

Anti-Aging Effects of Retinol and Alpha Hydroxy Acid on Elastin Fibers of Artificially Photo-Aged Human Dermal Fibroblast Cell Lines

M. Jarrar, S. Behl, N. Shaheen, A. Fatima, R. Nasab

Abstract—Skin aging is a slow multifactorial process influenced by both internal as well as external factors. Ultra-violet radiations (UV), diet, smoking and personal habits are the most common environmental factors that affect skin aging. Fat contents and fibrous proteins as collagen and elastin are core internal structural components. The direct influence of UV on elastin integrity and health is central on aging of skin especially by time. The deposition of abnormal elastic material is a major marker in a photo-aged skin. Searching for compounds that may protect against cutaneous photo-damage is exceedingly valued. Retinoids and alpha hydroxy acids have been endorsed by some researchers as possible candidates for protecting and or repairing the effect of UV damaged skin. For consolidating a better system of anti- and protective effects of such anti-aging agents, we evaluated the combinatory effects of various dosages of lactic acid and retinol on the dermal fibroblast's elastin levels exposed to UV. The UV exposed cells showed significant reduction in the elastin levels. A combination of drugs with a higher concentration of lactic acid (30 -35 mM) and a lower concentration of retinol (10-15mg/mL) showed to work better in maintaining elastin concentration in UV exposed cells. We assume this preservation could be the result of increased tropo-elastin gene expression stimulated by retinol whereas lactic acid probably repaired the UV irradiated damage by enhancing the amount and integrity of the elastin fibers.

Keywords—Alpha Hydroxy Acid, Elastin, Retinol, Ultraviolet radiations.

Abbreviations—UV: Ultra violet; RA: Retinol Acetate; LA: Lactic Acid; DMEM: Dulbecco's modified Eagle's medium; ELISA: Enzyme linked Immunosorbent Assay.

I. INTRODUCTION

BASED on an estimate proposed by the global industry analysts in February 2009, the global anti-aging products market may reach to \$291.9 billion by 2015. Immense amount of researches have been focused in determining the factors that cause aging and the agents that would prevent or reverse the process.

With the rapidly growing market of anti-aging products, indicative of a financially sound future for the cosmetic

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biotechnology research industry, it is of key importance to research over the different combinations of promising naturally occurring anti – aging agents such as retinoids and alpha hydroxy acids on the health and integrity of vital cells in skin.

A variety of mammalian cell lines have been used over the past, such as human vena saphena cells and chick embryonic vascular smooth muscle cells for studying and testing of such agents [1], [2]. We chose Human Dermal Fibroblast cell lines primarily for the fact that out of the many factors contributing towards aging, photoaging has always been the inevitable factor affecting the dermis layer of human skin leading to degradation of the dermis.

Photo aging (dermatoheliosis) is a term used for the characteristic changes to skin induced by chronic UV exposure [3]. Exposure of human skin to UV radiation, sunlight being the main source, has a profound effect on the skin causing premature skin aging, skin cancer, and a host of skin changes. Human epidermis plunks meticulously on a matrix of collagen and elastin fibres and degradation of this matrix leads to skin sagging and wrinkling [4]. Exposure to ultraviolet light rays from sunlight accounts for 90% of the symptoms of premature skin aging. These rays are a primary mutagen that can only penetrate through the epidermal (outermost) layer of the skin and resulting in DNA mutations. These mutations arise due to chemical changes, primarily the formation of what called cyclobutane pyrimidine dimers and the photoproducts formed between adjacent pyrimidine bases. These mutations may be clinically related to specific signs of photoaging such as wrinkling, increasing in elastin and collagen damage, dermal thinning and reduced collagen synthesis [5].

A coordinate increase in elastin, fibrillin, and versican mRNAs has been demonstrated in fibroblasts derived from photodamaged skin, as compared to those from normal skin from the same individuals [6]. However, this may not implicate the health and levels of their protein products.

Most studies usually emphasize the promotion of collagen synthesis and value strategies of prevention of its degradation since collagen accounts for 30% of the protein content in the human body [7]. However, there are rather few studies that provide a more detailed insight as well as enough attention towards elastin synthesis and drugs that would enhance or degrade it. Elastin fibres are bundles of a stretchy protein called elastin produced by the ELN gene present in fibroblast cells in humans. These fibres provide the elastic nature of skin

i.e. allows the skin to return back to its original shape after stretching or pinching.

Retinol has been long contributing its therapeutic ability into the treatment of dermatoses including photoaging for more than two decades [8]. It is proposed to increase elastin protein levels and elastin fibres density in cultured dermal fibroblasts [8].

Retinoids strongly absorb UV light, and thus appear as potential photosensitizers. Even though, the standing of retinoids as inducers of photosensitivity comes mainly from in vitro studies, very few in vivo studies report severe photosensitivity reactions after application of topical retinoids.

On the other hand alpha-hydroxy acids belong to a special group of nontoxic organic acids that are naturally found in fruits [9]. The main cosmetic actions of these acids on the skin are increased exfoliation, moisturization and dermal thickness.

As lactic acid is a large molecule; derived from sour dairy products and fermented fruits and vegetables; it does not penetrate as deeply and hence much less likely to cause irritation as other glycolic acids. Primarily for this reason LA is the best candidate of AHA's to use especially for sensitive prone skin. However, the benefits of LA are manifold, and its other rejuvenating properties also make it the clear choice for mature or prematurely aged (damaged) skin [10].

Analysis of experimental and clinical data has suggested theories for the mechanism of action of alpha-hydroxy acids topically applied to the skin. Lactic acids reduce the epidermal calcium ion concentration and remove calcium ions from the cell adhesions by chelation. This results in a loss of calcium ions from the cadherins of the desmosomes and ultimately causes a disruption of their adherence, which results in skin shedding/flaking [11], [12]. Hence, they stimulate the growth of new skin, resulting in a rejuvenated, fresher complexion.

Looking over previous researches and for the importance of retinoids and Alpha hydroxy acids in treatment of photo-aged dermis, we choose to study the effects of the combination of retinol acetate and lactic acid elastin fibres of UV exposed human dermal fibroblast cell lines in vitro.

II. MATERIALS AND METHODS

A. Dermal Fibroblast Cells and Culture Initiation

Human dermal fibroblast cells were purchased from Himedia™. The primary cell cultures were maintained in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal bovine serum, 2mM L-glutamine and antibiotics at 37°C. The cells were passaged and sub-cultured 4 times were then utilized for radiation experiments. The cells were plated onto six plates with six wells each, with each well containing 2ml of DMEM media and 4000 cells. The cells were incubated for 24 hours prior to exposure to UV.

B. Exposure to UV Radiation and Treatment with Retinol Acetate and Lactic Acid

Prior to application of the drugs, the well plates containing the cells were exposed to UV at 254 nm at a distance of 20 cm from the UV source except for the two control plates. After

exposure to UV, the drugs were applied in the 6 wells as shown in Fig. 1.

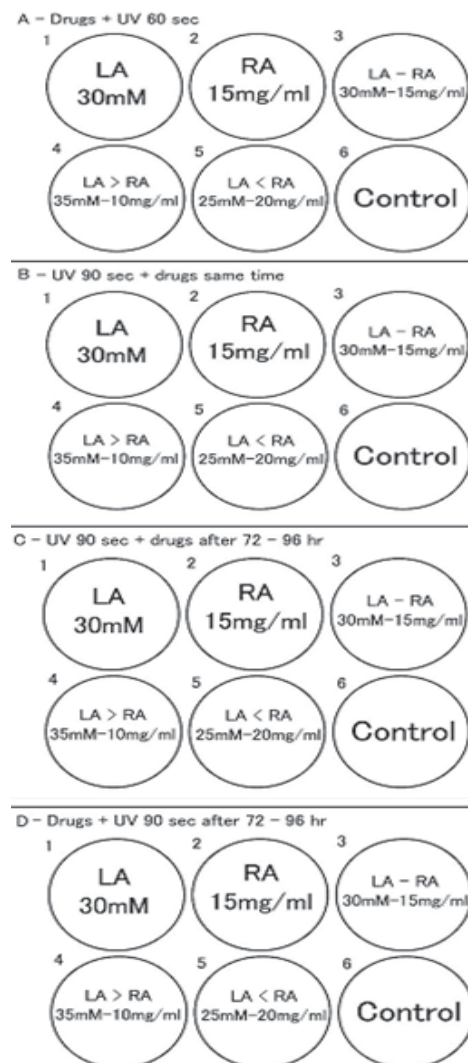


Fig. 1 Nested well plates showing different concentrations of the drugs and UV exposure

C. Cell Count and Morphology

The cells were periodically viewed under the microscope before and after photographed and treatment with drugs. They were fed every 4-5 days. After a period of 3 weeks of treatment with drugs and 3 feeding sessions, samples from each well of the 6 plates were taken and counted for an approximate number of cells using the haemocytometer. Cell counts in all treated wells were almost similar 2400±100 and that for all controls were 3000±50.

D. Quantification of Elastin by Enzyme Linked Immunosorbent Assay

This procedure was performed using the commercially available Human Elastin (ELN) ELISA kit (SHANGHAI YEHUA Biological Technology) and as per the manufacturer's instructions. The detection rate for this kit is

3.12 pg/ml-200 pg/ml and the minimum detectable dose of human ELN is typically less than 0.78 pg/ml.

Briefly, samples were prepared by using PBS to dilute the cell suspension. They were then degraded by freeze-thaw cycles repeated 3 times for 5 minutes each. They were then placed into the plate and incubated at 37 °C for 60 minutes. Each well was filled with washing solution (1X) and kept for 30 seconds and drained. This was repeated 5 times. Then 50 µl of chromogen reagent A was added to each well followed by 50 µl of chromogen reagent B and the plate was shaken gently and incubated at 37 °C for 10 minutes away from light for color development. 50 µl of stop solution was added to each well to stop the reaction. The absorbance of each plate was then measured at 450 nm using ELISA reader.

III. RESULTS AND DISCUSSION

Periodic microscopic examination of the dermal fibroblast cells revealed substantial change in the morphological characteristics of the cells after exposure to UV and drug treatments.

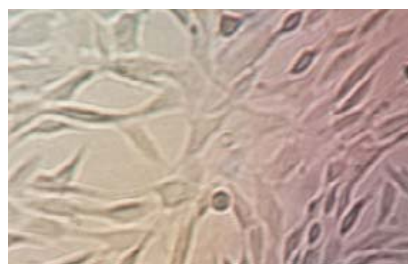


Fig. 2 (a) Cells after the 4th passage of subculture

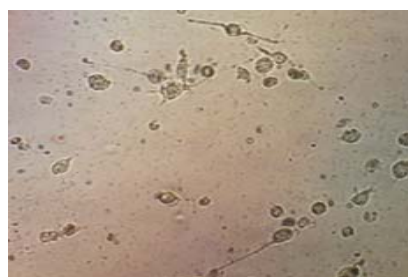


Fig. 2 (b) Cells exposed to UV for 90 sec and treatment with 15mg/ml Retinol acetate

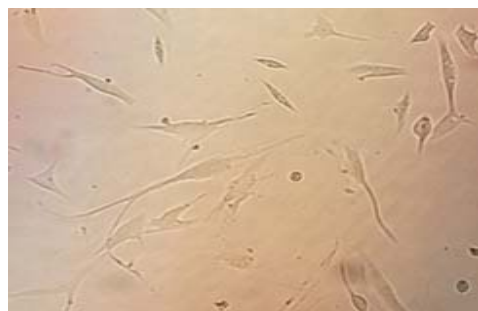


Fig. 2 (c) Cells exposed to UV for 90 sec after 72 hours of application of Lactic Acid

The cells after the 4th passage of subculture but before UV exposure and drugs treatments appeared to be attached, large and flat with elongated protruding from the body of each cell, creating the spindle-like appearance of the cell. The nucleus in the body of the cell appeared oval (Fig. 2 (a)).

After exposure to UV for 90 sec and treatment with 15mg/ml Retinol acetate appeared shorter and rounded with enlarged nucleus and in suspension (Fig. 2 (b)).

View of the cells exposed to UV for 90 sec but after 72 hours of application of Lactic Acid showed that some cells appear small and rounded while others retain the normal fibroblast shape but shorter than usual (Fig. 2 (c)).

Furthermore, we used ELISA to establish relations between elastin levels and UV effect by measuring the absorbance in each well and co-relating it with the number of cells. UV exposed cells showed significant reduction in elastin levels but not comparable with minimal reduction in cell count (Fig. 3).

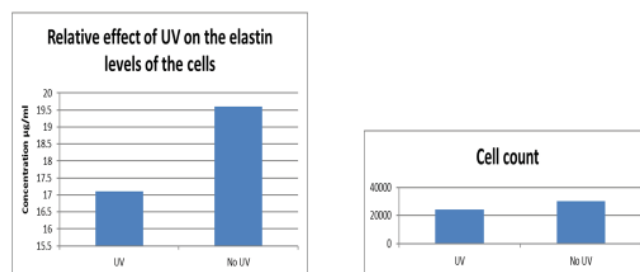


Fig. 3 Elastin level and cell count comparison between cell samples that were exposed to artificial UV and cell samples that were not exposed to artificial UV

When we compared the elastin concentration in dermal fibroblast cells that were exposed to UV for 60 sec and with different concentrations of the two drugs at the same time, there was increase in the elastin levels of the samples especially when treated with combination of high concentration of LA of 35mM and low concentration of 10 mg/ml of RA as compared to the control as shown in (Fig. 4).

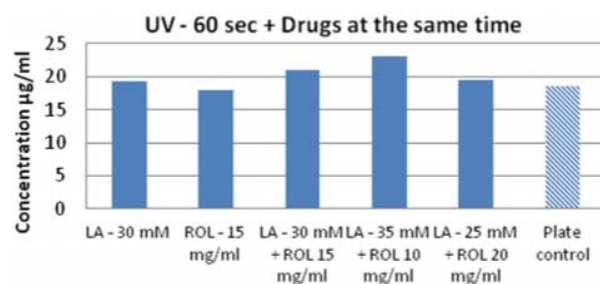


Fig. 4 Elastin Concentration in dermal fibroblast cells that were exposed to UV for 60 seconds and treated with LA and RA.

Comparing the elastin levels of cells that were exposed to UV only and for 90 sec to that with drug treatments revealed and showed a significant reduction in the elastin levels due to UV exposure and it could be a major contributor towards elastin degradation. Relating it to the cell number reduction, the reduction was not correlating. Different drug combination

treatments and comparisons before and after UV exposures showed remarkable results in elastin levels (Figs. 5 (a)-(c)).

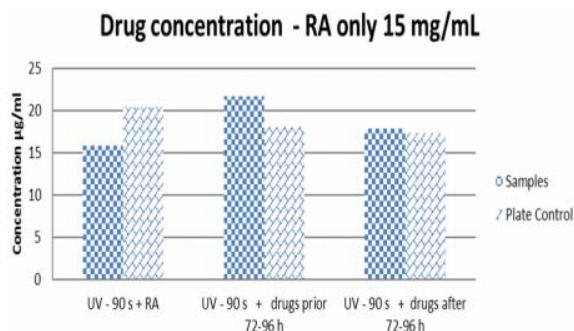


Fig. 5 (a) Elastin levels of cells treated with 15 mg/ml RA

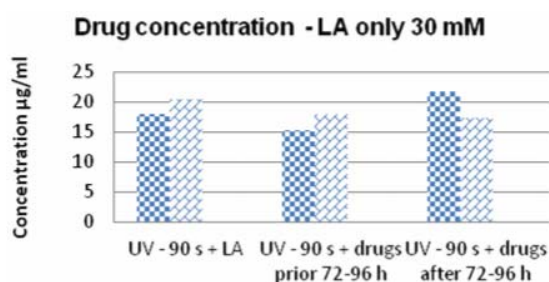


Fig. 5 (b) Elastin levels of cells treated with 30 mg/ml LA

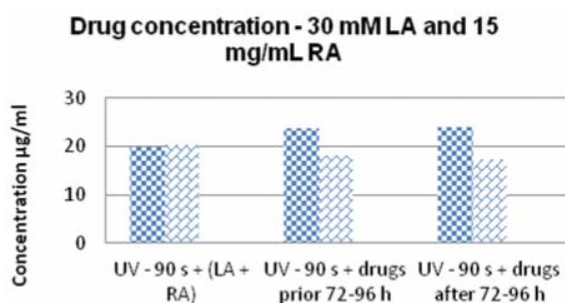


Fig. 5 (c) Elastin level in cells treated with 30mM of LA and 15mg/ml RA

At a concentration of 15 mg/ml RA an increase in elastin levels in the cells was noticed especially when the drug was applied prior to UV exposure, showing its possible preventive potential towards UV exposed aging (Fig. 5 (a)).

On other hand, treating cells with 30mM of LA only showed an increase in elastin levels of the cells when the drug was applied 72-96 hours after the UV exposure, suggesting possible repairing UV damaged cells (Fig. 5 (b)).

More interestingly, the combination of 30 mM LA and 15 mg/ml RA resulted in the best overall increase of elastin levels whether the drugs applied prior or after of 72 h of UV 90 sec exposure but not at the same time (Fig. 5 (c)). These results also hold true when cells were exposed to UV for 60 sec as seen before in (Fig. 4). These conditions suggest such combination of these 2 drugs in the right ratios has great potential in their ability in repairing elastin levels of cells that have been exposed to UV for a shorter period of time.

In conclusion, the UV exposed cells showed significantly reduced the elastin levels compared to non UV exposed cells. A combination of the two drugs with a higher concentration of lactic acid (30-35 mM) and a lower concentration of retinol (10-15mg/mL) showed to work better in maintaining elastin concentration of UV exposed cells. We assume this enhancement or protection effect could be the result of increased tropoelastin gene expression stimulated by retinol where as lactic acid probably repaired the UV irradiated damaged elastin by enhancing the amount and integrity of the elastin fibres. Even though, there were controversial previous studies debating RA effect on elastin gene expression [1], [2], we think this is not true or may be at least not in the same extent on the protein level. Furthermore, we think that further studies of differential elastin gene expression with different treatment combinations of these two drug is essential to consolidate better understanding of their effect. Finally, we believe the combination of these 2 drugs in the right ratios would have better therapeutic or preventive effects in skin care agents than any one unaided by the other.

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