium Iodide (PI), 5 µL of each, were added to 100 µL of cell suspension (10^5 cells/mL) in binding buffer. Samples were left in

the dark for 15 min and 400 µL of "1x Binding Buffer" was added. The samples were analyzed by flow cytometry using a Coulter EPICS XL flow cytometer® (Coulter Electronics, Hialeah, Florida, USA). Data was acquired using the SYSTEM II™ (v. 2.5) software. The cytogram of FITC fluorescence in log scale *versus* PI fluorescence in log scale allows the identification of non-apoptotic cells (Annexin V-FITC negative, PI negative), early apoptotic cells (Annexin V-FITC positive, PI negative), late apoptotic cells (Annexin V-FITC positive, PI positive) and dead cells (Annexin V-FITC negative, PI positive). FCM data was analyzed by FlowJo software® (Tree Star Inc., Ashland, OR).

Analysis of Apoptosis Regulating Genes by RT-PCR

Total RNA extraction: At least 1x 10^5 HaCaT cells were rinsed twice with sterile PBS after removal of culture medium. After rinsing, cells were lysed in 1 mL TRIzol® reagent (Life Technologies, Saint Louis, MO, USA) and after 5-min room temperature incubation, 200 µL chloroform were added to each sample, shaken on vortex for 10 s, and incubated at room temperature for 2 min. Phase separation was achieved by centrifugation at 12,000 *g* for 5 min at 4 °C in Phase-Lock Gel Heavy tubes (5 Prime 3 Prime, Inc., Boulder, CO, USA). The aqueous phase was mixed with 1 volume 70 % ethanol and RNA was further purified using RNeasy Mini Kit columns® (Qiagen, Hilden, Germany) following the manufacturer's recommendations. The total RNA was quantified by spectrophotometry at 260-280 and 230-260 nm (Nanodrop Spectrophotometer ND-1000®, Thermo Fisher Scientific, Wilmington, DE, USA).

cDNA synthesis: 1 µg total RNA was reverse-transcribed using the Omniscript RT Kit® (Qiagen, Hilden, Germany) in a reaction mixture containing 1 µM Oligo(dT)18 primer, 5 mM dNTPs, reaction buffer and RT enzyme according to the manufacturer's instructions. After the enzymatic reaction (incubation at 37 °C for 1 h), cDNA samples were prediluted in milliQ water (1:20).

Quantitative RT-PCR (qPCR): For qPCR, primers were used complementary to the genes coding for Bcl-2 and Bax. SDHA (succinate dehydrogenase) was used as the reference

gene. The target genes and corresponding oligonucleotide primer sequences (5' to 3') were:

Bcl-2: GGAGGATTGTGGCCTTCTTT and GCCGGTTCAGGTACTCAGTC;
Bax: GACGGCCTCCTCTCCTACTT and CAGCCCATCTTCTTCCAGAT;
SDHA: CTGCAGAACCTGATGCTGTGT and GGATGGGCTTGGAGTAATCG.

Primer design was performed using Primer3 (44) and primer specificity was confirmed using the In-Silico PCR UCSC Genome Browser (45). The final individual qPCR reactions contained iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA), 1.5 µM each primer and 1:4 (v/v) prediluted cDNA (1:20). Two qPCR technical replicates were performed per sample in the iQ5 Bio-Rad thermal cycler. The qPCR program included 1 min denaturation at 95 °C, followed by 60 cycles at 94 °C for 5 s, 58 °C for 15 s, and 72 °C for 15 s. After qPCR, a melting temperature program was performed. Mean PCR efficiencies and cycle thresholds were determined from the fluorescence data using the algorithm Real-Time PCR Miner (46). Relative gene expression of cell samples relative to SDHA was calculated using the Pfaffl method (47).

Cell Cycle Analysis by Flow Cytometry

After complexed lycopene exposure and UV-B irradiation as described before, the cells were harvested with Accutase® and centrifuged at 1157 *g* for 5 min at 4 °C. The supernatant was removed and the cells were washed in PBS and suspended in 1 mL of 85 % ethanol at 4 °C and kept at -20 °C until analysis. After that, the cells were centrifuged twice at 1157 *g* for 5 min at 4 °C and suspended in 800 µL PBS and vortexed. Samples were then filtered on a nylon mesh (50 µm pore size) to the analysis tubes. PI, a DNA intercalating fluorochrome, and RNase were added to the samples (50 µL each one) and vortexed. The mixture was incubated 20 min at room temperature and then analyzed by flow cytometry using a Coulter EPICS XL flow cytometer®. The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm for excitation. Data was acquired using the SYSTEM II™ (v. 2.5) software. Integral fluorescence together with fluorescence pulse height and width emitted from nuclei

was collected through a 645 dichroic long-pass filter and a 620 band-pass filter and converted on 1024 ADC channels. Prior to analysis the amplification was adjusted so that the peak corresponding to G0/G1 was positioned at channel 200. This setting was kept constant. The results were obtained in the form of three graphics: linear fluorescence light intensity (FL), forward angle light scatter (FS) *versus* side angle light scatter (SS) and FL pulse integral *versus* FL pulse height. This last cytogram was used to eliminate partial nuclei and other debris, nuclei with associated cytoplasm and doublets (these events have a higher pulse area but the same pulse height as single nuclei). Cell cycle analysis was performed using the FlowJo software® and applying the Dean-Jett Model.

Reactive Oxygen Species (ROS) Quantification by Flow Cytometry

ROS ($O_2^{\cdot -}$ and $^{\cdot}OH$) generation was assayed by Coulter EPICS XL flow cytometer® using the fluorescent probe 2,7-dichlorodihydrofluorescein diacetate (H_2DCF-DA) which upon acetate cleavage is oxidized to fluorescent dichlorofluorescein (DCF) by hydrogen peroxide. After complexed lycopene exposure and UV-B irradiation as described before, the medium was replaced by serum-free α-MEM containing 10 μM H_2DCF-DA for 30 min, at 37 °C in dark. Cells were washed with PBS, trypsinized with Accutase® and collected for analysis. ROS formation was estimated by the median fluorescence intensity (MFI) parameter using the FlowJo software®.

Statistical Analysis

The results are reported as mean $\pm$ SD of at least three replicates/treatment. In addition, at least three independent assays were performed for each analysis. The results of all these experiments were statistically analyzed by Analysis of Variance (ANOVA with All Pairwise/Non Pairwise Multiple Comparison Procedures) using SigmaPlot 11.0 software®. The differences were considered statistically significant when $p < 0.05$.

Results

HaCaT Cell Growth and Confluence under normal culture conditions

HaCaT cell growth and confluence under normal culture conditions until 120h are represented on **Fig.2A.** As it can be observed, the exponential phase extends until approximately 72 h and the full confluence can be maintained more than 1 week. The selected confluence for complexed lycopene exposure and UV irradiation in this experiment was attained at 24 h and 48 h, respectively.

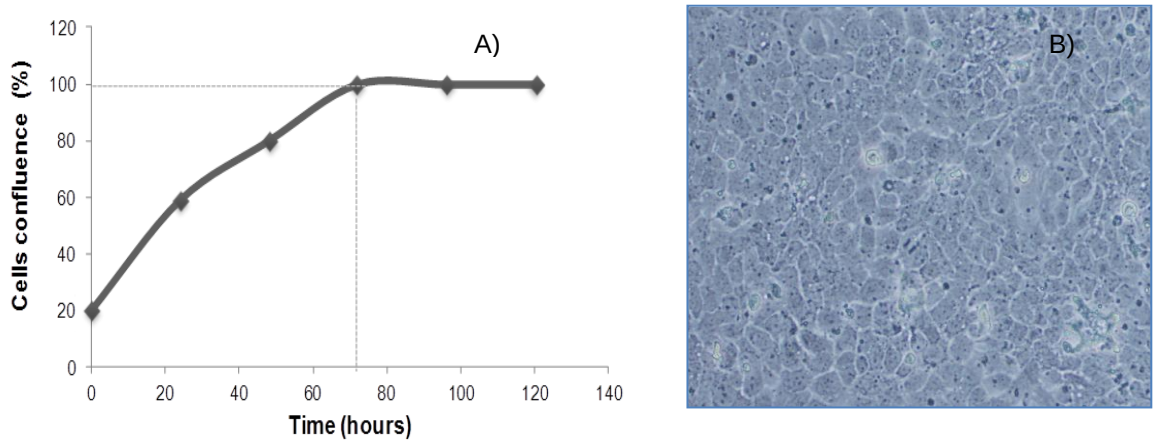


Fig.2- A) HaCaT cells growth and confluence curves under normal culture conditions for 120 hours.
B) HaCaT cells observed by phase-contrast microscope (total magnification 10x10x).

Under phase-contrast microscopy, the cells displayed typical intermediate phenotype of polygonal cells interspersed with giant often multinucleated cell and single morphology (**Fig. 2B**).

Effect of UV-B doses on Cell Metabolic Activity by MTT Assay

As theoretically expected, increasing UV-B dose resulted in decreased cell metabolic activity (**Fig.3**). UV-B irradiation resulted in distinct morphological changes in HaCaT cells, as irradiated cells became round and detached from the surface of the plate.

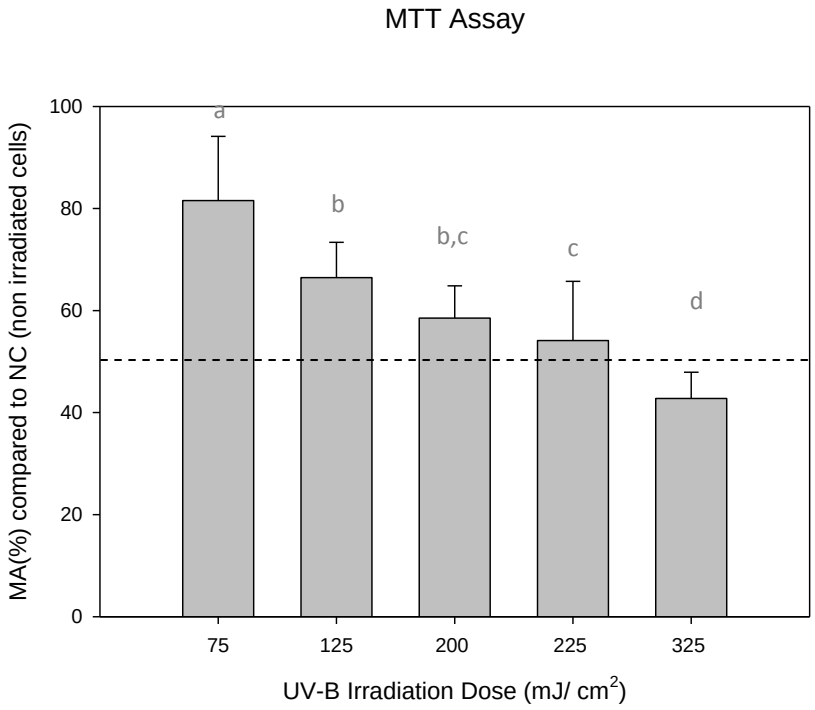


Fig.3- Effect of different UV-B doses on cell Metabolic activity (MA) determined by MTT Assay. Results are expressed as percentage (mean ± SD) of cell MA compared to non irradiated cells (NC). Statistical analysis: One Way Anova with All Pairwise Multiple Comparisons by Holm-Sidak method: statistical differences between the samples are represented by different letters when $p<0.05$.

According to MTT results, the UV-B condition leading to, approximately 50 % of inhibition of cell MA, was 225 mJ/cm^2. Therefore, this was the chosen UV-B condition for the following studies.

Complexed lycopene up to 15 μM did not affect the enzyme activity (MTT assay) of non-exposed cells, and only 20 μM led to significant decrease in metabolic activity in these cells (**Fig.4**). Comparing the effect of complexed lycopene exposure between non irradiated and irradiated cells (225 mJ/cm^2), it is evident that, at doses higher than 10 μM, complexed lycopene decreases the viability of irradiated cells (**Fig**.**4**).

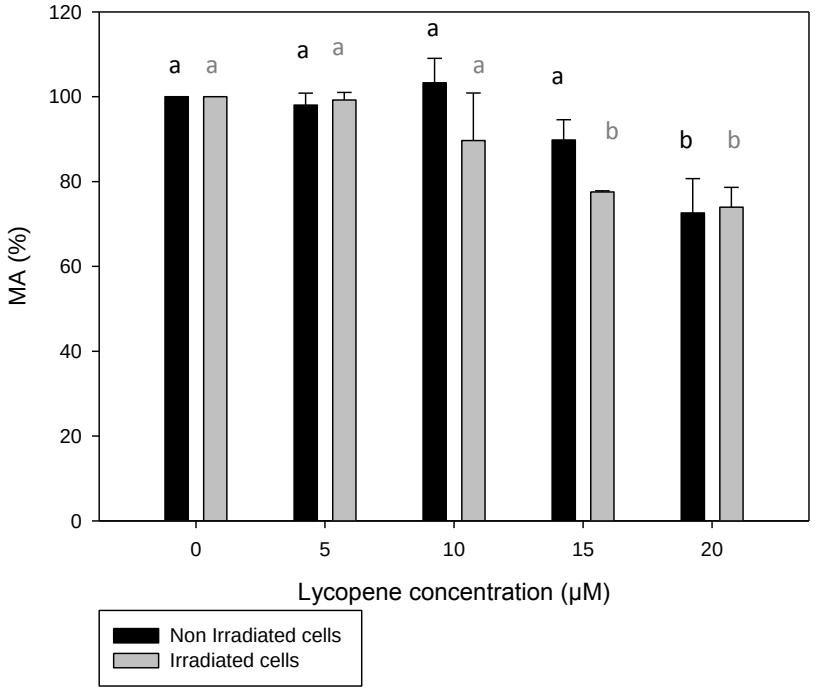


Fig.4- Effect of complexed lycopene exposure on UV-B irradiated (225 mJ/cm^2) and non irradiated HaCaT cells on cell MA measured by MTT assay. Results are expressed as percentage (mean ± SD of 3 independent experiments with 6 replicates each one). Statistical analysis: One Way Anova with Multiple Comparisons *versus* Control Group (Holm-Sidak method): statistical differences between the samples within non irradiated and irradiated groups (in respect to cells not exposed to lycopene) are represented by different letters when $p < 0.05$.

The higher decrease in MA in irradiated cells treated with higher lycopene concentrations was further confirmed by a higher number of additional MTT experiments. In addition, previous experiments also demonstrated that exposure to the vehicle (CD) alone did not significantly alter the MA rates of non irradiated cells (data not shown).

According to these results, we decided to choose an intermediate complexed lycopene concentration (10 µM) whose effects have been previously established in with other cell lines (48-50). At this lycopene concentration, the cell metabolic activity (and viability) was higher than 50 % and was not significantly different from the negative control (non

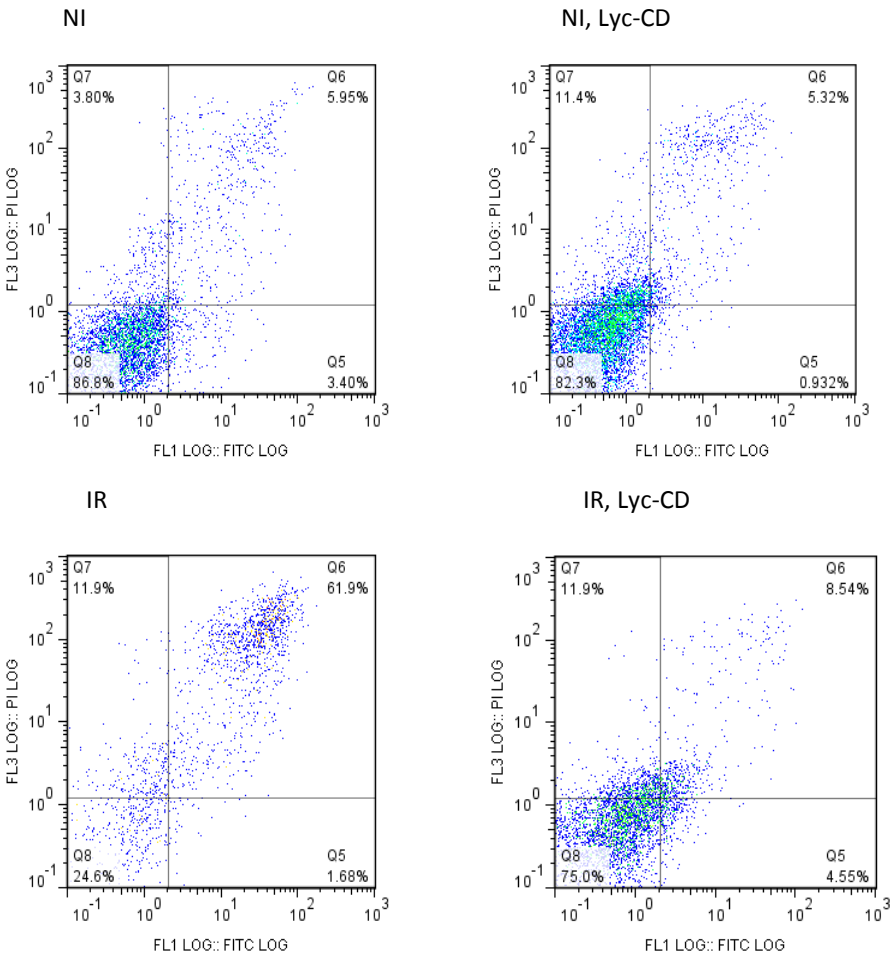
exposed cells from irradiated group). Higher lycopene concentrations (e.g. 20 µM) could decrease the cell viability due to a prooxidant effect.

Annexin V Assay

Annexin assay differentiates subpopulations that are necrotic, apoptotic or viable. Comparative analysis of these subpopulations in irradiated (225 mJ/cm^2) and non irradiated HaCaT cells, treated with 10 µM complexed lycopene, was performed using the Annexin V assay (**Fig.5**).

As shown in **Fig**.**5**, within non irradiated cells, complexed lycopene alone did not affect ($p > 0.05$) the percentage of necrotic, apoptotic and viable cells compared to the control (non exposed cells). Contrarily, within non exposed cells, irradiation alone increased the percentage of both early and late apoptotic cells ($p < 0.05$), and decreased the percentage of viable cells compared to the control. On the other hand, irradiated cells previously exposed to complexed lycopene showed a decrease of cells in apoptosis compared to irradiated cells alone ($p<0.05$), while the number of dead cells increased.

a)



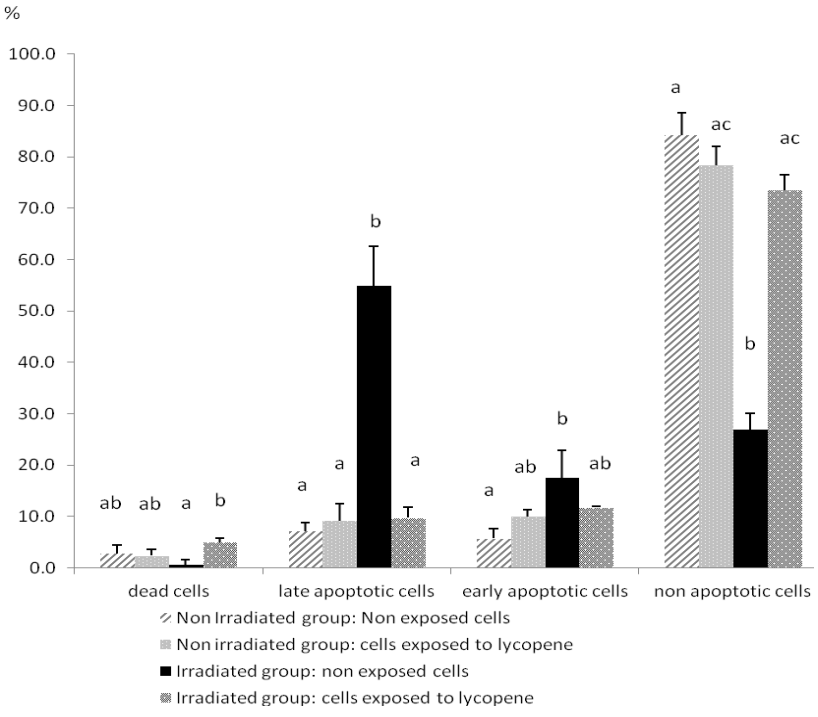


Fig.5- a) Representation of Annexin V-FITC Dot-Plots Gating (FL1 LOG *vs* FITC LOG) above, and on the right, graphic results of UV-B (225 mJ/cm^2) irradiated (IR) and non irradiated (NI) HaCaT cells exposed to 10 µM complexed lycopene (Lyc-CD); **b)** apoptotic results are expressed as percentage (mean ± SD, n = 3) of: **non apoptotic or viable cells**, Q4: Annexin- FTIC (-) and PI (-); **early apoptotic cells**, Q3: Annexin- FTIC (+) and PI (-); **late apoptotic or dead cells**, Q2: Annexin- FTIC (+) and PI (+) and **dead cells**, Q1: Annexin- FTIC (-) and PI (+). Statistical analysis: One Way Anova with All Pairwise Multiple Comparison Procedures (Holm-Sidak method): means with different letters are significantly different ($p < 0.05$).

Also complementary data showed that irradiated cells previously exposed to the vehicle used to solubilize lycopene (CD) presented, once again, results quite similar to those of non irradiated cells (**Fig.6**).

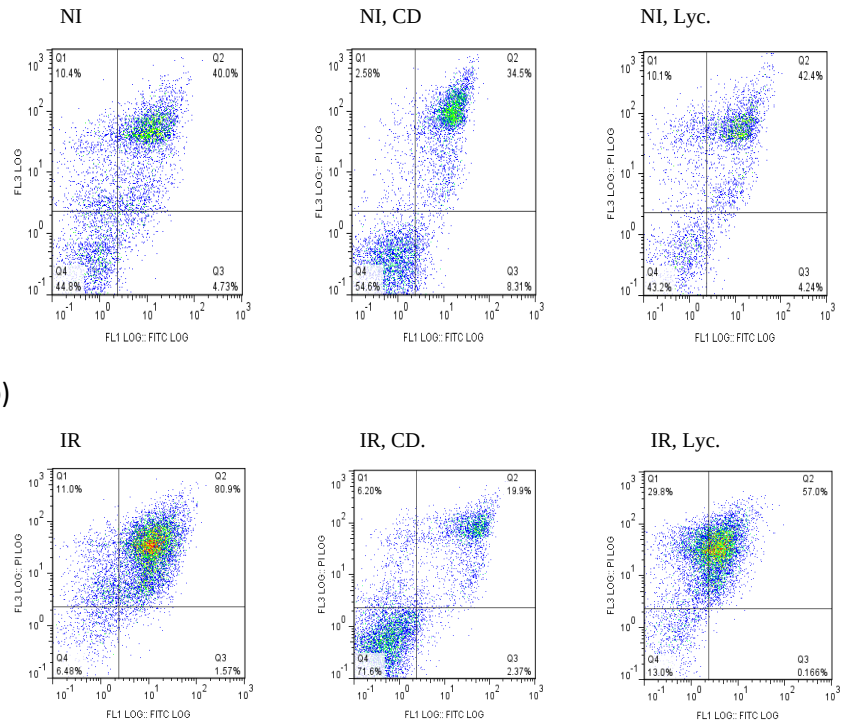


Fig.6- Representation of Annexin V-FITC Dot-Plots Gating (FL1 LOG *vs* FITC LOG) of UV-B (225 mJ/cm^2) irradiated (IR) and non irradiated (NI) HaCaT cells exposed to 10 µM complexed lycopene (Lyc-CD) and only to the vehicle (CD) correspondent to a complementary experiment.

Analysis of Apoptosis Biomarkers and Gene Expression by RT-PCR

UV-B irradiation has been shown to increase levels of apoptosis biomarkers, specially the proapoptotic proteins Bax, thereby inducing apoptosis in the skin cells. The pretreatment of complexed lycopene seemed to maintain the modulatory effects of UV-B towards Bax/Bcl-2 ratio (3.5 ± 1.4) in a way that favored apoptosis (**Fig.7**). However, it should be noted that the number of samples was not enough to detect statistical differences between the samples in each group (irradiated and non irradiated cells).

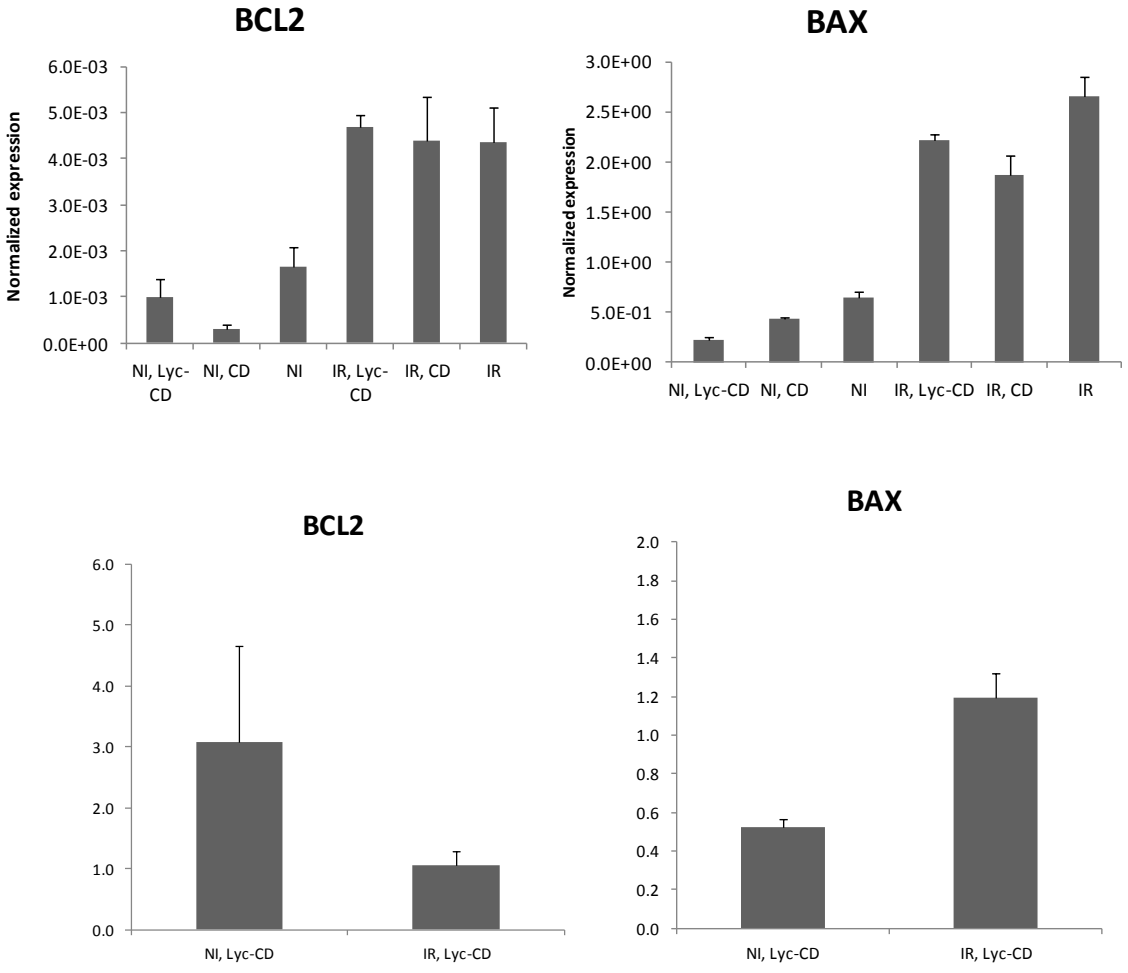


Fig.7- - Representation of Bcl-2 and Bax expression. A) and B) expression values normalized to SDHA reference gene; C) and D) expression values in HaCaT cells exposed to 10 µM complexed lycopene (Lyc-CD) normalized to the SDHA reference gene and relative to each CD vehicle control. Cells were irradiated with 225 mJ/cm^2 UV-B (IR) and non irradiated (NI). Results are expressed as mean ± SEM of two technical replicates from one independent assay.

Cell Cycle Analysis

The **Fig.8** shows representative histograms of cell cycle of HaCaT cells after 10 µM complexed lycopene exposure and UV-B irradiation (225 mJ/cm^2).

Cell cycle analysis shows that complexed lycopene exposure alone did not significantly ($p > 0.05$) affect the dynamic of cell cycle in comparison to control cells. Comparatively to non irradiated and non exposed cells, irradiation induced a decrease in the percentage of cells in the G0/G1 phase of cell cycle specially in complexed lycopene and CD exposed cells (P = 0.011 and 0.008, respectively) (**Fig.8**). Although the S and G2 phases were not significantly affected by any of those treatments, it can be observed a higher synthesis (S phase). Furthermore, in irradiated and complexed lycopene exposed cells, an increase in the percentage of cells in sub-G0/G1 was detected, often associated with apoptotic events.

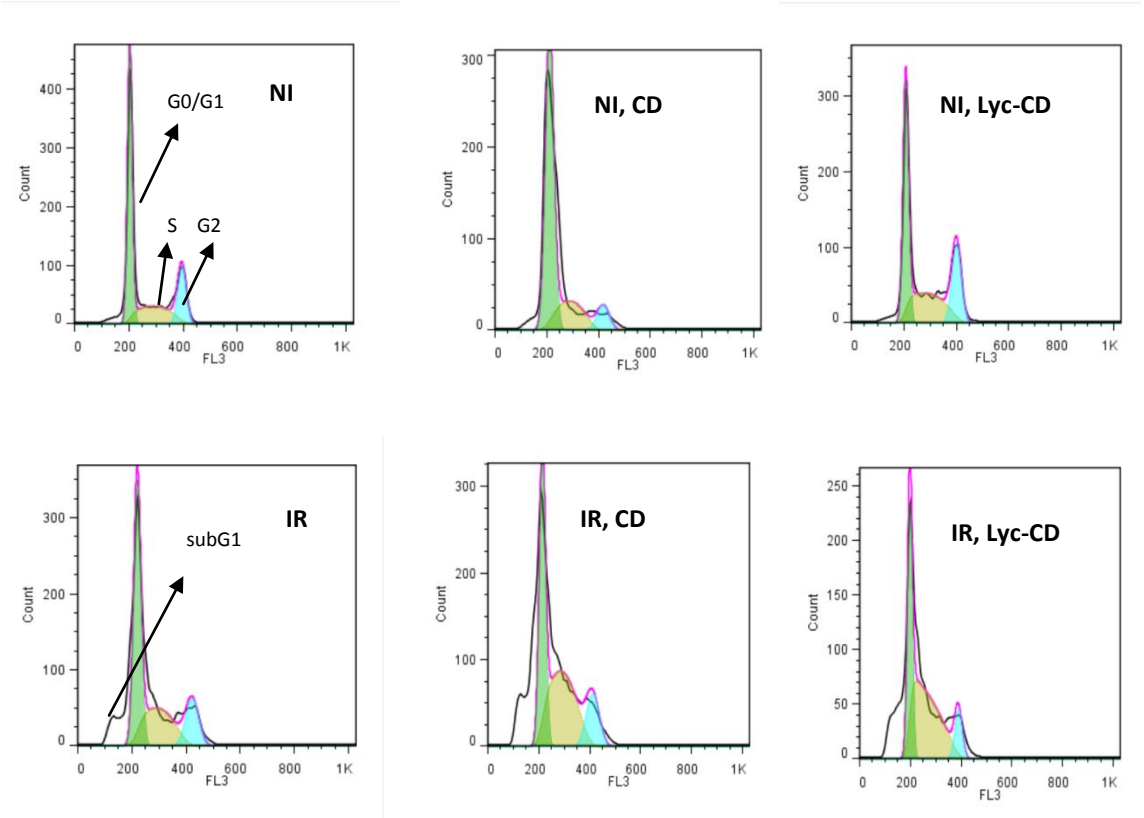


Fig.8- Representation of cell cycle histograms and graphic results of UV-B (225 mJ/cm^2) irradiated (IR) and non irradiated (NI) HaCaT cells exposed to 10 µM complexed lycopene (Lyc-CD) and to the respective controls.

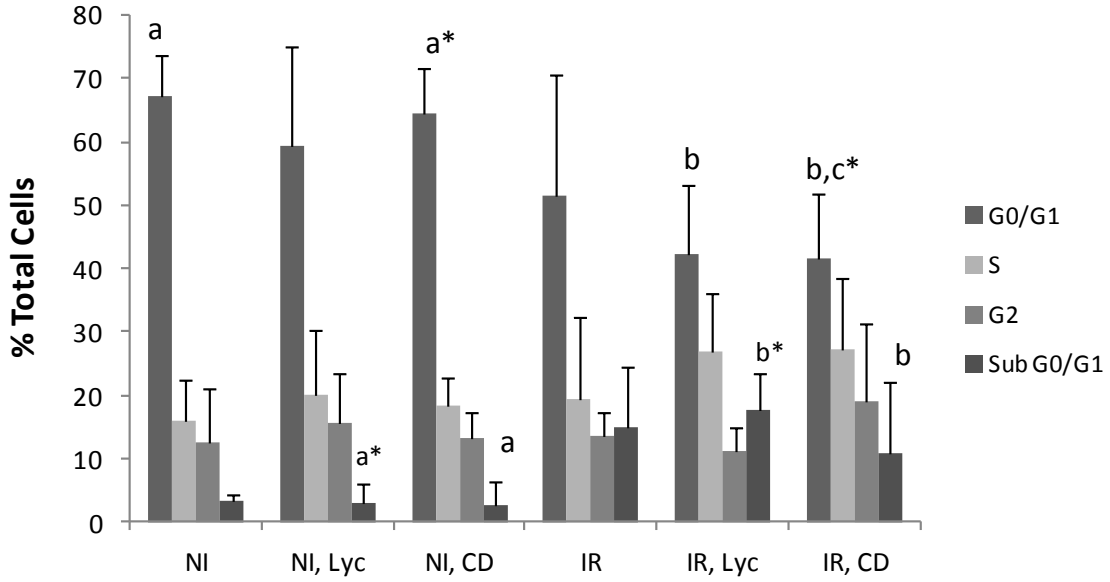


Fig.9- Representation of cell cycle results of UV-B (225 mJ/cm^2) irradiated (IR) and non irradiated (NI) HaCaT cells exposed to 10 µM complexed lycopene (Lyc-CD) and the respective controls. Results are expressed as percentage (mean ± SD). Statistical analysis: One Way Anova with All Pairwise Multiple Comparison Procedures: means with different letters are significantly different ($p < 0.05$). In this case, only the statistical differences were marked in order to simplify the results representation.

Reactive Oxygen Species (ROS) Analysis

Analysis of oxidative stress damage of HaCaT Cells after 10 µM complexed lycopene exposure and UV-B irradiation (225 mJ/cm^2) was performed by determining reactive oxygen species (ROS) by flow cytometry (FCM).

The results of irradiated and non irradiated cells are represented in **Fig**. **10**. Irradiated and non irradiated cells presented a different ROS profile. In fact, two peaks were observed in irradiated cells against one in non irradiated cells. The first peak (1) may correspond to “non viable” cells which had a higher count number than “viable” cells marking DCF-DA (peak 2), as it would be expected considering to the UV-B dose used. In this case, the Median Fluorescence Intensity (MFI) corresponded only to the cells represented on the 2nd peak and it was much higher than MFI from non irradiated cells, as theoretically expected.

Complexed lycopene appears to attenuate the ROS production in non irradiated cells, as theoretically expected. However, the same did not happen in irradiated cells. In addition, CD influenced the ROS level of the cells in each group when compared to the control (NI/IR).

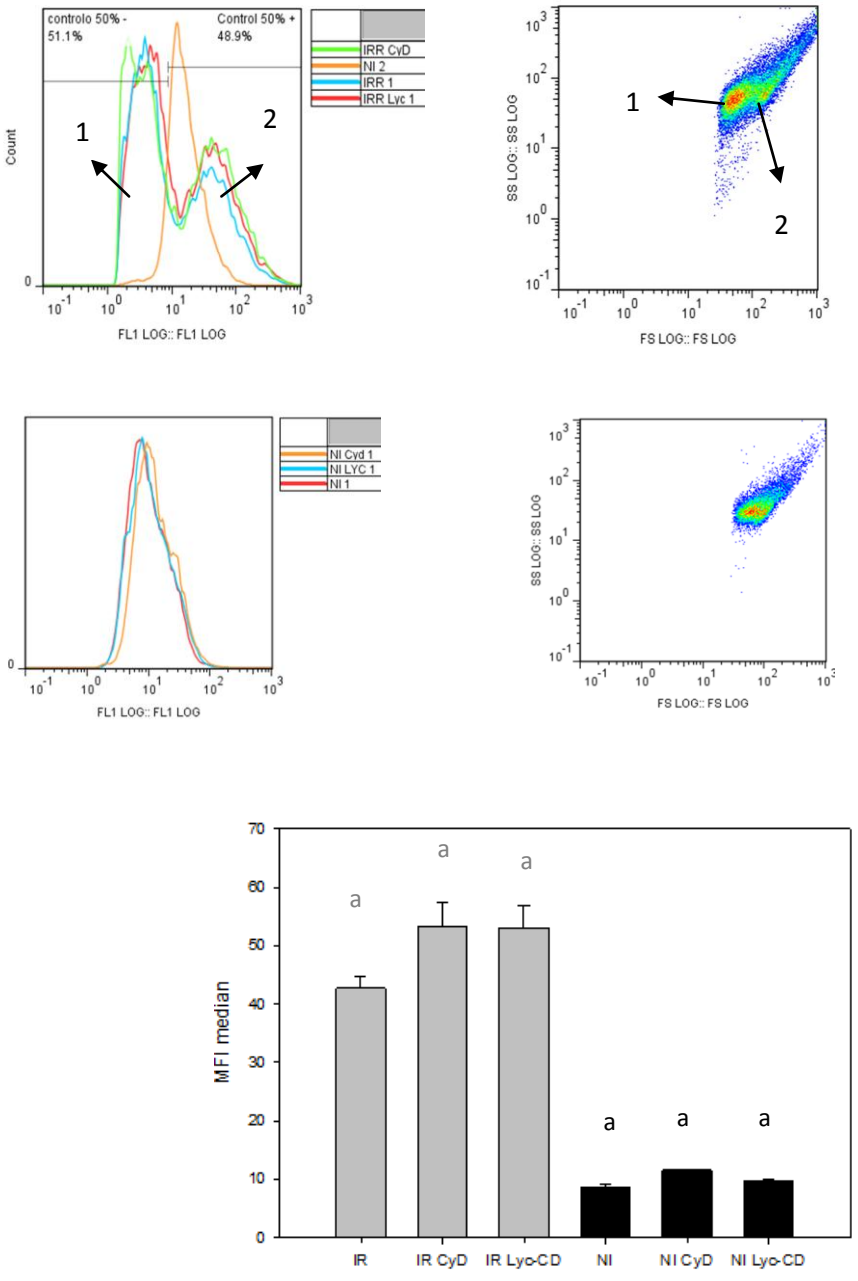


Fig.10- Median Fluorescence Intensity (MFI) histograms, dot-plots gating (SS LOG *vs* FS LOG) and graphic results of HaCaT UV-B irradiated cells, IR (225 mJ/cm^2) and non irradiated (NI) exposed to 10 µM complexed lycopene (Lyc-CD) and to the respective controls marking DCF-DA obtained by FCM. Statistical analysis: One Way Anova with Multiple Pairwise Comparisons: medians with different letters are significantly different ($p < 0.05$) within each group (irradiated and non irradiated cells).

Discussion

In this work, we used **HaCaT cells**, a spontaneously transformed human epithelial cell line from adult skin which maintains full epidermal differentiation capacity. This cell line is immortal (> 140 passages) but remains nontumorigenic, and it is aneuploid (hypotetraploid) with unique stable marker chromosomes indicating monoclonal origin (13). As performed in this experimental work, further investigation needs to include studies dealing with normal cells, their transformation into malignant cells, and the association between malignant cells and the surrounding normal cells in order to determine the cytotoxicity in both cell populations.

The level of lycopene normally observed in human plasma is on the order of 0.5 µM even with dietary supplementation (21, 48, 49). Therefore, for therapeutic purposes (assuming topical application) we used a range above these normal plasma levels (5 up to 20 µM). According to MTT results, 20 µM revealed to be toxic in *in vitro* studies (**Fig.4**). Thus, **10 µM lycopene nominal concentration** selected for further studies, supporting the selected dose used in other *in vitro* studies (48-50).

The option to use **cyclodextrins** for lycopene solubilization and photoprotection was based on data resulting from previous studies (**Table 1**) on parameters/conditions affecting the delivery of lycopene to cells, including the solubilization, stabilization and cellular uptake by using other vehicles.

It is noteworthy that cell culture studies are usually carried out under abnormal conditions known as "**culture shock**", where cells are exposed to high oxygen tension and to free metal ions in the medium (28, 51). Thus, it must always be taken into account if the study compound reacts with the cell medium. Different half-life values of lycopene under standard cell culture are reported in the literature, e.g., 12-20h (39). Our experiments were conducted at 24 h in order to guarantee the lycopene cellular uptake.

The antioxidant action of carotenoids is related to their ability to trap free radicals and quench singlet oxygen. However, depending on the redox potential of lycopene and the

surrounding environment, its antioxidant activity may shift to prooxidant activity (52, 53). In fact, the HaCaT cells medium (DMEM) contains ferric nitrate ($Fe(NO_3)_3.9\ H_2O$) and Fe(III) can react with excess neutral carotenoids, such as lycopene. Ferric ions have been proposed to degrade carotenoids by the following mechanism (54, 55): Fe^{3+} + carotenoid => Fe^{2+} + carotenoid$^{\cdot+}$. Although there are many Fe chelators that inhibit the reactions of Fe, oxygen, and their metabolites (56), these chelating agents may also mediate toxicity by stimulating Fe-mediated oxygen radical generation). The chelating agent used for HaCaT trypsinization was ethylenediaminetetraacetic acid (EDTA) which can induce Fenton-chemistry mediated radical damage. In fact, the autoxidation of Fe(II) enhanced by EDTA was observed by others (57, 58). Therefore, **in order to prevent lycopene oxidation by Fe (III), another cell culture medium (α-MEM) was used** without this element during cells exposure to lycopene. A normal cellular growth was observed. Furthermore, it should be also noted that the solution with the highest lycopene dose had a **suitable osmolarity** lower than 320 mOsm/ kg.

During storage, special precautions were taken such as the **protection of lycopene from temperature, light and air**. However, during lycopene exposure, cell culture was maintained at 37 °C in a humidified incubator with 5 % CO_2 atmosphere. Even considering other experimental conditions reported in the literature (59) that comprise the addition of lycopene solutions to the cell culture medium under N_2 environment, we preferred to maintain our protocol to avoid perturbing the cell culture with other variable.

Apoptosis is the best-characterized type of programmed cell death because of its importance in development, homeostasis, and pathogenesis of different diseases, such as cancer. Cells respond to specific apoptotic signals by initiating intracellular processes that result in characteristic physiological changes. Among these changes are externalization of phosphatidylserine to the cell surface, depolarization of mitochondrial membranes, cleavage and degradation of specific cellular proteins, compaction and fragmentation of nuclear chromatin, loss of cell membrane integrity, and cellular shrinkage. We studied one of the earliest apoptotic events, i.e., the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. As

Annexin V has high affinity to PS, when conjugated with a fluorescent probe as FITC, allows the identification of early apoptotic cells. Mitochondrial dysfunction also occurs early in apoptosis and is accompanied by a decreased membrane potential and the release of mitochondrial proteins such as cytochrome c and Smac/DIABLO. This leads to the activation of caspases (early apoptosis) and DNA fragmentation (late apoptosis) (60, 61).

Moreover, Bowen *et al.* (62) have found that HaCaT cell line (which harbors two mutant p53 alleles) are more susceptible to apoptosis than normal keratinocytes *in vitro*, possibly because of aberrant signaling pathways resulting from long-term culture (63, 64).

In this work, we may have observed a "false negative" **Annexin result (Fig.5 and 6)** using CD as lycopene vehicle (data not shown), as mentioned before and attending to the other assays results. In fact, it is well known that CD can complex with lipids, such as cholesterol, from the skin cells (65, 66). George *et al.* (67) demonstrated that cholesterol located in the plasma membrane of HaCaT cells is required for lipid raft domain formation and activation of UV-B induced apoptosis through Fas-receptor activation. Depletion of cholesterol by methyl-β-cyclodextrin reduces Fas aggregation which is accompanied with a reduced apoptotic, but increased nonapoptotic death of UV-B-irradiated HaCaT cells (67). In addition, free CD molecules (also present in complex mixture) might also complex with phosphatidylserine (PS) also present in lipid rafts in disrupted membrane cells, decreasing the binding to Annexin V- FITC, thereby causing technical interference with this assay.

Besides cholesterol depletion from cells membranes, free CD molecules might also complex with PS in disrupted membrane cells, decreasing the binding to Annexin-FITC (**Fig.11**). In fact, the complex was not filtered, which means that we have all species in the complex solution (lycopene $_{insoluble}$ + CD $\leftrightarrow$ lycopene- $CD_{soluble}$).

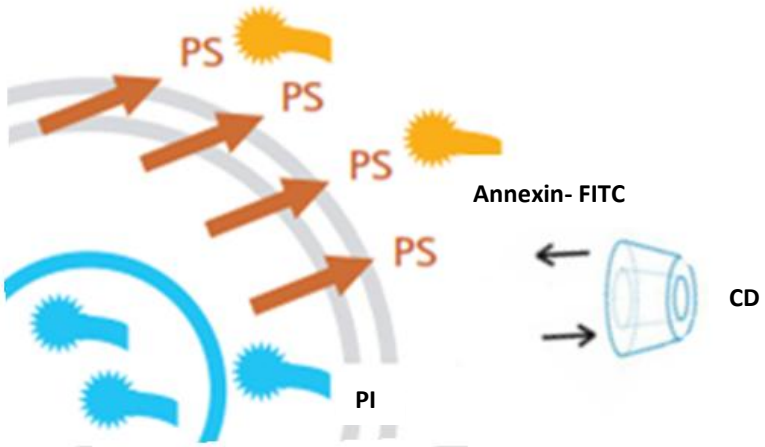


Fig.11- Representation of cell membrane in apoptotic cells and hypothetical interaction with Annexin-FITC + PI and CD (adapted (60)).

In another parallel work, THF/BHT diluted in FBS was also used to solubilize lycopene. In fact, Lin *et al.* (28) demonstrated that FBS is likely responsible for the improved stability and cellular uptake of lycopene (28). As with complexed lycopene, cells exposed to lycopene vehiculated in THF-BHT-FBS and irradiated also showed a decreased viability compared to non exposed irradiated cells (results not shown).

Regarding the **apoptosis biomarkers**, Bcl-2 family proteins can modulate mitochondrial permeability through oxidative phosphorylation during apoptosis. Changes in the Bax/Bcl-2 ratio suggest a corresponding change in mitochondrial permeability to release apoptogenic molecules from the mitochondria to the cytosol (5). In fact, exposed irradiated cells presented a higher Bax/ Bcl-2 ratio (**Fig.7**).

Cell Cycle can be divided into two distinct stages and controlled by a series of cell cycle regulators, including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitors. The first stage is the interphase (G1, S, and G2 phases) in which cells are active, growing, and DNA is being replicated. The second is the "mitotic phase" (M phase) in which cell division takes place. Keratinocytes have a mean intermitotic time of 22–24 h *in vitro* (14).

UV-B irradiation activates diverse cellular responses in human cells, such as cell cycle arrest, DNA repair and apoptosis through signal transduction pathways (16). Previous studies have shown that UV radiation can cause cell cycle arrest both at G1 (68) and G2 phases (69) of cell cycle. However, in our study, irradiation did not affect significantly the percentage of cells on S and G2 phases (**Fig.8 and 9**). On the contrary, the percentage of cells in G1 phase decreased in irradiated cells, specially those previously exposed to complexed lycopene or the respective vehicle alone (cyclodextrins). Cyclin D regulates the transition from G0 to early G1 phase, while cyclin E regulates the transition of the cell from late G1 phase to S phase; p21 and p27 CDK inhibitors bind and inhibit the activity of cyclin E/CDK2 complex, blocking cell cycle progression in G1 phase. In fact, after UV irradiation, the half-life of the tumor suppressor (p53) appears to be extended which will induce the p21 CDK inhibitor leading to G1 phase arrest or cell death by apoptosis. G2-phase checkpoint control does not appear to be affected (70). It has been reported that the growth inhibition of lycopene on MCF-7 breast cancer cells was also associated with decreased G1-S cell cycle progression, decreased cyclin D1 expression, and stabilization of p27 in the cyclin E-CDK complex (7, 71). Cell cycle arrest increases the time available for DNA repair before DNA replication and mutation fixation (5). Regarding the cell cycle results obtained with CD, it has been reported that methyl β-CD inhibits cell growth and induces cell cycle arrest via a prostaglandin E2 independent pathway in Raw264.7 macrophage cells (72). Thus, a control assay with CD alone is mandatory in these studies.

UV radiation results in an increased generation of **ROS** that interact with proteins, lipids and DNA, overwhelming the antioxidant defense mechanisms of the cells.

The epidermis is composed mainly of keratinocytes, which are rich in ROS detoxifying enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and in low-molecular-mass antioxidant molecules (73). Although skin spontaneously responds to increased ROS levels, this response may not be sufficient to prevent the progression of skin cancer (4). Despite the extensive evidence implicating ROS in oxidative DNA damage, little is known about its involvement in DNA damage of keratinocytes, which

are the most relevant cell type in non-melanoma skin cancer. The non-radical singlet oxygen and the hydroxyl radical are the major damaging oxidative species that can be generated inside cells during normal aerobic metabolism and by processes such as photosensitization (74). The major DNA oxidation products include 8-oxo-7-hydro-deoxy-guanosine (8-oxodG) and 2,6-diamino-4-hydroxy-5-for-mamidopyrimidine (75).

According to the ROS determination (**Fig. 10**), the UV-B irradiation significantly altered the cells population. This fact could be observed by the appearance of two peaks in the histograms of DCF-DA of irradiated cells. The first peak might correspond to dead cells, which had a very weak signal of DCF (compatible with autofluorescence). In this case, the DCF might not be activated and metabolized within the cell (no fluorescence), and / or the cells might not retain the DCF because of the loss of membrane integrity. Complexed lycopene increased the ROS level in irradiated cells. Shaw *et al.* (5) have also obtained a significantly decrease of SOD activity (MnSOD) after treatment with sanguinarine, enhancing UV-B-mediated oxidative stress in HaCaT cells. However, according to Onoue *et al.* (76), ROS data might not always provide a reliable indication for the capacity of a chemical to participant in a photogenotoxic cascade. This fact could be more realistic when irradiation is used in experiments. In fact, the photodegradation of lycopene may contribute to cytotoxicity, including oxidative damage. For example, apo-6´-lycopene, 2-methyl-hepte-6-one, as well as further reaction products, are formed during irradiation of lycopene (33, 77). Nagao (78) found that oxidized metabolites of lycopene but not lycopene itself can inhibit cell growth and stimulate apoptosis in a different cell line (HL-60). In another study, lycopene in human prostate cancer cells inhibited cell growth, but the oxidized mixtures displayed markedly more potent growth inhibition (78, 79). On the contrary, some recent studies suggested that lycopene or lycopene metabolites may, as β-carotene and its metabolites do, enhance carcinogenesis.

In general, similar results were obtained by Shaw *et al.* (5) who suggested that sanguinarine (also a botanical antioxidant) may protect skin cells (also HaCaT cell line) from UV-B- mediated damages via apoptotic elimination of damaged cells that escaped from the programmed cell death. These are clearly important observations since

apoptosis is a mechanism of defense and acts by opposing the creation of damaged and preoneoplastic cells, and expansion to a clone. Once mutations arise, apoptosis also removes preoneoplastic cells that are aberrantly proliferating due to genetic defects.

However, further investigation into the dose effect of lycopene, as well as a further understanding of the metabolism of apo-10′-lycopenoids on carcinogenesis are still needed (7).

Conclusions

According to data obtained from all experimental assays (Scheme 1, in annex to this chapter), we demonstrate here that complexed lycopene up to 10 μM does not show metabolic toxicity (MTT assay) under standard cell culture conditions. On one hand, at non toxic dose (10 μM) complexed lycopene does not affect the profile of apoptotic, necrotic and viable cells, nor shows cytostatic effects despite slightly increasing the ROS content. However, cells previously exposed to complexed lycopene when irradiated with metabolically damaging UV-B dose, show a distinguishing switch in the dead : apoptotic : viable subpopulations compared to non exposed-irradiated cells. On the other hand, exposed irradiated cells showed a decrease in G0/G1 phase. In fact, the increased sub-G0/G1 phase, and a trend for S-phase delay (even not statistically different) could contribute to this cell cycle change.

Therefore, complexed lycopene might play a corrective role or cytotoxic effect in photodamaged and preneoplastic keratinocytes, while allowing other keratynocytes to accelerate repairing mechanisms becoming viable. However, future studies will be performed regarding the analysis of apoptosis regulating genes by RT-PCR to further confirm these results (with statistical significance). In addition, the same studies will be repeated with other exposure conditions, i.e., other lycopene vehicle and a lower UV-B dose.

Acknowledgments

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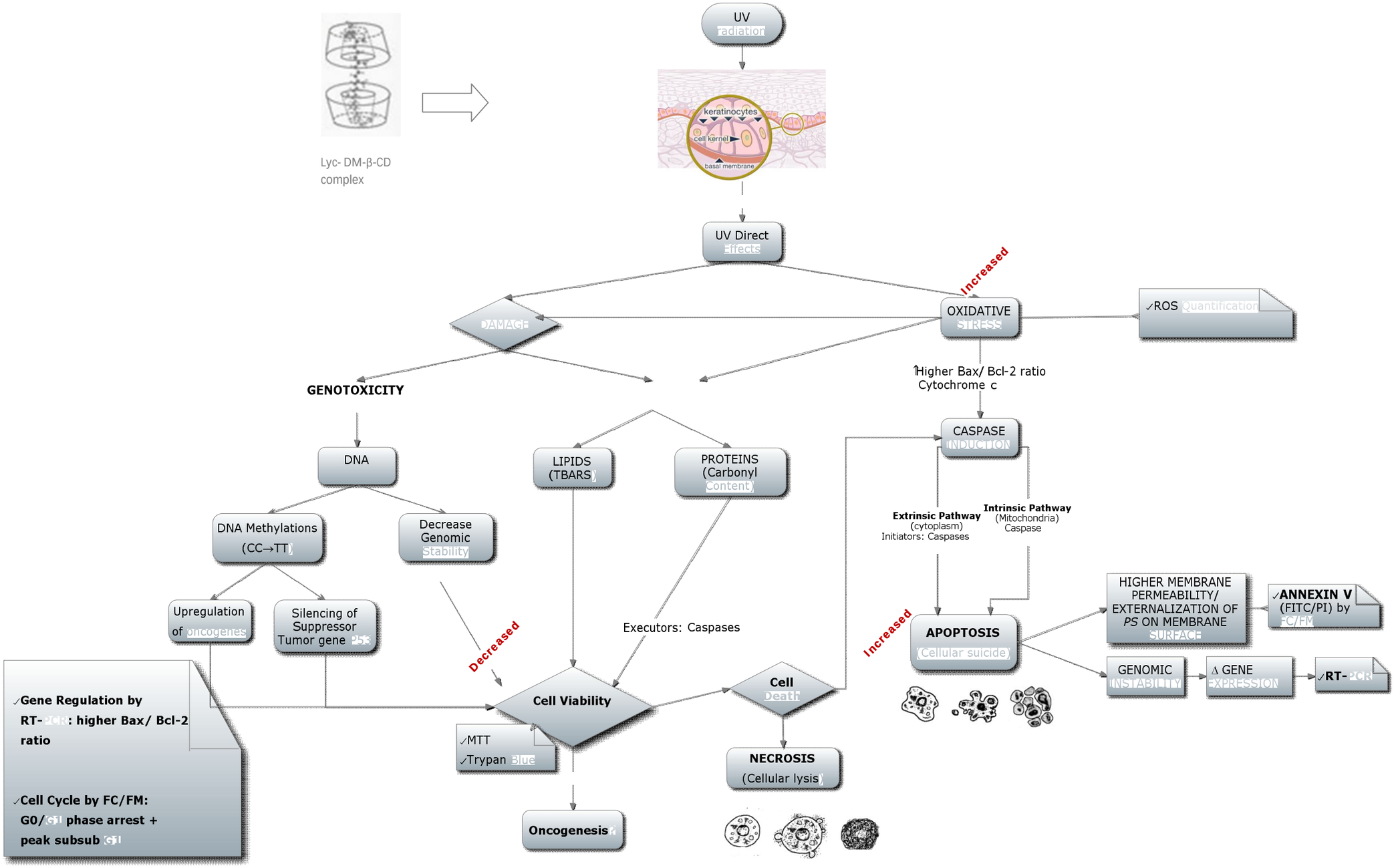

Scheme 1 - Proposal model of the effects of UV irradiation on skin cells and analytical assays used to study those effects.

Conclusions and Future Perspectives

In summary, tretinoin was incorporated in free and in complexed form (with dimethyl-β-cyclodextrins) in ultradeformable vesicles (transfersomes) formulations. Lycopene was incorporated in transfersomes and ethosomes, attending to the results previously obtained with tretinoin. Tretinoin and lycopene share similar physicochemical properties, mainly high hydrophobicity and chemical instability to air and light. In fact, lycopene was incorporated in the most stable formulation tested for tretinoin, transfersomes with 20 % lipid concentration.

Carrier-mediated tretinoin and lycopene have improved the dermal drug delivery. Tretinoin chemical stability was only achieved by incorporating this drug into transfersomes made of a high phospholipidic concentration. In addition, this last formulation appears to be a promising delivery system to promote cutaneous delivery of tretinoin because it exhibited predominant dermal delivery and showed no toxicity.

Regarding the lycopene extraction process, a rapid, simple and economically advantageous process was achieved, providing a lycopene-rich extract. This lycopene source may be, thus, taken as a good alternative to the expensive commercially available lycopene to integrate topical formulations. Extracted lycopene loaded-deformable lipidic carriers (transfersomes and ethosomes) were able to inhibit ear swelling mediated by ROS generated within the skin by anthralin application. Finally, according to data obtained from *in vitro* photocarcinogenesis experiments, lycopene might play a corrective role/cytotoxic effect in photodamaged cells.

A number of pharmaceutical challenges are still to be met. However, the experimental data collected in this work provides evidence of the significance of delivery systems on tretinoin and lycopene on prevention and/or treatment of skin disorders.

Future experiments regarding optimization of lycopene extraction process, stability studies of lycopene-loaded vesicular nanocarrier formulations, and the effect of surfactant on physical properties and stability of those formulations are also planned. Additional *in vitro* experiments could be performed in order to further confirm the chemopreventive role of lycopene in photodamaged skin cells with different exposure conditions (e.g., HaCaT irradiation to a UV-B single dose of 25 mJ/cm^2 before and/or after 6 h of cells exposure to 5 µM extracted lycopene, vehiculated in 0.05 % THF-0.0005 % BHT- 1 % FBS system). Other alternative assays could also be used, such as the clonogenic assay, detection of chromosomal breakage by cytokinesis-blocked micronucleus cytome, and SOD and total antioxidant capacity assays, in order to achieve the cell viability, apoptosis and oxidative stress of exposed cells, respectively. In addition, the analysis of more upstream/downstream damage biomarkers will be useful to better understand the cellular mechanisms behind the photodamage process. At least, the role of lycopene in *in vivo* conditions regarding the irradiation protocol could be more explored using animal models. The hypothesis to test the benefits of an antioxidant combination (e.g. lycopene and vitamin E) is still open.

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