UNIVERSITY OF MEDICINE AND PHARMACY
" CAROL DAVILA" BUCHAREST

Fatty acids as potential modulators of cell proliferation and migration in tumor cell lines

SUMMARY OF THESIS

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Introduction

Fatty acids are a group of lipid compounds that are essential for all organisms. They can be classified as either saturated or unsaturated. ω-3 and ω-6 are two polyunsaturated fatty acids (PUFA) which are recognized as pivotal in regulation of various biological functions. They can be an energy source, as well as structural elements for cell membranes or signaling molecules for many different processes, including gene expression. Although vital for homeostasis, neither linolenic acid (ω-3) nor linoleic acid (ω-6) can be synthesized in vivo, thereby constant uptake through food is essential – hence the designation of *essential fatty acids* (EFA) (1, 2). Multiple studies focus on evaluating the way natural compounds alter and influence gene expression at a systemic level (transcriptomic, proteomic or metabolomic), by elucidating the exact mechanism of action of these substances. Although the effects of ω-3 and ω-6 have been described, and will be reviewed summarily in this dissertation, some aspects regarding their precise mechanism of action both in salutary physiological processes, as well as pathological ones, remain unclear.

During these last few years, interest in natural compound research has increased, both in cancer research and in the field of antibiotics, with the hopes of finding new anti-tumor or anti-microbial compounds respectively (3). Although FA are an important component of the bioactive compounds extracted from sea-buckthorn, their biological effects have not been as well researched. Sea-buckthorn oil is rich in both saturated and unsaturated FA, with large quantities of palmitoleic oil (POA), which is known for its beneficial effects on the skin (4). POA shows antioxidant and anti-tumor effects on normal and tumor cells, including dysplastic skin cells (5-7). Most studies on cell proliferation and regeneration use polyphenols, or whole natural compounds, such as whole sea-buckthorn oil, while relatively few explore the effect of FA extracted from these sources in isolation.

The research which serves as the basis for this dissertation has been conducted in the National Institute for Research and Development in Pathology and Biomedical Sciences „Victor Babeş", Bucharest, Laboratory of Biochemistry and Proteomics, of which I am a part of since 2018 as a research assistant. The main objective was to directly contribute to development of new technologies in the biomedical and pharmaceutical fields, through rapid and efficient transfer of knowledge towards industries involved in producing and commercializing biological products for health, as well as integrating these compounds into new or optimized products. The research conducted in our laboratory has been supported by projects PNIII.P2-2.1-PED-2019-3141, number 382/2020, PN 19.29.01.04 and COP A 1.2.3./

grant ID:P_40_197/2016. Both projects have been implemented in order to develop new biotechnologies based on proteomics, epigenetics, immunotoxicology and imaging, for use in novel regenerative therapies and nanobiotechnology. The mechanism of action and biological signaling of various molecules were studied, with the hope of gaining useful insight into new biopharmaceutical compounds that target specific biological processes, including intracellular and intercellular delivery systems. Various products, systems and methods based on biotechnologies have also undergone preclinical tests, and preparations have been made for future clinical testing.

The main goal of this research is to evaluate the *in vitro* effects of sea-buckthorn oil and its fatty acids on proliferation and inflammation, both in normal and in tumor cells, as well as possible epigenetic modifications that may be induced, by studying cytotoxicity, cell viability and morphology, transport protein expression, migration pattern, tissue repair, cellular inflammation and DNA methylation in tumor cells.

The main hypotheses of this dissertation are as follows:

I. Sea-buckthorn seed oil is an appropriate source of fatty acids, that shows regenerative potential on skin cells;
II. Sea-buckthorn seed oil is an appropriate source of fatty acids, that shows anti-inflammatory effects on skin cells;
III. Sea-buckthorn seed oil is an appropriate source of fatty acids, that has regenerative effects on skin cells following exposure to ultraviolet rays type A (UVA);
IV. Saturated fatty acids have a pro-tumoral effect on melanoma tumor cells;
V. ω-3 fatty acids have anti-proliferation effects on melanoma tumor cells;
VI. Fatty acids modulate genomic DNA methylation in melanoma tumor cells;

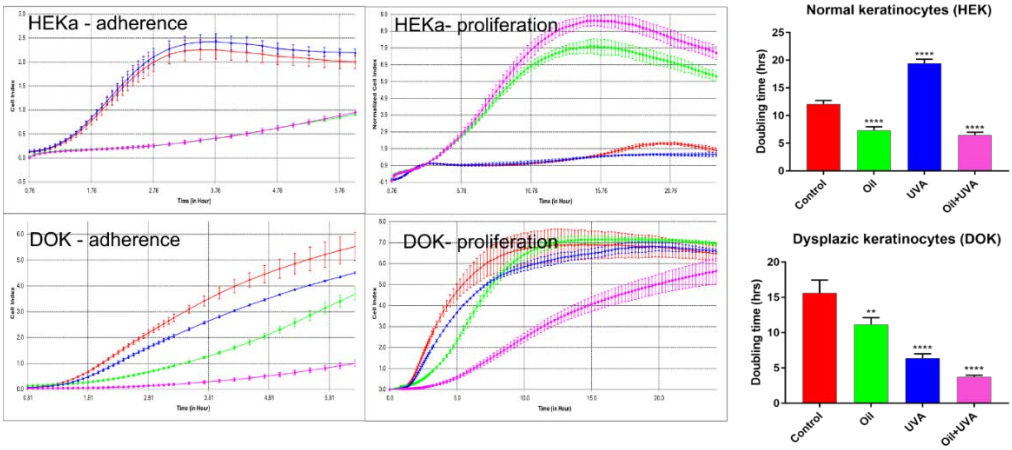
The main objectives of this doctoral dissertation are:

- Evaluation of cell viability after fatty acid treatment and the cytotoxic effect of fatty acids
- Evaluation of the regenerative effect of sea-buckthorn oil on normal and dysplastic cells
- Analysis of global DNA methylation in tumor cells and DNMT enzyme activity following treatment with saturated and polyunsaturated fatty acids

This dissertation presents experimental data regarding the regenerative properties of fatty acids extracted from sea-buckthorn oil, and analysis of their modulatory effects on inflammation and tumorigenesis, both on normal skin cell lines and on dysplastic and tumor skin cell lines. In order to reach the aforementioned objectives, normal fibroblast and keratinocyte cells, dysplastic keratinocyte cells and melanoma cells have been developed into cell cultures as per the instructions provided by the manufacturer and MTS and LDH cytotoxicity and cell viability tests and cell morphology analysis have been performed. Lipid inclusion morphology in both treated and irradiated cells has been evaluated using Oil Red coloration and phase contrast microscopy with UV/TxRed filter. Proliferation and cell migration has been followed using Nikon Biostation and the xCelligence RTCA DP platform. Interleukins and growth factors were assessed by xMAP and ELISA, for analysis of genomic DNA extraction and global 5-mc methylation and post-treatment activity of the DNMT implicated in methylation, ELISA and immunofluorescence were employed. The results of the research involved in this dissertation will be presented through the view of selected figures extracted experiments.

The first study involved the potential proliferative of sea buckthorn oil on normal and dysplastic keratinocytes - under basal conditions and after UVA irradiation. The results attained are:

1. Sea buckthorn oil stimulates the proliferation of normal and dysplastic keratinocytes
2. Sea buckthorn oil inhibits the migration of normal and dysplastic keratinocytes
3. Normal keratinocytes do not express CD 36 under basal conditions
4. UV irradiation of normal and dysplastic keratinocytes increases CD36 expression



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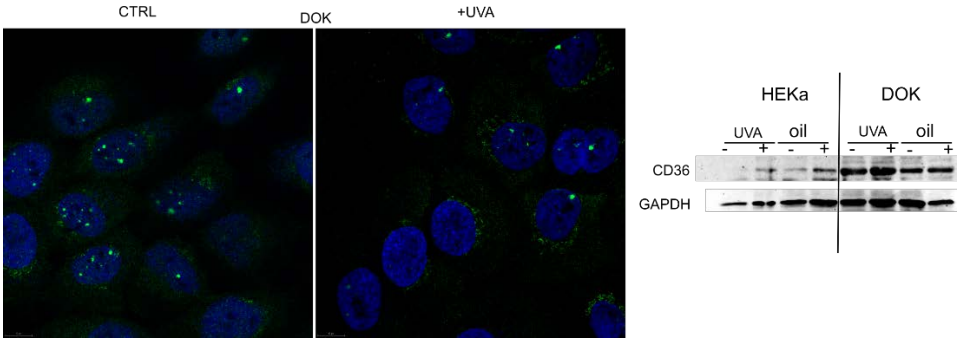

Figure 1. a) UVA irradiation of normal keratinocytes inhibits proliferation, but oil treatment stimulates proliferation in basal conditions but also after irradiation. The oil stimulates the proliferation of dysplastic keratinocytes, without this treatment showing beneficial effects. Cell impedance monitoring via xCelligence. b) CD36 is expressed in dysplastic keratinocytes, but not in normal ones, and the expression increases after irradiation. Immunofluorescence and Western Blot for CD36.

The study compares the effect of sea buckthorn seed oil on normal and dysplastic human keratinocytes, starting from the premise that normal skin is made up of both cell types, the proportion of which changes with age and exposure to harmful environmental factors.

Sea buckthorn fatty acids have been shown to have the ability to improve the post-inflammatory response resulting from UV exposure. Moreover, they could mitigate the effects of sunburn by activating the regenerative processes of the healthy skin (8). UVA exposure affects these populations of cells differently, from a proliferative point of view, and cells migrate to the injured areas. Previously, dysplastic keratinocytes have been shown to be less capable of migrating to the denudated area than normal keratinocytes, being more affected than the latter by UVA treatment (9). With regard to cell proliferation, UVA exposure induced cell cycle arrest in normal keratinocytes (10, 11); this result, discussed in (18), may lead to an increase in cell doubling time, a change observed by real-time videomicroscopy and cell impedance experiments. Dysplastic cells do not have this protective character; are prone to proliferation following UV irradiation, and treatment with sea buckthorn seed oil stimulates this harmful behavior. One possible mechanism involved in stimulating the proliferation of dysplastic cells after treatment with sea buckthorn seed oil could be CD36-mediated lipid metabolism. Fatty acid transporters, including CD36, are overexpressed in tissues with increased fatty acid metabolism (12). Fatty acids are able to activate PPAR receptors (14), transcription factors that activate CD36 gene transcription (13). It has previously been shown that tumor cells alter their lipid metabolism (14) to promote cell survival and proliferation. A

study conducted by Pascual et al. showed that CD36+ cells are directly related to the lipid-rich diet and the metastatic potential is based on lipid metabolism. In this study, CD44+ cells isolated from human oral squamous carcinoma exerted a distinctive ability to overexpress both CD36 fatty acid receptor genes and genes involved in lipid metabolism, thereby accelerating metastases progression (14).

Conclusion: whole sea buckthorn oil, although considered in the literature as having beneficial effects on the skin, can worsen the proliferation of dysplastic cells and protect them from the effect of UVA irradiation, possibly by increasing the expression of the CD36 receptor.

The second study involved analysis of proliferation, migration and inflammation after treatment with purified fractions of sea buckthorn oil in normal keratinocytes and dermal fibroblasts. The results are as follows:

1. The palmitic acid-rich fraction stimulates cell proliferation in normal keratinocytes and normal dermal fibroblasts
2. The fraction rich in palmitic acid has anti-inflammatory effect in normal keratinocytes and normal dermal fibroblasts
3. The palmitic acid-rich fraction stimulates VEGF expression in normal keratinocytes in a time-dependent manner

Sea buckthorn seed oil has many benefic effects on skin health, such as wound healing (15, 16), regulating the amount of sebum, and improving conditions in atopic dermatitis (4). Some of these effects are ascribed to palmitoleic acid, an unsaturated fatty acid found in the pulp of sea buckthorn but absent in seeds (15, 17). However, fatty acids in sea buckthorn oil can also contribute to the health of the skin. For example, palmitic acid, found in large amounts in the seeds of sea buckthorn, is known to be abundant in healthy human skin; it contributes to an effective lipid barrier and is used by cells as a precursor of fatty acids with saturated carbon chain. It is used in cosmetics for the skin and considered safe for administration. However, fatty acids are not studied, being overlooked, most studies focus on unsaturated fatty acids (ω-3 and ω-6). Also, the fatty acid composition of the different sea buckthorn derivatives varies depending on the area, the harvest season and even from cultivate to cultivate (mentioned in the general part of the work).

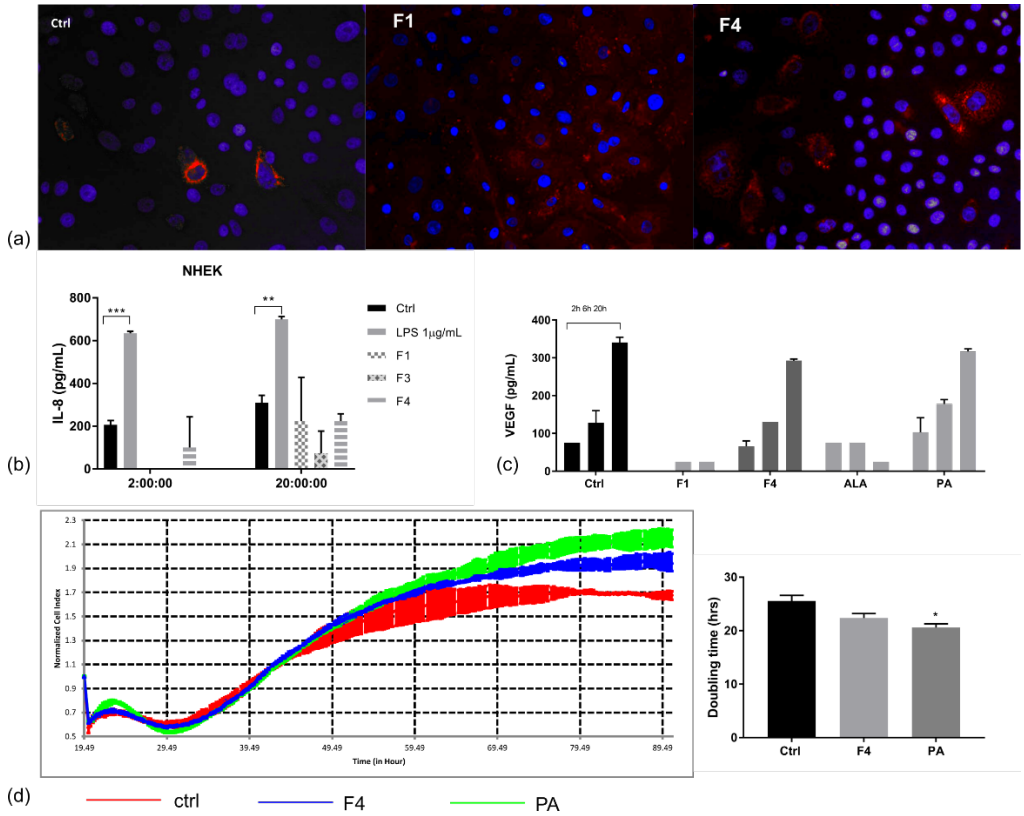

Figure 2. a) Up-take up purified fractions rich in ALA and PA in normal keratinocytes evaluated by Oil Red. b) Treatment with purified fractions does not induce an inflammatory response, evaluated with IL-8 value with ELISA technique. c) VEGF expression assessed by the xMAP technique shows that F4 and PA stimulate growth factor in a time-dependent manner compared to control. d) PA stimulates the proliferation of normal keratinocytes by decreasing the doubling cell time by xCelligence.

Regardless of these variations, data related to the method of purification of sea buckthorn seed oil in fractions enriched with saturated and unsaturated fatty acids whose effects on the proliferation of epidermal cells have been tested are presented in the study. Four fractions, enriched in α-linolenic acid, linoleic acid, oleic acid and palmitic acid, were separated, fractions that do not affect cell viability at a low concentration at the micromolar level (50 µM or less) as well as their takeover at the cellular level. In the study it is shown that purified fractions, in addition to palmitoleic acid, can be used to develop skin-related products, despite variations in the chemical compositions of the seed oil.

Of the four purify fractions, ALA and PA – enriched fractions did not alter the cell morphology of normal keratinocytes and did not cause inflammation in normal keratinocytes and dermal fibroblasts (assessed by the release into the cellular environment of inflammatory cytokines - IL-6 and TNFα). The production of IL-8 was noted in both types of cells at levels

like control. It is known that keratinocytes are a rich source of IL-8, which stimulates migration, and is useful to induce wound healing (18).

Another cytokine associated with cell proliferation is VEGF, also produced by keratinocytes, which acts by autocrine signaling and stimulates the proliferation of keratinocytes It is worth noting that VEGF overexpression is associated with psoriasis and tumor pathology (19) therefore, a balanced expression is desirable following the application of a treatment. The fraction enriched in palmitic acid reduced the expression of VEGF, therefore, this fraction does not affect the mechanism mentioned above. Finally, of the two fractions, the PA-enriched fraction supported keratinocytes and proliferation of fibroblasts, possibly by supporting cellular metabolism. This effect became apparent after about 30 h of incubation, with a noticeable effect at 48 h.

Conclusion: of the purified fractions from sea buckthorn seed oil, the fraction rich in palmitic acid supports cell proliferation, without stimulating inflammation or VEGF synthesis. This fraction is suitable for treatment intended for the skin, requiring additional in *vivo* tests . Despite the impossibility of extracting palmitoleic acid separated from α-linolenic acid from the seeds of sea buckthorn le, we have demonstrated that other fractions can be used to develop skin care-related products.

The third study involved analysis of proliferation, migration and methylation of genomic DNA after fatty acid treatment in human melanoma cells. The results obtained are as follows:

1. Docosahexaenoic acid has a strong antiproliferative effect on melanoma and normal epidermal fibroblasts
2. Palmitic acid inhibits the migration of the tumor line from melanoma
3. Palmitic acid has antiproliferative effect on melanoma but also on normal epidermal fibroblasts
4. Palmitic acid increases the methylation level of DNA without altering the activity of DNMTs
5. α-linoleic acid has antiproliferative tumor effect and does not influence normal epidermal fibroblasts
6. α-linoleic acid decreases the methylation level of DNA, with a decrease in the activity of DNMTs

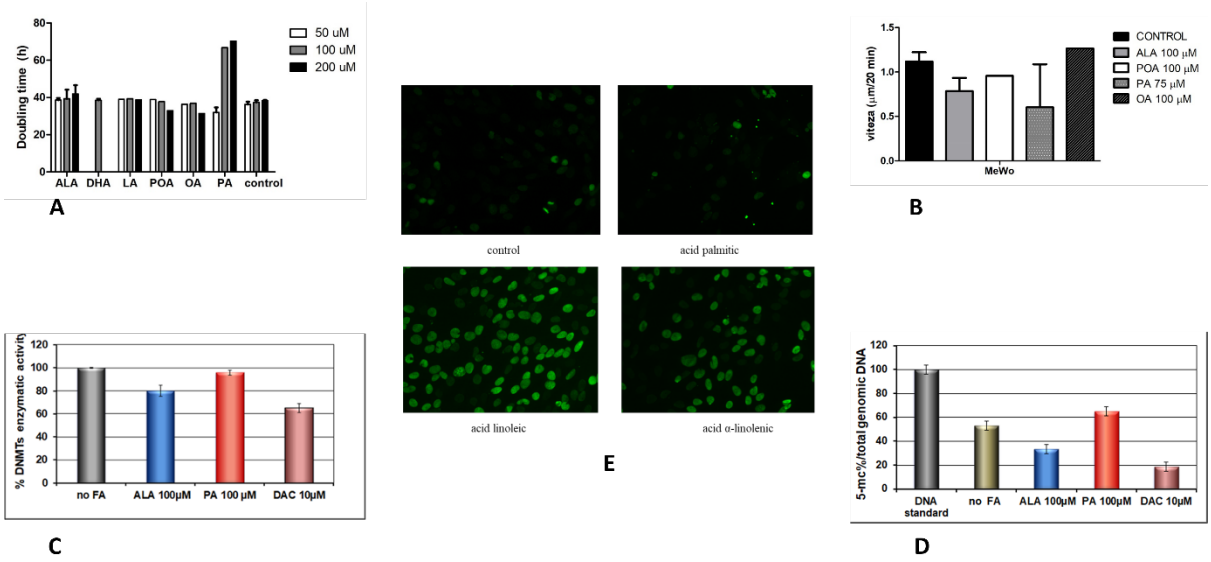

Figure 3. a) Palmitic acid inhibits the proliferation of concentration-dependent melanoma evaluated by measuring cell impendance at xCelligence. b) Palmitic acid decreases the migration of the cellular front evaluated by scratch test. c) Palmitic acid does not change the activity of DNMTs. d) and e) Palmitic acid increases the methylation of the DNA of cancer cells evaluated by ELISA and Immunofluorescence.

Conclusion: Palmitic acid has antiproliferative effect on the tumor line of melanoma, by inhibiting the proliferation and migration of cells, as well as by decreasing the possibility of migration / metastasis. The proposed possible mechanism is to increase global methylation, methylation of which is not generated by the activation of DNMTs. However, the antiproliferative effect also occurs at the normal cell line, but at high concentrations of fatty acids, without modulating the methylation of DNA.

An important counterparts of bioactive compounds extracted from sea buckthorn, fatty acids have been less explored in terms of biological effects. Sea buckthorn plant is enriched in saturated and unsaturated fatty acids, having beneficial effects is proven on normal skin, but also after UV exposure. This study presents data obtained after testing of sea buckthorn oil and component fatty acids on cell regeneration and skin, inflammation, and epigenetic modulation of melanoma. Sea buckthorn seed oil stimulates the proliferation of normal and dysplastic keratinocytes under basal conditions but also after UVA irradiation, irradiation leading to increased CD36 expression. Purified fractions of sea buckthorn seed oil rich in palmitic acid have a proliferative effect on normal epidermal cells. Thus, the effect of this oil and fractions on cell lines was preclinically tested, being prepared for clinical testing of these possible products with use in cosmetology but also as supplements. Also, α-linoleic acid is a bioactive nutrient with possible capacity as a positive epigenetic modulator, unlike palmitic

acid. It is necessary to evaluate in future studies the expression of mRNA of DNMTs involved in DNA methylation, as well as the analysis of post-transcriptional changes in histones H3 and H4 for the investigation of the mechanism of action of fatty acids as epigenetic modulators.

Conclusions and future perspectives

The main purpose of this thesis is to investigate the effects that some natural compounds could have on cellular proliferation with the purpose of identifying possible candidates with antitumoral effects in vitro. Starting from the actual state of knowledge (for example: cellular activity of polyphenolic plant extracts, the antioxidant activity of various plant extracts), I've identified fatty acids, saturated and polyunsaturated acids: palmitic acid, oleic acid, linoleic acid, α-linolenic acid, palmitoleic acid and docosahexaenoic acid as biological active compounds. These fatty acids are less studied in literature regarding their antiproliferative activity. Many studies related to fatty acids explore the role in inflammation, partly mediated by a membrane receptor capable of taking long-carbon fatty acids from the external environment and transporting FAs into the cell- CD36. As a result, sea buckthorn oil was selected, a compound derived from a plant that is also widespread in our country, rich in fatty acids and whose composition has been frequently studied as a source of compounds with regenerative action. Regarding the flow of experiments, we chose cells of epidermal origin, because the results obtained in vitro have the fastest translatability to a topical application in vivo. The original research activity was divided in three layers:

i) the study of the whole sea buckthorn oil on normal and dysplastic cells regarding proliferation and migration,

ii) the study of the fatty acids isolated from oil on normal and dysplastic cells regarding proliferation and migration,

iii) the study of the antiproliferative effects of these fatty acids on tumor cells.

The results initially confirmed the data from the literature regarding the regenerative effects of sea buckthorn oil for epithelial cells, but additionally highlighted the aberrant response of the dysplastic cells in the same experimental conditions, standard and after UV irradiation. As for the next step, it was necessary to evaluate whether the effects obtained are due to the mixture of active compounds in the sea buckthorn oil or just one particular compound, therefore all fractions of purified fatty acids from the sea buckthorn oil were tested. Thus, palmitic acid exhibits proliferative effects on normal keratinocytes, in agreement with the literature, and, in addition, does not stimulate the production of pro-inflammatory cytokines. This study was extended to normal dermal cells, fibroblasts, which after treatment

with palmitic acid in concentrations below 100 μM migrate more rapidly, proliferate and in this way can contribute to the healing of a wound. On the contrary, at high concentrations (between 100-200 μM) an inhibitory effect is observed, which is also present in the case of melanoma cells. As part of the investigation of the mechanism involved in this process, palmitic acid treatment has been observed to increase DNA methylation, without altering the action of proteins that catalyze the transfer of a methyl group from donors to cytosine. Finally, we compared the effects of this acid, which is a saturated fatty acid, with those of unsaturated fatty acids, frequently mentioned in the literature as having, among other properties, beneficial cardiovascular effects, which could be purified from sea buckthorn oil. Among the tested fatty acids, α-linoleic acid, at high concentrations, had an inhibitory effect on melanoma cell proliferation, but favored migration by decreasing overall DNA methylation. In conclusion, concerning the aggressiveness of the malignant cell behavior, a paradigm shift or change regarding essential fatty acids may be necessary, in which saturated fatty acids are to be considered as having beneficial effects, on certain cell lines, depending by the cellular phenotype and the distribution of receptors involved in lipid metabolism. However, considering the systemic effects, the current clinical use is limited to topical applications, but the wide bioavailability, anti-tumor effect and lack of toxicity on normal cells support the advancement of future research on spheroids, organoids and in vivo models. Next, the results of the study are presented according to the hypotheses presented in the thesis:

i. Sea buckthorn oil stimulates the proliferation of normal and dysplastic keratinocytes

ii. Sea buckthorn oil has an important effect of inhibiting the migration of dysplastic keratinocytes

iii. Sea buckthorn oil inhibits the migration of normal keratinocytes

iv. Sea buckthorn oil treatment increases the proliferation of UV-irradiated normal keratinocytes

v. Sea buckthorn oil treatment increases the proliferation of UV-irradiated dysplastic keratinocytes

vi. Irradiation does not affect uptake and formation of lipid inclusions

vii. Normal keratinocytes do not express CD 36 under basal conditions

viii. UV irradiation of normal and dysplastic keratinocytes increase CD36 receptor expression

ix. The fractions purified from sea buckthorn oil rich in palmitic acid and α-linolenic acid do not show cytotoxicity, same effects as standard fatty acids

x. The fraction rich in palmitic acid stimulates cell proliferation of normal keratinocytes and normal dermal fibroblasts

xi. The fraction rich in palmitic acid has an anti-inflammatory effect on normal keratinocytes

xii. The α-linolenic-rich fraction does not stimulate VEGF expression in normal keratinocytes and normal dermal fibroblasts

xiii. The palmitic acid-rich fraction modulates VEGF expression in a time-dependent manner, similar to control in normal keratinocytes and normal dermal fibroblasts

xiv. Selected fractions decrease IL-8 secretion in normal keratinocytes

xv. Selected fractions do not alter IL-8 secretion in normal dermal fibroblasts

xvi. The fraction rich in palmitic acid stimulates the migration of normal dermal fibroblasts after 24 hours of treatment

xvii. Fatty acids form perinuclear lipid inclusions in melanoma tumor line

xviii. Docosahexaenoic acid has a strong antiproliferative effect on melanoma and normal epidermal fibroblasts

xix. Fatty acids, except linoleic acid, stimulate the migration of the normal fibroblast line in wound healing assay

xx. Palmitic acid inhibits melanoma tumor line migration and alters cells morphology

xxi. Oleic acid stimulates the migration of the melanoma tumor line in wound healing assay

xxii. Palmitic acid has an antiproliferative effect concentration-dependent on melanoma but also on normal epidermal fibroblasts

xxiii. Palmitic acid inhibits trans-well migration of melanoma tumor cells

xxiv. Palmitic acid increases the level of DNA methylation without changing the activity of DNMTs

xxv. α-linoleic acid has a concentration-dependent tumor antiproliferative effect and does not influence normal epidermal fibroblasts

xxvi. α-Linoleic acid decreases the level of DNA methylation, with decreased activity of DNMTs.

Following these results, it is necessary to pursue with the evaluation of the enzymatic activity of DNMTs by ELISA method as well as the expression of DNMTs by immunofluorescence and Western Blot, the evaluation of the mRNA of DNMTs by q-RT-PCR, as well as the analysis post-transcriptional modifications of histones H3 and H4 (ELISA, immunofluorescence and Western Blot) to investigate the mechanism of action of fatty acids

as epigenetic modulators. It is also necessary to assess the effect in vitro, including spheroids for the melanoma tumor line or skin organoids to further test the anti-inflammatory, anti-proliferative effects and also as adjuvants in the treatment of metastases. Expression of the CD36 receptor can also be inhibited by using sulfo-N-succinimidyl oleate to study the effect of palmitic acid treatment and how methylation is modulated under these conditions, preliminary experiments in this purpose being already tested on monocytes, but also on the melanoma tumor line. Considering the training acquired over years of study, I can edit tumor cell lines, including melanoma lines, which can be genetically edited CD36 K.O. through the CRISPR-Cas9 system, to study the function of this receptor in tumor cell proliferation and migration.

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