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EXPERIMENTAL OBSERVATIONS OF CHANGES IN EPIDERMOLYSIS BULLOSA AND OTHER SKIN INJURIES
ABSTRACT

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CONTENT

Keywords: epidermolysis bullosa, pentacyclic triterpenes, membrane transporters, skin lesions, UV radiation, physiological parameters of the skin.

Experimental observations on changes in epidermolysis bullosa and other skin lesions

The doctoral thesis addresses the research topic of identification regarding the changes in genetic disorders with skin fragility that are characterized by structural abnormalities that may include: peeling, blisters, erosions, ulcers, wounds or scars - a disorder called epidermolysis bullosa (EB); as well as the identification of changes in other skin lesions, induced by UV radiation.

The doctoral thesis is structured in two parts: the general part and the special part. The general part of the doctoral thesis includes three important chapters that contain the latest scientific information from the literature, regarding the changes in epidermolysis bullosa and other skin lesions. The special part consists of the experiments carried out practically, within the Pharmaco-Toxicological Research Center, Faculty of Pharmacy, UMFVBT, led by Prof. Univ. Dr. Farm Cristina Dehelean. The experiments are divided into four chapters and the result complements the information in the literature, and lead to the completion of the clinical picture of innovative treatment strategies for both epidermolysis bullosa and other skin lesions.

I. General Part

I.1. Experimentally approached skin pathology

This chapter provides an overview of skin structure and functions, basic pathophysiology, and some of the important skin toxicity considerations. A major objective for the toxicological evaluation of a dermal drug is to fully characterize systemic and toxicokinetic toxicity, thus covering the potential for the most unfavorable situations. Systemic toxicity studies may be required if low or minimal systemic exposure is achieved by dermal administration in dermal toxicity studies. Due to the fact that dermal medical products are often used on skin exposed to light (face, arms), the assessment of the phototoxic potential of the developing medicinal product may also be

justified. Also in this chapter we brought to the core information on dermal carcinogenicity studies, for dermal medicinal products intended for chronic and / or intermittent recurrent use. To reach the main topic of the doctoral thesis, namely: epidermolysis bullosa, I choose to describe the dermatological manifestations in inflammatory bowel disease, classifying mucocutaneous lesions, after which I focused on developing the information found in the literature on epidermolysis bullosa. Also in this chapter, are presented other pathologies that generate skin lesions, such as: hyperplasia , hyperkeratosis, inflammation, skin irritation and corrosion, hypersensitivity, skin pigment change concluding this chapter with the latest general information on skin cancer.

I.2. Innovative therapies for skin lesions and epidermolysis bullosa

Chapter 2 of the theoretical part, briefly describes the clinically approached therapies, both in the case of skin lesions and those approached in the case of epidermolysis bullosa. Rapid scientific developments in recent years have given rise to various promising therapeutic strategies for the treatment of epidermolysis bullosa. The diagnosis and management of various forms of epidermolysis bullosa are complex and require interdisciplinary collaboration. Severe forms of this disease are most in need of medical support, and most research efforts focus on developing therapies for these forms. Both curative therapies and symptom relief therapies are being developed. A number of therapies are under preclinical development, and the first one have already reached the clinical trial stage, either in phase 1/2 or in phase 3.

I.3. Ethical aspects regarding the experimental models research

This chapter describes the ethical aspects regarding the experiments on animal, ie the rules for animal care standards, mentioning the 3 "R" principles to be considered, regardless of the field of experimental animal research. All animal experiments detailed in the thesis were designed and implemented after taking into account the health of laboratory animals (mice), as well as after evaluating the influences that the experiments may have on animal welfare. For setting the animal welfare standards, the animal

welfare criterion was taken into account, which prevailed over their mere survival in the unfavorable conditions of the experiment. The studies described in the doctoral thesis were carried out in compliance with the principles imposed by Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 regarding the protection of animals used for scientific purposes, including those relating to the competence of researchers, such that animal welfare was fully ensured.

II. Special Part

The general purpose of the doctoral thesis was to identify changes in induced epidermolysis bullosa, on an experimental animal model, and in other skin lesions, respectively the design of therapeutic formulations that can be used successfully in treating these skin lesions.

In order to achieve the purpose of the thesis, the following objectives were taken into account, including their specific activities:

a) induction of lesions similar to the lesions given by epidermolysis bullosa, on animal model, using female SJL mice, by applying the active immunization method (100 µg type VII recombined mouse collagen (GST-mCVIICr)/50 µL injection/mouse, twice every 3 weeks);
b) evaluation the toxicity of raw material in the polyurethane industry, namely - the synthesis catalyst reaction . Regard to this, we have developed polyurethane particles , which do not contain any traces of catalyst, in order to establish safety use, in terms of toxicity. To evaluate the toxic and irritative nature induced by the catalyst content in these transmembrane transporters, we performed non-invasive evaluations of skin parameters using another animal model (BALB/c mice). Measurements of skin parameters were performed using a professional MPA5 monitoring system equipped with a Mexameter®MX18 probe to determine melanin and erythema levels, and a Corneometer®CM825 probe to assess changes in corneal hydration;

c) development and treatment of other skin lesions, similar to epidermolysis bullosa lesions, on animal model. Regard to this, we induced solar lesions to the animal model (SLJ female mice) using UV radiation. The treatment consist, use of synthesized oleogels, containing betulin and lupeol. The effectiveness of these oleogels against sunburn was investigated by measuring the skin parameters of the animal model, using the professional monitoring system MPA5, to which, this time, the Mexameter®MX 18 probe was attached to record melanin and erythema levels, while transepidermal water loss (TWL) and skin hydration were measured using Tewameter®TM 300 and Corneometer®CM 825 probes, respectively.

II.1. Therapies used experimentally

This chapter refers to phytomedicine preparations that have proven to be an interesting alternative to beneficially influence the various stages of wound healing. Wound repair is an extremely complex biological process, practically divided into three overlapping phases: inflammation, new tissue formation and remodeling. Wound healing requires the integration of many complex cellular and molecular events, which can be targeted in many ways, leading to either accelerated or delayed healing, the latter of which can lead to complex chronic wounds. An impressive number of studies, published in the literature, demonstrating the curative potential of pentacyclic triterpenes through various biological activities. Birch bark is known as a traditional medicinal remedy since the time of the North American Indians who wrapped their wounds in birch bark to speed up wound healing. Due to this aspect, an oleogel-type medicine (Episalvan) was first approved worldwide in 2016 (EU/1/15/1069/001), and contains purified dry extract from the bark of *Birch pendula* and *B. pubescens*, or hybrids of both species, rich in betulin as active ingredient (80%). The indication with which this drug was placed on the market was to treat superficial skin wounds (epidermis and upper dermis) and second-degree burns on adult skin. Till date, there has been no drug approved in Europe with proven efficacy in accelerating wound closure.

II.2. Common non-invasive measurements applied for experimental skin pathology

In general, whether we are talking about new synthetic products or based on natural extracts, aimed at cleansing and caring the skin or about new transdermal transporters such as polymeric carriers (liposomes) or based on inorganic nanocapsules (gold, silver, carbon or various metal or non-metallic oxides), they require testing of their properties in experimental investigations evaluating the irritant, moisturizing, emollient, etc.

In the last decades, skin parameters have become quantifiable through the use of a series of professional probes developed by the German manufacturer Courage-Khazaka (Köln, Germany). These probes are connected via a digital MPA (multi-probe adapter) interface to computers or laptops and use specialized software to measure with great precision the changes that have occurred on the surface of the skin.

II.2.1. Biophysical evaluation and the impact of the catalyst on the properties of polyurethane drug carriers

Due to the fact that transdermal drug delivery systems have generated widespread interest as a preferred alternative to oral drug administration and hypodermic injections, another chapter of the doctoral thesis refers to the assessment of the toxicity of a raw material in the polyurethane (PU) industry, namely the synthesis catalyst reaction . Polyurethanes (PU's) are a class of macromolecular compounds that act as transmembrane transporters for a wide variety of biologically active substances. Moreover, the diversity of applications of these macromolecular compounds has increased exponentially in recent decades, due to applications in the medical field, namely: catheter tubes, hospital bedding, wound dressings, bone reconstruction implants, artificial hearts, surgical drains, intraaortic balloon pumps, dialysis devices, non - allergenic gloves etc. Regard to this, the study presents various samples of transmembrane quarries based on polyurethane microstructures, in the synthesis of which an organic mixture of hexamethylene diisocyanate and isophorone diisocyanate dissolved in acetone alongside an aqueous phase containing ethyleneglycol, 1,4-

butanediol and polyethylene glycol at different concentrations of DABCO (1,4-Diazabicyclo [2.2.2] octane), were used. The results obtained indicated that DABCO used as a catalyst does not structurally influence the transmembrane carrier. The toxicity and irritation induced by the catalyst was quantified by non-invasive skin parameter assessments using 6 BALB/c mice and a professional MPA5 monitoring system. Significant differences were observed in terms of erythema and skin hydration, the influence of the catalyst being dose-dependent. In conclusion, synthesized polyurethane particles, which do not contain traces of catalyst, can be considered much safer in terms of toxicity.

II.3. Aspects related to the experimental production of epidermolysis bullosa

II.3.1. Challenges and limitations in the development of an animal model of epidermolysis bullosa using SJL mice

This chapter describe a method for inducing epidermolysis bullosa on the experimental animal model (female SJL mice), namely: the active immunization method, using recombinant mouse collagen type VII (GST-mCVIICr). The results obtained indicates that subcutaneous administration (footpad and tail base) of 100 μg of recombinant type VII mouse collagen (GST-mCVIICr) followed by an injection containing recombinant antibody emulsion on day 21 post-immunization, did not induce specific clinical signs of EB development in any of the immunized mice. In conclusion, under the conditions of this experimental protocol, SJL female mice have been resistant to the development of clinical disease, but further studies will be performed to obtain a reliable and reproducible model. However, this study could be considered as a lesson on the critical role played by the type of selection of the mouse strain when performing experiments on animal models.

In addition, to achieve this goal, we performed an *in vitro* pharmacotoxicological investigation on the biocompatibility of oleogels containing betulin and lupeol, on a human healthy skin cell line (immortalized human keratinocytes

- Ha-CaT), within the Pharmaco-Toxicological Research Center, within the Faculty of Pharmacy. This investigation was supplemented by *in vitro* evaluation of the potential of oleogels for wound healing and skin reepithelialization, by applying the Wound Healing (Scratch) assay. To complete the toxicological profile, the two oleogels with active substance contents were also tested *in ovo*, in the same research center, in order to establish the potential irritant profile of the formulations prepared, on the embryonated chorioallantoic membrane (HET-CAM assay).

II.4. Physico-chemical studies of betulin and lupeol release from pharmaceutical formulations for skin pathology

II.4.1. Oleogel formulations for topical release of betulin and lupeol into skin lesions - preparation, physico-chemical characterization and pharmacotoxicological evaluation

In order to achieve this goal, oleogels with betulin and lupeol have been synthesized, with applicability in sunburn, due to the fact that sunburn and sun protection are a field of research in which more and more is being invested due to the growing number of skin cancers. The study refers to the assessment of the effectiveness of oleogels with betulin and lupeol against sunburn induced by UV radiation, by using the experimental animal model (SJL female mice) that were initially exposed to UV radiation. The results obtained showed effective efficacy of betulin and lupeol oleogels, in the mice that were treated with both samples, registering clearly changed values compared to the mice from the control group or those treated with Blank oleogel - without content of active substance. It should be noted that this was one of the few studies in which skin parameters were measured both during the period of UV irradiation (when an extraordinary deterioration of each parameter studied can be observed) and in the subsequent period, the treatment or recovery period of skin. In conclusion, it can be said that oleogels with pentacyclic triterpenes, especially betulin and lupeol, can be used successfully as vehicles for sun protection.

II.5. General conclusions and personal contributions

The objectives proposed in the thesis were fully met, resulting the following conclusions:

- regarding the transmembrane carriers based on synthesized polyurethane microstructures, after characterization, we quantified the toxic and irritative character induced by the catalyst, by non-invasive evaluations of the skin parameters of Balb/c mice and found significant differences in erythema and skin hydration, the results show that the influence of the catalyst used in the synthesis is dose dependent. In conclusion, synthesized polyurethane particles, which do not contain traces of catalyst, can be considered much safer in terms of toxicity.
- regarding the study on the induction of mice lesions similar to the lesions given by epidermolysis bullosa, the experiment failed, given that no specific signs were observed for the development of this disease, ie mice did not develop blisters, erosions, alopecia, crusts or scar. A possible explanation for the non-development lesions specific to epidermolysis bullosa after immunization could be related to genetic variations that occurs in mice strain. Based on data from the literature, a possible factor that led to the failure of this experiment was that the mice used in our study were not inbred mice.
- due to this fact that nanoemulsions and nanogels are the most suitable vehicles for drug administration, due to their lipophilic nature, optical clarity and surface, the last objective proposed in the thesis was to obtain and characterize oleogels with betulin and lupeol, which have applicability in solar burns. After physico-chemical characterization of oleogels, the results obtained were correlated with *in vitro* biocompatibility and wound healing property, due to the fact that biocompatibility is mandatory for topical formulations. The viability results indicated a lack of cytotoxicity of the three formulations, with or

without active substance content and an adequate *in vitro* biocompatibility on HaCaT cells (healthy human keratinocytes). Testing of oleogels on the chorioallantoic membrane revealed the lack of irritant potential of the tested formulations, the irritation scores obtained for oleogels containing betulin and lupeol being lower than 1. The *in vivo* results obtained (using SJL mice) indicate the benefits of oleogels with betulin and lupeol on the skin, characterized by low erythema and increased skin moisture (both parameters were significantly changed compared to the control group and the group of mice treated with oleogel without active substance).

The novelty of the doctoral thesis consists in the fact that we have managed to synthesize semi-solid formulations with betulin and lupeol content, much lower (0.3%) than the product already approved on the market (80%), a formulation that demonstrates a good biocompatibility *in vitro*, as well as a lack of toxicity *in ovo* and *in vivo*. In addition, regarding to the potential effect on skin re-epithelialization, betulin oleogel exerted the strongest wound-healing activity *in vitro*.

The original character of the doctoral thesis is supported by 4 scientific papers published in ISI journals, of which 2 papers in ISI journals with an impact factor over 1 and two scientific papers in ISI journals with an impact factor over 4. In both the published scientific papers I am the first author, in one scientific paper I have an equal contribution with the first author, and I am co-author in the last scientific paper.