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Sex differences and pathology status correlated to the toxicity of some common carcinogens in experimental skin carcinoma

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ABSTRACT

The increased susceptibility of men as compared to women to develop different types of cancer, including skin cancer, is well known; however, the mechanisms involved in this process are still a matter of debate. This study aimed to obtain animal models of photo-chemically-induced skin carcinogenesis by exposure to ultraviolet radiation B (UVB) coupled with topical applications of a tumor initiator (7,12-dimethylbenz(*a*)anthracene, DMBA) and a tumor promoter (12-O-tetradecanoylphorbol-13-acetate, TPA) in order to characterize the gender disparities regarding the skin lesions developed by the female and male SKH-1 hairless mice included in this study. Histopathological analysis confirmed the presence of malignant lesions in both cases, in female and male mice, following chronic exposure (24 weeks) to the noxious effects of the carcinogens applied, whereas the tumors in male mice had a more severe histological grade. In addition, tumor incidence, size and multiplicity were higher in male mice than in female mice.

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1. Introduction

Non-melanoma skin cancers are considered the most prevalent type of cancer in Western populations and the reported incidence of this type of cancer is approximately 100 cases to 100,000 individuals in Europe. The family of non-melanoma skin cancers, also known as the "white skin cancers", includes squamous cell carcinoma (SCC), and basal cell carcinoma (Fartasch et al., 2012; Burns et al., 2013).

A topic of great interest is represented by the role of gender disparities in the development of cancer. According to the American Cancer Society reports, males present a 3-fold increased risk of developing SCC as compared to females (Thomas-Ahner et al., 2007). Similar data were observed in other types of pathologies, including melanoma, tobacco-induced lung cancer, myeloid leukemia, multiple sclerosis and diabetic cardiomyopathy (Joosse et al., 2011; Oberyszyn, 2008; Ngo et al., 2014).

Since the cellular and molecular processes involved in gender differences in skin cancer development, progression and survival are not yet fully characterized, numerous studies have used animal models in order to shed some light into this topic. Animal experimental models are currently used to study the chemoprevention, as well as the treatment of different types of human cancer, including non-melanoma skin cancer (Steele and Lubet, 2010). An ideal animal model for anticancer and chemoprevention tests should comply with several mandatory requirements, such as: similar pathology to human cancers, genetic and histological features as well as molecular pathways resembling human cancers and, most importantly, the development of the pathology within a relatively short period of time, depending on the cancer type (Steele et al., 2005).

The skin cancer animal model protocol employed to generate non-melanoma skin cancers is usually conducted in a two-stage chemically-induced process using topically applied DMBA (7,12-dimethylbenz[a]-anthracene) as tumor inducer and TPA (12-O-tetradecanoylphorbol-13-acetate) as promoter during a period of time

Abbreviations: DMBA, 7, 12-dimethylbenzanthracen; TPA, 12-O-tetradecanoylphorbol acetate; UVB, ultraviolet radiation type B; SCC, squamous cell carcinoma; TEWL, transepidermal water loss.

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Table 1
Modifications of different characteristics at the meaningful points of the experiment.

Tumors characteristics	Animal groups			
	Group 1, F UVB, acetone	Group 2, M UVB, acetone	Group 3, F UVB, DMBA, TPA	Group 4, M UVB, DMBA, TPA
10 weeks of experiment				
Weight (g) (average)	27.1	32.9	28.1	34.6
Tumor incidence (%)	0	0	0	0
Tumor multiplicity (average)	0	0	0	0
Tumor yield (mm)	none	none	None	none
24 weeks of experiment				
Weight (g) (average)	28.4	34.3	29.4	35.4
Tumor incidence (%)	0	0	100	100
Tumor multiplicity (average)	0	0	5.3	3.7
Tumor yield (mm)	none	none	>1 mm	>1 mm

F - female mice; M - male mice.

ranging from 10 to 25 weeks (Abel et al., 2009; Ishikawa et al., 2010).

An important issue to be considered when establishing a chemically induced animal model is the intrinsic toxicity of the chemical agent, at local as well as at systemic level. Studies have shown that DMBA is rapidly absorbed through the skin (Sanders et al., 1986) thus affecting internal organs, mainly the liver. Its cutaneous absorption could lead to the development of various types of cancer, including lymphoma and leukemia (Wei et al., 2002; Kozma et al., 1993). This aspect carries a great relevance in terms of animal survival throughout the experiment.

Recently, given the high relevance of the role of ultraviolet radiation type B (UVB) in the occurrence of skin cancers in humans, a new animal model was developed using SKH-1 hairless mice that were repeatedly exposed to UV radiation for a variable period of time, with or without previous DMBA topical application (Jiao et al., 2014; Chillampali et al., 2010).

The objective of our study was to monitor and analyze the gender disparities from a histopathological point of view in a mouse model of photochemically-induced skin carcinoma using SKH-1 hairless mice. We also evaluated the physiological skin parameters (skin hydration, erythema and transepidermal water loss (TEWL)) as co-markers for the development of skin tumors.

2. Materials and methods

2.1. Chemicals

DMBA and TPA were purchased from Sigma (Germany) and used as acetone solutions (0.025% DMBA and 15 nmol TPA, respectively, in acetone). Acetone of analytical purity (99.92%) was purchased from SC Chimreactiv SRL (Bucharest, Romania) and used as received.

2.2. Experimental design of the two-stage skin carcinogenesis animal model

Adult SKH-1 female (weight 24 ± 1 g) and male (weight 30 ± 1 g) mice at the age of 10 weeks were divided into 4 groups (n = 8 mice/group) as follows: group 1 - female control group (UVB exposure and acetone topical application); group 2 - male control group (UVB exposure and acetone topical application); group 3 – females - UVB exposure and DMBA and TPA topical application; group 4 – males - UVB exposure and DMBA and TPA topical application. Since acetone was the solvent for the two chemical agents, groups 1 and 2 were considered as the control groups during the experiment. The protocol for the skin carcinoma animal model was conducted according to the literature with several modifications. Briefly, the protocol involved two steps: 1) an initiation step – the first 2 weeks of the experiment – consisting of: UVB radiation for 3 min (total dose around 200 J/m^2 UVB radiation) followed after 30 min by the topical application of DMBA once a week, 200 μl/mouse, and 2) a promotion step - until the end of the experiment – consisting of: UVB radiation for 3 min (total dose around 200 J/m^2 UVB radiation) followed after 30 min by topical application of TPA (200 μl/mouse) – twice a week. UVB was generated using VL-6.M/6W (312 nm wavelength) tubes (Vilber Lourmat, France). The same procedure was followed for the control groups using UVB radiation and the topical application of acetone (200 μl/mouse).

All experimental procedures were conducted in accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes. The experimental protocol was approved by the Committee for Ethics Research of the "Victor Babeş" University of Medicine and Pharmacy, Timisoara, Romania. The mice were kept in standard conditions: room temperature 22.5 ± 2 °C, humidity around 55% and 12 h/12 h light/dark cycle. The animals were fed with a standard diet *ad libitum* (see Fig. 1).

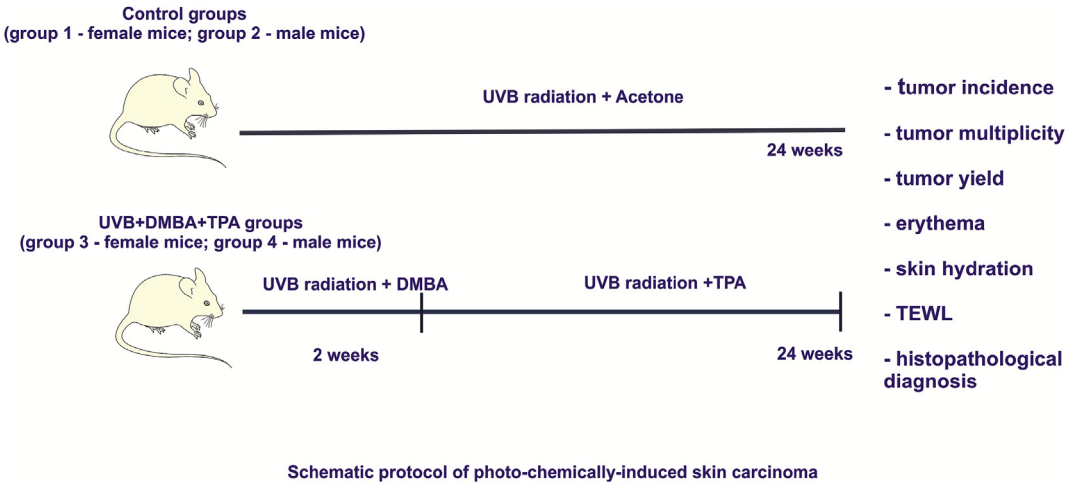

Fig. 1. Schematic protocol of photochemically-induced skin carcinoma (this image contains elements from Servier Medical Art).

2.3. Tumor assessment

The mice were monitored weekly for tumor assessment. Tumor dimensions were determined using a caliper when the tumors reached >1 mm. Tumor incidence (the percentage of mice that developed at least one tumor after exposure to the carcinogens), tumor multiplicity (number of tumors/mouse), tumor yield and mice weight were recorded weekly until the end of the experiment.

2.4. Non-invasive skin parameter measurements

Measurements of the physiological skin parameters were conducted using a Multiprobe Adapter System (MPA5) purchased from Courage-Khazaka, Germany. The erythema values were recorded using a Mexameter® MX 18 (Courage-Khazaka) probe in order to achieve quantitative results regarding the content of hemoglobin. The skin hydration values were recorded using Corneometer CM 825 probe (Courage-Khazaka). The transepidermal water loss (TEWL), an indicator of the integrity of the skin barrier, was measured using the probe Tewameter® TM300 (Courage-Khazaka).

2.5. Histopathological evaluation

For the evaluation of the precancerous skin lesions as an early assessment, at week 10 of the experiment, some mice of both genders were sacrificed for histopathological analysis. At the end of the experiment (week 24), all mice were sacrificed. All animals used in this study were sacrificed under anesthesia. At both time points, clinically normal skin and tumor samples were harvested from all the mice. The specimens were fixed in 4% v/v buffered formaldehyde and embedded in paraffin. Three micrometer-thick serial slides were cut using a Leica Rotary Microtome RM2255 and stained with hematoxylin and eosin (H&E) and Giemsa stain. The microscopic examination was conducted using a Leica Light Microscope DM750; images were captured using a Leica DMShare System.

2.6. Statistical analysis

The results were expressed as the means ± standard deviation, and the differences among the means were evaluated using the one-way ANOVA test followed by Tukey's Multiple Comparison post-test. Significance was ascribed for p-value less than 0.05. Levels of significance are indicated by the number of symbols, for example, $^{*}p = 0.01$ to 0.05; $^{**}p = 0.001$ to 0.01; $^{***}p < 0.001$. Data are presented as the mean ± SE.

2.7. Compliance with ethics requirements

Authors declare that all procedures involving animals complied with the specific regulations and standards; this study was evaluated and approved at first by the Ethics Committee of the "Victor Babes" University of Medicine and Pharmacy Timisoara, Romania.

3. Results

3.1. Tumor incidence and multiplicity

The measurements of tumor incidence, tumor multiplicity and tumor yield as well as mice weight are presented as mean values in Table 1.

After 10 weeks of the experiment, no tumors were noticed either in the control groups (1 and 2) exposed to UVB and acetone, or in the mice treated with UVB, DMBA and TPA (groups 3 and 4). The skin of the male mice from groups 2 and 4 exhibited indurated plaques, small vesicles and was rough on palpation.

At the end of the experiment, at week 24, the tumor incidence in groups 3 and 4 (exposed to UVB and topically treated with DMBA and TPA) was 100% (Table 1); in one case, in the group of female mice treated with UVB and acetone, extensive blisters were reported.

Tumor multiplicity average values differed between the groups, being higher for the male mice as compared to the female mice exposed to the same carcinogens (5.3 *vs.* 3.7, respectively) (Table 1). Moreover, among the male mice, the most affected ones were those exposed to UVB, DMBA and TPA.

The mean weight values of the male mice were higher as compared to those of the female mice; the mice body weight was not influenced by exposure to the three carcinogens used in this study (Table 1).

3.2. Measurements of the physiological skin parameters

Our results indicated that during the experiment all three parameter values (erythema, transepidermal water loss, and skin hydration) were changed as a consequence of UVB exposure and topical application of acetone, DMBA and TPA. Exposure to UVB and topical application of acetone in the control groups (group 1 and 2) led to an increase of the erythema values, with a higher degree in the female group (group 1) (Fig. 2).

The measurements of erythema in the groups of mice exposed to UVB + DMBA + TPA (group 3 and 4) revealed a statistically significant increase in the female group (group 3) as compared to the males (group 4). When we compared the values recorded for groups 3 and 4 with those of the control groups (groups 1 and 2), we noticed an increase in the values recorded for group 3 as compared to group 1, but of no statistical significance, whereas the male mice exposed to UVB + DMBA + TPA (group 4) exhibited significantly higher values of erythema as compared to the males in the control group (group 2) (Fig. 2).

Another skin physiological parameter assessed in the present study was the skin hydration, also known as the water content of *stratum corneum*. Our results showed a decrease in the skin hydration values in groups 3 and 4 (mice exposed to UVB + DMBA + TPA) as compared to the control groups (groups 1 and 2) (Fig. 3). The male mice from group 4 exhibited a significant decrease in skin hydration as compared to the control group (group

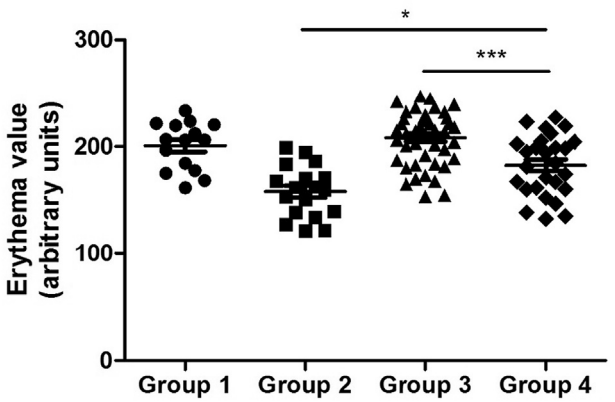

Fig. 2. Erythema values recorded during the experiment: Group 1 – female control group (UVB + acetone), Group 2 – male control group (UVB + acetone), Group 3 – females exposed to UVB + DMBA + TPA and Group 4 – males exposed to UVB + DMBA + TPA. Each value is the mean of 3 different measurements. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.0001$ with One-way ANOVA and Tukey's Multiple Comparison Test.

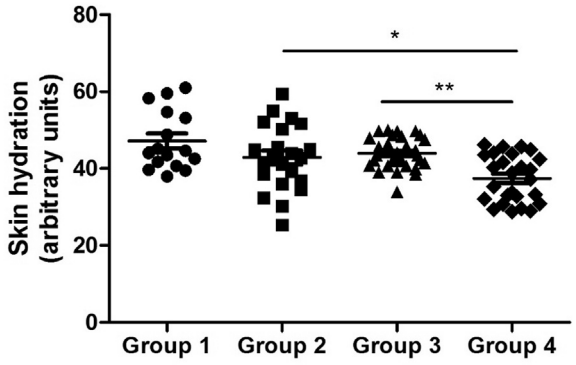

Fig. 3. Skin hydration (water content of *stratum corneum*) recorded during the experiment: Group 1 – female control group (UVB + acetone), Group 2 – male control group (UVB + acetone), Group 3 – females exposed to UVB + DMBA + TPA and Group 4 – males exposed to UVB + DMBA + TPA. Each value is the mean of 3 different measurements. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.0001$ with One-way ANOVA and Tukey's Multiple Comparison Test.

2). The female group (group 3) exhibited a slight decrease in skin hydration as compared to the control group (group 1); however, the results showed no statistical significance (Fig. 3).

Transepidermal water loss values indicated an increase in all groups of mice in this study; however, the changes were more prominent in the groups exposed to UVB, DMBA and TPA (Fig. 4).

3.3. Histopathological aspects of the lesions developed after 10 weeks of the experiment

Following 10 weeks of exposure, the histopathological cutaneous modifications of the mice exposed to UVB and acetone (groups 1 and 2) were minimal, in terms of inflammation signs. By comparing the two groups, the female mice exposed to UVB and acetone exhibited a slight modification of the normal epidermal histology (Fig. 5, 1a-d); however, in the reticular dermis, a moderate number of mast cells, disposed in groups, was noticed, indicating the presence of local inflammation. Also, the medium-sized blood vessels of the profound vascular plexus exhibited hyperemia. Interstitial edema was observed between the striated muscle cells under the skin.

The skin of the male mice (group 2) after 10 weeks of exposure showed signs of response to the tissue toxicity similar to those

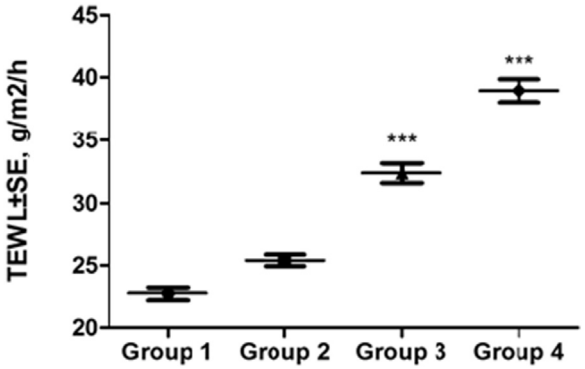

Fig. 4. Transepidermal water loss recorded during the experiment: Group 1 – female control group (UVB + acetone), Group 2 – male control group (UVB + acetone), Group 3 – females exposed to UVB + DMBA + TPA and Group 4 – males exposed to UVB + DMBA + TPA. Each value is the mean of 3 different measurements. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.0001$ with One-way ANOVA and Tukey's Multiple Comparison Test.

reported in the female mice (group 1) (Fig. 5, 2a-d). The epidermis of the skin of the male mice exhibited more pronounced inflammatory signs than that of the female mice, such as spongiosis, marked acanthosis, hypergranulosis, hyperorthokeratosis and hydropic vacuolization of all keratinocytes' layers. In the reticular dermis only isolated mast cells were observed.

After 10 weeks of the experiment, in groups 3 and 4 (exposed to UVB, DMBA and TPA) the histopathological modifications were more pronounced compared to groups 1 and 2 (exposed to UVB and acetone), maintaining the differences between female and male mice. Furthermore, when comparing the groups of male mice, the histopathological modifications reported in the male mice exposed to UVB + DMBA + TPA (group 4) were more prominent than those observed in the male mice exposed to UVB + acetone (group 2).

In the female mice (group 3), the skin exhibited similar modifications as those observed in the female mice exposed to UVB and acetone, but more pronounced (Fig. 5, 3a-d). Apart from the hydropic vacuolization of the keratinocytes, we noticed premalignant lesions such as mild dysplasia with the loss of cellular polarity in the basal layer of the epidermis. Mast cells, lymphocytes and macrophages were noticed as inflammatory cells. Hyperemia of the arterioles and venules as well as edema between striated muscle cells was also recorded.

The skin of the male mice exposed to UVB radiation, DMBA and TPA (group 4) was the most severely affected (Fig. 5, 4a-d), as compared to both the female mice exposed to the same carcinogens (group 3) and the male mice exposed to UVB and acetone (group 2). The epidermis showed hydropic degeneration of keratinocytes and premalignant lesions consistent with marked dysplasia. In all animals, intraepidermal bullae were reported. The mast cells were increased in number and dimensions while the edema between muscle cells was intense.

Within this experimental model, tumor papillomas occur relatively late, after a minimum of 10 weeks, when their number becomes detectable in the groups exposed to both potent chemical carcinogens (DMBA, TPA) and UVB.

3.4. Histopathological aspects of the lesions at the end of the experiment

The panel of lesions after 24 weeks of exposure was consistently different. All the mice presented with tumor lesions, with differences between the females and males. As a rule, the skin harvested from the female mice exhibited a smaller number of lesions than that of the male mice, with low malignant potential or even benign forms.

The female mice from group 1 exposed to UVB and acetone developed only premalignant lesions (Fig. 6, 1a-c) consisting of hypertrophic actinic keratosis with moderate dysplasia. Also, in the reticular dermis the number of mast cells was increased.

The male mice from group 2 exposed to UVB and acetone developed both benign lesions such as seborrheic keratosis and cutaneous horns, and also malignant lesions such as verrucous carcinoma (Fig. 6, 2a-c).

Apart from squamous lesions, the male mice exposed to UVB and acetone also developed numerous bullae (Fig. 7, a-h). In one case, in the group of female mice exposed to UVB and acetone, at 24 weeks of exposure, we noticed the presence of mastocytosis with extensive blisters (Fig. 7, a1-h1).

The female mice exposed to UVB, DMBA and TPA developed both premalignant lesions such as actinic keratosis and also malignant lesions such as verrucous carcinoma (Fig. 8).

The male mice exposed to UVB, DMBA and TPA developed benign lesions such as seborrheic keratosis, premalignant lesions such as keratoacanthoma and cutaneous horns and malignant

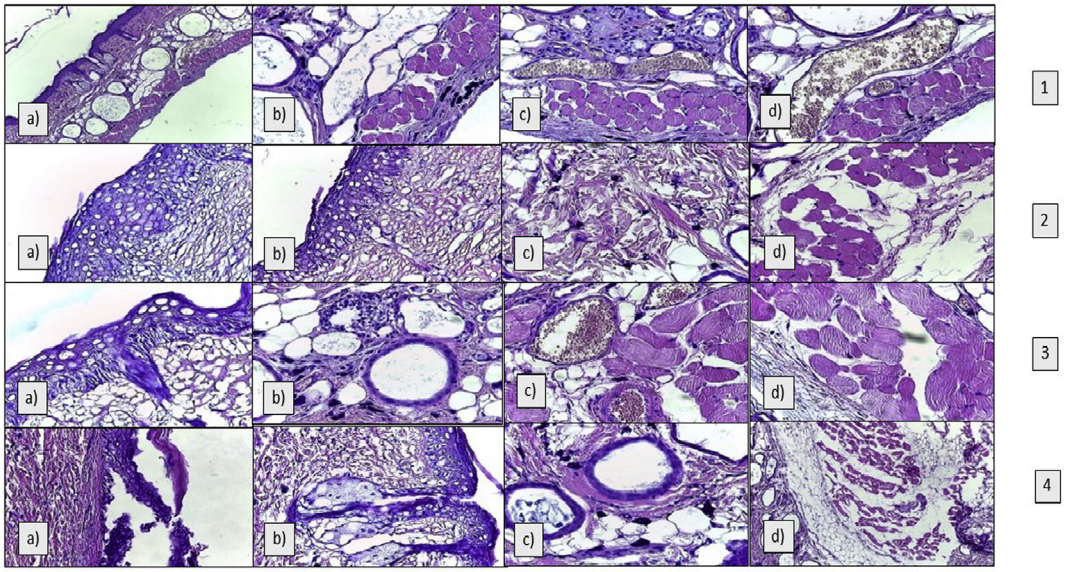

Fig. 5. 1a-d. Female mice exposed to UVB and acetone, H&E stain: a. skin with minimal histological modification, *original magnification* (*OM*) ×40; b. groups of mast cells in the connective tissue and some edema between striated muscle cells, *OMx100*; c-d. hyperemia of postcapillary venules and arterioles, *OMx100*; 2a-d. Skin of the male mice exposed to UVB and acetone showed accentuated modifications at epidermal level than female mice, H&E stain: a-b. hydropic degeneration of keratinocytes from all layers of the epidermis, *OMx100*; c. isolated mast cells in the connective tissue, *OMx100*; d. edema between striated muscle cells, *OMx40*; 3a-d. Skin of female mice exposed to UVB, DMBA and TPA, H&E stain, *OMx100*: a. mild dysplasia of the epidermis and hydropic vacuolization of keratinocytes; b. increased number of inflammatory cells in the dermis (mast cells, lymphocytes, macrophages); c. hyperemia of the arterioles and venules; d. edema between striated muscle cells; 4a-d. Skin of the male mice exposed to UVB, DMBA and TPA showing the most severe histopathological modifications compared to other three groups, H&E stain: a. bulla formation, *OMx100* b. hydropic degeneration of keratinocytes and loss of cellular polarity in the basal and spinous layers of the epidermis, *OMx100*; c. increased number of mast cells, *OMx100*; d. intense edema between muscle cells, *OMx40*.

lesions such as verrucous carcinoma and keratinizing squamous cell carcinoma with striated muscle invasion (Fig. 9).

4. Discussion

4.1. Etiology of squamous cell carcinoma

Squamous cell carcinoma was described as a type of tumor that develops mostly in the elderly as a consequence of the cumulative noxious effects of the solar radiation during a lifetime. Of the sunlight wavelengths, UVB is responsible for 75% of skin cancers, while UVA is responsible for only 25% (Oberyszyn, 2008). In humans, the frequency of non-melanoma skin cancers is higher in males than in females, presumably due to habitual differences between males and females; however, some authors have experimentally proven that male mice exposed to the same quantity of UVB as female mice develop more skin tumors than female mice (Thomas-Ahner et al., 2007).

Moreover, many chemicals may be involved in skin carcinogenesis, in particular polycyclic aromatic hydrocarbons and phorbol esters; UVB radiation or any added carcinogens may increase the occurrence of the pathological process. Simultaneously, these associated agents may lead to higher systemic toxicity; therefore, the assessment of the benefit (in terms of successful experimental model)/toxicity ratio is highly relevant particularly in view of the fact that humans are also exposed at the same time to carcinogenic

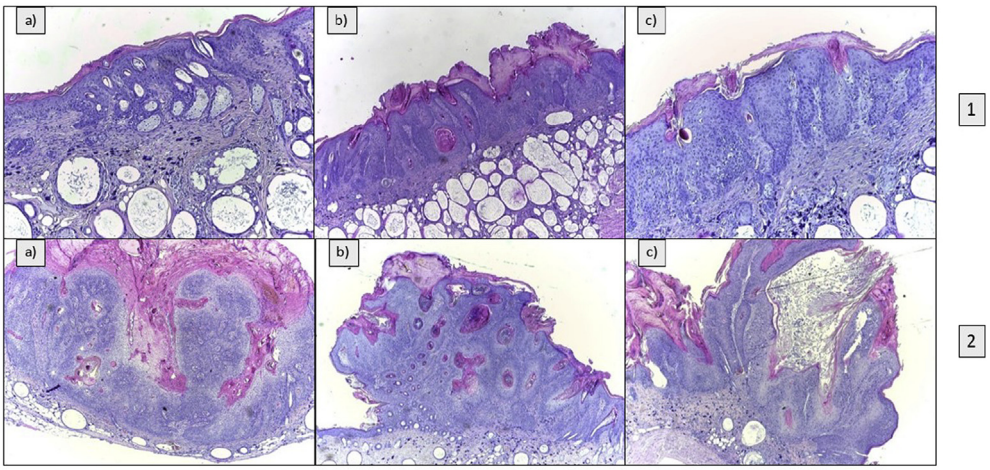

Fig. 6. 1a-c. Female mice exposed to UVB and acetone developed hypertrophic actinic keratosis, with moderate dysplasia, H&E stain: a. *OMx40*; b, c. *OMx100*; 2a-c. Male mice exposed to UVB and acetone developed: a. verrucous carcinoma; b. seborrheic keratosis; c. cutaneous horns, H&E stain, *OMx40*.

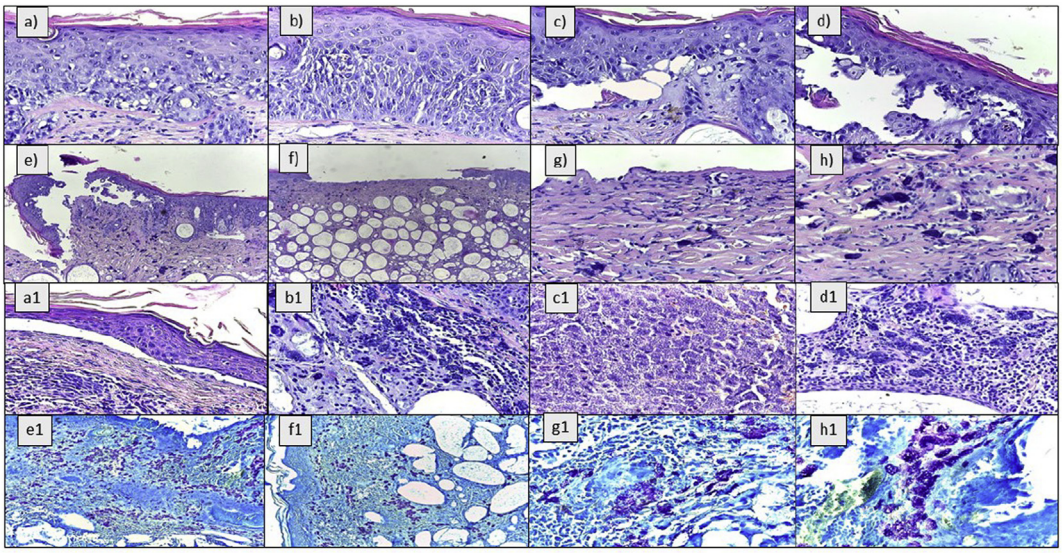

Fig. 7. a-h. Male mice, UVB and acetone exposure: a. interface dermatitis; b. spongiosis in the spinous layer of the epidermis; c-e. area of acantholysis in the spinous layer of the epidermis with bulla formation; f-g. exulceration of the epidermis; h. hypertrophic mast cells in the dermis; a-h. H&E stain: a-d, h - *OMx400*; e, g – *OMx100*; f – *OMx40* a1-h1. Mastocytosis developed by one female mouse treated with UVB and acetone: a1. bullous lesion resulting after a suprabasal split; b1-h1. groups of degranulated mast cells admixed with lymphocytes and macrophages; a1-d1. H&E stain: a1 - *OMx40*; b1-d1 – *OMx100*; e1-h1. Giemsa stain: e1-f1 – *OMx4*; g1- *OMx100*; h1 – *OMx400*.

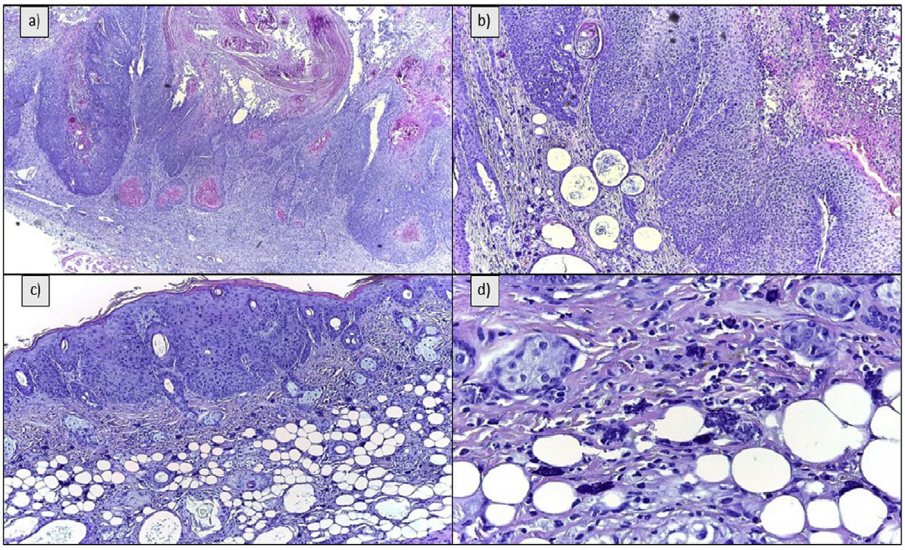

Fig. 8. Female mice exposed to UVB, DMBA and TPA showed: a. verrucous carcinoma; b. verrucous carcinoma, many mast cells and atretic hair follicles in the dermis; c. hypertrophic actinic keratosis, many mast cells in the dermis; d. hypertrophic mast cells filled with basophilic granules admixed with melanophages; H&E stain: a-c - *OMx40*, d – *OMx100*.

chemical compounds and UVB radiation combined.

4.2. Animal model

The SKH-1 hairless mouse strain is the most common strain used in experimental studies on skin carcinogenesis, wound healing, acute and chronic inflammation. This mouse model offers several advantages such as easy exposure to ultraviolet radiation accompanied by the visualization of the skin response and evaluation of the toxicological/pharmacological effects after the topical application of various agents (Benavides et al., 2009).

In a recent study, Kim and colleagues (Kim et al., 2012) proved that the *Hairless* (*Hr*) gene carried by the SKH-1 mice is involved in the regulation of SCC development by suppressing the NF-kB signaling pathway after UVB irradiation. These findings led to the conclusion that SKH-1 mice display features coupled with an enhanced proliferation and cellular dysregulation, features that may explain the molecular mechanisms of their inherent tumor susceptibility (Kim et al., 2012).

Budan et al. revealed the upregulated expression of several oncogenes thus indicating the tumorigenesis process after DMBA administration to mice (Budan et al., 2009); in 2012, Juhasz et al. demonstrated the early impact of DMBA on microRNA expression in vital organs of experimental mice, 24 h after DMBA application (Juhasz et al., 2012). Other studies (Osaka et al., 1997; Sugyiama et al., 2002) have focused on the development of leukemia in Long-Evans rats by DMBA treatment. DMBA is metabolized in the liver through Cyt P450-mediated oxidation to 3,4-dihydrodiol-1,2-

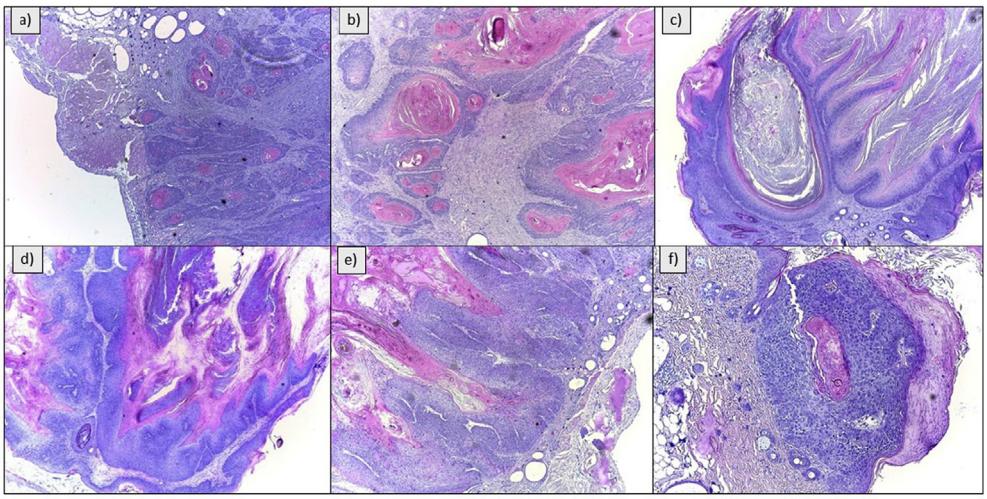

Fig. 9. Skin lesions developed by the male mice exposed to UVB radiation, DMBA and TPA (group 4): a-b. keratinizing squamous cell carcinoma with striated muscle invasion; c. cutaneous horn; d. keratoacanthoma; e. verrucous carcinoma; f. seborrheic keratosis; H&E stain: a – *OMx40*, b-f – *OMx100*.

epoxide, a more active carcinogenic compound (Savas et al., 1997); a very challenging issue was the determination of the cause of organ toxicity produced by DMBA, particularly bone marrow toxicity: the formation of liver active metabolites versus DMBA activation in the targeted organs. Heidel et al. proved that lymphoblastoma appeared following chronic DMBA administration as the result of reactive DMBA metabolites generated at extrahepatic level, such as bone marrow (Heidel et al., 2000).

Based on the literature and our own data (Ciurlea et al., 2011; Soica et al., 2014) regarding the use of SKH-1 hairless mice as animal models for skin disorders, we developed a new animal model of skin carcinogenesis by combining the deleterious effects of UVB, DMBA and TPA at the skin level.

To the best of our knowledge, this animal model stands as a novelty in the research field, as previous studies have only referred to animal models of skin carcinoma obtained by the association of UVB radiation and TPA (Katiyar et al., 1997), UVB radiation and DMBA (Katiyar et al., 1997; Yamada et al., 2008), UVB alone (Katiyar et al., 1997) or DMBA and TPA (Kowalczyk et al., 2013) respectively.

4.3. Skin carcinogenesis

Skin carcinogenesis is a two-step process that involves an initial inflammatory stage followed by a tumorigenic phase (reviewed by Neagu et al., 2016).

4.3.1. Acute and chronic inflammation

Inflammation is a common process that can be observed in many organs being triggered by a harmful agent (pathogen, physical or chemical irritant) and represents a *sine qua non* step in the healing process. It affects the tissue balance through the action of a large number of cytokines and chemokines released by normal connective tissue residents such as mast cells, fibroblasts or by acquired connective tissue participants such as activated macrophages.

The clinical signs of cutaneous acute inflammation were described over 2000 years ago being known as Celsus tetrad (calor, rubor, tumor and dolor). Virchow, in the late 19th century, completed the signs list with *functio laesa*. To quantify these signs, erythema, skin hydration (water content of the *stratum corneum*) and transepidermal water loss were assessed in this study due to their key roles in maintaining the skin barrier functionality intact.

Our data reported a more pronounced erythema following exposure to UVB + acetone or UVB + DMBA + TPA in the female groups as compared to the male groups of mice. These results are consistent with the observations stated by Thomas-Ahner and colleagues which revealed that male mice exhibited a reduced inflammatory response as compared to females after exposure to the same dose of UVB, this response being assigned to the increased skin thickness and myeloperoxidase activity in men (Thomas-Ahner et al., 2007). In another study it was shown that hairless male mice exposed to UV radiation exhibited a less intense, but slower sunburn inflammatory edema followed by the decrease of the epidermal expression of pro-inflammatory IL-6 as compared to females. Moreover, the males were unresponsive to the photoimmune protective effects of UVA; these facts could be considered as a possible explanation for the gender disparities regarding the development of skin cancers (Reeve et al., 2012).

The evaluation of skin hydration in our experiment showed a pronounced decrease in hydration in the groups of mice exposed to UVB + DMBA + TPA, a statistically significant reduction being observed in the group of male mice (group 4). In general, males have higher hydration values and sebum content and lower pH than females (Boelsma et al., 2003). Our results pointed out that the *stratum corneum* was more affected in the male group by the activity of the three carcinogens, results that are consistent with the data from the histopathological analysis.

Transepidermal water loss is considered a marker of skin barrier function integrity. Rissmann and coworkers demonstrated that the topical application of acetone (aprox. ten wipes of acetone) on male SKH-1 hairless mice dorsal side induced a significant increase in TEWL values (65 ± 10 g/h/m^2), values that are more than 6-fold higher than the normal TEWL values reported for male SKH-1 male mice (around 10 g/h/m^2) (Rissmann et al., 2009; Soica et al., 2014). Another study showed that exposure to UV radiation was associated with increased TEWL values and an affected skin barrier (Goto et al., 2011). Based on these observations we could state that our results are in agreement with the data found in the literature regarding the increased values of TEWL especially in the groups that were exposed to UVB + DMBA + TPA.

The skin physiological parameter values recorded during the experiment were confirmed by the results of the histopathological diagnosis that demonstrated the vasodilation of vessels with hyperemia, extravasation of leukocytes and plasma with consequent

edema. Plasma contains chemokines and cytokines that act as chemoattractant for neutrophils and lymphocytes. The reactive oxygen and nitrogen species produced by neutrophils during the fight with infections may lead to DNA damage that promotes carcinogenesis.

From the connective tissue, mast cells and macrophages are other active participants in the process of inflammation. In the mice dermis we identified many hypertrophied large mast cells, some of them filled with granules. After 10 weeks of the experiment the most frequent observed inflammatory cells were numerous mast cells noted in the dermis of all mice, in contrast with the findings of other authors who highlighted that the inflammatory infiltrate was composed of lymphocytes and macrophages (Arwert et al., 2010). Similar to the data published by several authors, the number of mast cells was slightly higher in the female than the male mice thus demonstrating a greater inflammatory response in the female mice to the harmful agents applied which is presumably the reason for which the female mice develop a lower number of tumors (Thomas-Ahner et al., 2007).

The exacerbation of edema can sometimes lead to blister formation at the dermoepidermal junction. Moreover, inflammatory dermatitis can sometime evolve to bullous lesions (epidermolysis bullosa, bullous lichen planus). After 10 weeks of the experiment all the male mice presented bullous lesions. As opposed to this, in the case of a female mouse, an exacerbated inflammatory reaction led to the appearance of diffuse cutaneous bullous mastocytosis; however, the responsible mechanisms require further investigations. Cutaneous mastocytosis is a disease characterized by the presence of numerous mast cells in the dermis and has an indolent course. The appearance of extensive bulla confers a more reserved prognosis to the disease (Golitz et al., 1984). There are no other data in the previously published papers about the occurrence of cutaneous mastocytosis after experimental UVB or chemical exposure.

4.3.2. Tumorigenesis

In response to a pro-inflammatory agent, a complex cytokine network developes and controls inflammation; however, the prolonged production of cytokines can create an immunosuppressive microenvironment that can generate tumorigenesis.

The evolution of skin carcinoma is a multistep process encompassing an initiation step (an irreversible process when DNA lesions occur leading to mutations while keratinocytes acquire the irreversible ability to form tumors); a promotion step – (a largely reversible process during which a clone of initiated keratinocytes expand to form a benign lesion); and the tumor progression step characterized by the presence of genetic modifications that transforms the premalignant lesions into a carcinoma (Rundhaug and Fischer, 2010; Benavides et al., 2009).

In the present study we evaluated some tumor characteristics such as tumor incidence, tumor yield and tumor multiplicity; our data indicated that after 24 weeks of the experiment all the mice (both females and males) exposed to the three carcinogens (UVB, DMBA and TPA) developed tumors whereas the number and yield of the tumors were higher in male mice (group 4) as compared to female mice (group 3).

Even if in the published literature many authors have described that, after UVB exposure, the mice developed only papillomas (considering these papillomas as pre-malignant lesions) that can regress or evolve to squamous cell carcinoma (Benavides et al., 2009; Thomas-Ahner et al., 2007), in the present study various other types of pre-malignant lesions were reported in the evolution of experimental squamous cell carcinoma.

Moreover, Benavides et al. (2009) and Thomas-Ahner et al. (2007) grouped papillomas in three classes: grade 1 - lesions consisting only of proliferated keratinocytes with no connective core, grade 2 - well-differentiated papillary mass and grade 3 - the mass exhibits finger-like projections in the dermis. In this study, we did not notice the presence of lesions that could be classified as grade 2 or 3 papilloma according to the Benavides scheme of diagnosis.

Benavides et al. (2009) contested the idea of Canfield et al. (1985) who described the development of keratoacanthomas after UVB exposure as cup-shaped lesions filled with keratin filaments. Sometimes, finger-like projections could be seen from the base of the cup to the dermis. These lesions were probably diagnosed as grade 3 papillomas or even microinvasive squamous cell carcinoma by Benavides et al. (2009). Since in humans after an evolution of months or years keratoacanthomas can transform into squamous cell carcinoma, we believe that it is appropriate to notice the presence of keratoacanthomas in experimental models.

Moreover, in humans, different histological types of actinic keratosis, with or without dysplasia (mild, moderate, severe), can be diagnosed as pre-malignant lesions many years before the appearance of squamous cell carcinoma.

Actinic keratosis is a lesion composed of proliferated and, sometimes, atypical keratinocytes, currently considered a preliminary step in the formation of squamous cell carcinoma (Leffell, 2000; Kramata et al., 2005; Rebel et al., 2012). The dysplasia noted in actinic keratosis is graded similarly to squamous intraepithelial lesions of the cervix (mild, moderate, severe or low/high grade squamous intraepithelial lesion); moreover, some authors consider cutaneous horns as a type of actinic keratosis (Duncan et al., 2008). Furthermore, a longstanding keratoacanthoma may develop into squamous cell carcinoma. Therefore, we believe it is important to signal the presence of all these lesions after UVB and chemical exposure.

None of the lesions observed in mice after 10 weeks of the experiment regressed spontaneously until the end of the experiment, contrary to the observations reported by Benavides et al. (2009).

We did not noticed the presence of microinvasive carcinoma as Benavides et al. (2009) described in their study, all the squamous cell carcinoma cases recorded in the present study being fully invasive. In humans, the majority of diagnosed squamous cell carcinoma is at grade 2 of differentiation. As opposed to this but similar to the findings of Canfield et al. (1988), all malignant tumors diagnosed in mice were at grade 1 of differentiation (Canfield et al., 1988). The differences in tumor histological grade in mice versus humans are linked to the period of evolution, the mice being sacrificed earlier following the appearance of lesions, while in humans the tumors evolve longer before the presentation of the patient to the dermatologist. Moreover, all invasive lesions evolved from a pre-malignant tumor and not directly from irradiated skin as Canfield et al. (1988) reported.

Similar results were obtained in an important study conducted by Thomas-Ahner et al. in 2007 on SKH-1 mice with UVB-induced skin carcinogenesis. The study clearly revealed that following equal exposure to UVB male mice develop more numerous and larger non-melanoma skin tumors than female mice also showing a higher histological grade than females thus suggesting a higher susceptibility to skin cancer (Thomas-Ahner et al., 2007). This study, as well as others (Syed and Mukhtar, 2012; Xu et al., 2012), have suggested that female mice possess a higher antioxidant capacity than males thus indicating a possible biological explanation for the experimentally proven gender differences in carcinogenesis at skin level. Also, another additional argument is the influence of sex hormones; estrogens may reduce the production of hydrogen peroxide and free radicals thus providing a protective activity in female organs including skin (Thomas-Ahner et al., 2007; Borras

et al., 2003).

In terms of the increased susceptibility of male mice to develop non-melanoma skin cancers, a possible explanation was provided by Mancuso et al. which showed that the endogenous estrogen had a protective role against squamous cell carcinoma development in female mice (Mancuso et al., 2009).

A probable cause of the gender bias in the development of skin cancer may be the different skin structure between genders: male mice have a 190% thicker dermis but a thinner epidermis and hypodermis than female mice which leads to the conclusion that male skin is 40% thicker than female skin (Dao and Kazin, 2007).

5. Conclusions

Animal models are frequently used by researchers in order to investigate the etymology of the disease as well as its evolution in a way that would be inaccessible in a human patient. Skin carcinomas can be easily studied through experimental models fact that makes those tumors an important tool in the study of carcinogenesis; however, due to their low capacity to metastasize, these tumors are not recommended for the study of metastasis development.

Non-melanoma skin cancer retains the gender dependency, being more aggressive in males; similar data were collected in experimental animal models developed by using SKH-1 mice, a strain extensively used in the study of skin disorders.

In the current study, male and female mice exposed to UV radiation developed less intense skin pathology and in a different manner than in previously reported papers. Male and female mice exposed to DMBA, TPA and UV radiation developed the pathology through a progressive and relatively rapid process. In addition, it was revealed that the coupling of several carcinogens, both chemicals and UV radiation, generates tumors with various growth rates regardless of gender, and accelerates the occurrence of skin cancer in experimental models. The signs of systemic toxicity were within admissible limits in terms of the potential use of the suggested experimental scheme in future practical applications.

During our experimental study, all malignant tumors developed from pre-malignant lesions. The pre-malignant lesions observed herein were similar to those observed in humans, with the exception of seborrheic keratosis-like lesions that are not considered precursors of skin cancer in humans. The histological features of the experimentally induced squamous cell carcinoma are similar to those observed in humans.

The results provide valuable information in terms of the changes that occur at histopathological level during the development of this type of skin cancer. Furthermore, these data may represent the background for testing new prophylactic therapies on skin cancer animal models.

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