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TITLE

In Vitro Extracellular Matrix Expression Analysis on the Anti-Aging Activity of Cosmetics on Primary Human Dermal Fibroblast

Study Sponsor : PT. Paragon Innovation and Technology
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The investigators, data collector, author and everyone involved in this study hereby certify the validity of the data presented in the report and has come to an agreement to the conclusions written at the end of the report.

The Head of Laboratory and Laboratory Coordinator also certifies that a quality control has been performed at each stage of the method described in a specific protocol. This control leads to accurate investigations to the tested product and to guarantee the reliability of analyzed data in accordance with this standard procedure. The present report constitutes a precise description of the performing of experimentation, processing of data and detailed procedure that have been used.

Any information concerning products to study such as patents, formulae, raw materials, manufacturing procedures etc. directly or indirectly provided by the sponsor of the study, will be regarded as confidential, will remain his property and will not be disclosed without his express consent.

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1. INTRODUCTION

Skin is the largest organ in the human body. As the organ that is exposed towards the environment, skin confers a cosmetic role in which clean and beautiful skin is perceived as a symbol of beauty and may have a positive influence on the social behavior of surrounding people as well as symbolize the perceived "health" of an individual. However, being exposed to the environment also made the skin vulnerable to damage, which resulted in wrinkles, loss of elasticity, dry skin, thickened epidermis, skin darkening, and discoloration. These conditions are also referred to as skin aging, and many researchers have now considered skin aging as a separate disease that would require more attention [1,2,3].

Many factors could contribute towards the skin aging process and these factors are generally divided into intrinsic and extrinsic factors. Intrinsic factors particularly involve age and the genetic makeup of an individual, while extrinsic factors typically involve radiation, chemicals, pollution, and toxins exposure, and are receiving more attention as skin aging prevention strategies. The combination of these factors accelerates the aging process and affects skin appearances as a whole. In particular importance towards the aging process is the level of extracellular matrix components such as protein (e.g. collagen, elastin) and naturally occurring hyaluronic acid, as these components received the highest impact of skin aging processes [2,3,4].

Throughout history, many attempts have been made to preserve skin's beauty and youthful appearance and to slow down the skin aging process. Skin aging prevention involves the development of various cosmetics that protect against damaging UV radiation, oxidative damage. Additionally, various cosmetics also induce extracellular matrix content synthesis to replenish extracellular matrix content that has been lost. The development of anti-aging cosmetic products is of particular interest due to the growing interest in having beautiful and healthy skin, as well as for the maintenance of healthy skin [2,3].

In this study, an anti-aging cosmetic from a well-known cosmetic company will be tested to examine their capability of inducing expression of collagen I, collagen III, elastin, and hyaluronic acid in primary human dermal fibroblast (HDF) cell lines. Fibroblast is a cell type which is responsible and contributes to the formation of connective tissue. It is responsible for producing extracellular matrices (ECM) and exist abundantly in the dermal layer of the skin [5,6,7]. ECM is responsible for the elasticity and firmness of the skin which are often attributed as the level of health and youthfulness of the skin. Fibroblasts in aged skin would have a lowered level of expression in the ECM production compared to young adult skin [8,9]. Based on these, it is possible to see whether the product is able to increase the production of ECM by measuring the expression level of several ECM markers after treatment of a skincare product. The expression level of ECM markers such as collagen I, collagen III, elastin, and hyaluronic acid are necessary to determine the level of skin aging. An increase in the production of the ECM would delay and prevent loss of elasticity and firmness of the skin which are usually the most prominent sign of aging.

AIM OF STUDY : to study the effectiveness of anti-aging cosmetic products towards dermal extracellular matrix expression by using enzyme-linked immunosorbent assay (ELISA) with HDF cells as a subject model

2. MATERIALS AND METHODS

a. In vitro model

- Model : Human Dermal Fibroblast (HDF) primary cell line
- Culture conditions : 37℃, 5% CO_2
- Culture medium : Fibroblast Growth Medium (FGM)
  : DMEM supplemented with:
    - Sodium bicarbonate
    - 2 mM L-glutamine
    - 1 mM sodium pyruvate
    - 1% (v/v) non-essential amino acids
    - Penicillin - Streptomycin 1% (v/v)
    - Fetal Bovine Serum (FBS) 10% (v/v)

b. **Test compound (group of experimental design)**

Sponsor	Product Reference	Reception Date of The Study	Aspect	Product Storage	Study Date
PT Paragon technology and innovation	Serum 0921 - E	30/9/21	Liquid	In a transparent bottle, protected from light	8/10/21 to 22/05/2021
	Serum 0921 - F		Liquid	In a transparent bottle, protected from light	
	Essence 0921 - A		Liquid	In a transparent bottle, protected from light	
	Essence 0921 - B		Liquid	In a transparent bottle, protected from light	
	Essence toner 0921 - A		Liquid	In a transparent bottle, protected from light	
	Essence toner 0921 - B		Liquid	In a transparent bottle, protected from light	
	Raw Mat 0921 - L		Powder	In a plastic clip, protected from light	
	Raw Mat 0921 - J		Paste	In a plastic clip, protected from light	
	Raw Mat 0921 - Y		Liquid	In a transparent bottle, protected from light	
	Raw Mat 0921 - D		Liquid	In a transparent bottle, protected from light	
	Raw Mat 0921 - C		Liquid	In a transparent bottle, protected from light	
	Raw Mat 0921 - R		Liquid	In a transparent bottle, protected from light	
	Raw Mat 0921 - A		Powder	In a plastic clip, protected from light	
	Raw Mat 0921 - P		Powder	In a plastic clip, protected from light	
	Raw Mat 0921 - K		Liquid	In a transparent bottle, protected from light	
	Raw Mat 0921 - U		Liquid	In a transparent bottle, protected from light	
	Raw Mat 0921 - I		Powder	In a plastic clip, protected from light	
	Raw Mat 0921 - S		Powder	In a plastic clip, protected from light	

	Raw Mat 0921 - E		Liquid	In a transparent bottle, protected from light	
	Raw Mat 0921 - B		Liquid	In a transparent bottle, protected from light	

c. Methods

c.1 Method origin

The *in vitro* extracellular matrix (ECM) analysis by using ELISA to determine the effect of test compounds on the ECM production of HDF cells was performed based on a previous study done by Dooley et al [10] wherein the cells were treated with certain test compounds, tested for their respective cytotoxic effects in order to determine the highest non-cytotoxic concentration to be used in subsequent treatment, and finally analyzed using ELISA to measure the ECM production. In this study, the aforementioned method was adjusted according to the kit manufacturer protocol.

c.2 Method Principle

The whole experiment utilized an *in vitro* cell culture method using primary HDF cells. The experiment was conducted in three biological replicates. The first step of the experiment was conducted to test the cytotoxicity of the test compounds towards the primary HDF cell.The cytotoxicity test also included various concentrations of the test compounds to determine the highest non-cytotoxic concentration that can be used on the cell. Following the cytotoxicity test, the effect of each test compound towards the ECM production was analyzed by utilizing an enzyme-linked immunosorbent assay (ELISA) of the target proteins of choice (Collagen 1, collagen 3, hyaluronic acid, elastin). Briefly, the cells were treated with the products and other treatments and then the level of ECM marker expression was compared with the non-treated group. The anti-aging effect of the test compounds can be deduced by analyzing the expression of the ECM as during skin-aging, the constituents of dermal ECM changes and the ECM production deteriorates, thus improvement of the ECM marker expression signify improvement in delaying aging and improving skin firmness and elasticity. The three primary components of the skin ECM that are mostly altered during skin-aging are collagen, elastin, and hyaluronic acid levels. Therefore, these ECM constituents have been the focus of many skin-aging and anti-aging studies [6-9].

c.3 Description of the method

<u>c.3.1. HDF cell culture</u>

The HDF cells were obtained from Stem Cell Institute Kalbe Farma Tbk, Indonesia. HDF cells were maintained in a T25 flask and cultured in a standard two-dimensional model. The cells were grown in Dulbecco's modified eagle medium (DMEM) containing L-glutamine and pyridoxine hydrochloride, supplemented with 1 mM sodium pyruvate, 3.7 g/L sodium bicarbonate, 1% penicillin-streptomycin, 1% non-essential amino acid, and 10% fetal bovine serum. The cells were maintained in a humidified atmosphere at 37°C in the 5% CO_2 incubator.

<u>c.3.2. Cytotoxicity test</u>

The cytotoxicity test was performed following the MTS assay protocol (CellTiter 96® AQ$_{ueous}$ One Solution Cell Proliferation Assay, G3582). Prior to the MTS assay, the cells were seeded to a 96-well plate with the density of 0.7 x 10^4 and grown until they reached approximately 80% confluence.

Along with the cell seeding process, treatment media were made by preparing five different concentrations of product, base/vehicle, and API **(Table c.3.2.1.)**. To determine the cytotoxic concentration of the tested product towards HDF cell lines and also to determine an optimum amount of

the product to be used, the amount of product applied to the skin must equal the amount applied to the cell culture.

The standard amount of serum/ essence/ toner to be applied to one's face is 4-5 drops [11, 12]. This number was converted to grams by weighing them. Because the treatment for ECM expression analysis will be conducted in a 24-well plate, the amount of product used in the face must be scaled down accordingly.

$$\text{Amount in 24 well plate} = \text{amount in face (mg)} / \frac{\text{average facial surface area (cm2)}}{\text{24-well plate surface area (cm2)}}$$

$$= \text{amount in face} / \frac{630}{1.9}$$

$$= \text{amount in face} / 331.6$$

Table c.3.2.1 The treatment media concentration of the moisturizer product, base, and API for cytotoxicity test.

	Concentration points of working solution				
	4X	2X	Normal usage	½ X	¼ X
Serum	1.88 mg/ml	0.96 mg/ml	0.47 mg/ml	0.24 mg/ml	0.12 mg/ml
Serum Base	1.68 mg/ml	0.84 mg/ml	0.42 mg/ml	0.21 mg/ml	0.11 mg/ml
Serum API	41.28%	20.64%	10.32%	5.16%	2.58%
Essence	1.483 mg/ml	0.7414 mg/ml	0.3707 mg/ml	0.1853 mg/ml	0.0927 mg/ml
Essence base	1.374 mg/ml	0.6872 mg/ml	0.3436 mg/ml	0.1718 mg/ml	0.0859 mg/ml
Essence API	29.4%	14.7%	7.35%	3.68%	1.84%
Toner	1.483 mg/ml	0.7414 mg/ml	0.3707 mg/ml	0.1853 mg/ml	0.0927 mg/ml
Toner base	1.374 mg/ml	0.6872 mg/ml	0.3436 mg/ml	0.1718 mg/ml	0.0859 mg/ml
Toner API	34.8%	17.42%	8.71%	43.55%	21.78%
Control Groups					
DMSO	1% DMSO in serum-free DMEM				
Negative control	Untreated cells in serum-free DMEM				
Blank	Serum-free DMEM without cells				

Using the formula above, the standard amount/ normal usage is converted to an amount equal to the surface area of a 24-well plate. This amount was then scaled down and up to create 5 concentration points as shown in table c.3.2.1. This study will also evaluate the expression level of just the base of the product and the active pharmaceutical ingredient (APIs) of the product.

Serial dilution was conducted to achieve the desired concentrations as listed in **table c.3.2.1**. The treatment media was sterilized through a filtration process with a 0.22 µm nylon syringe filter and the sterilized media were transferred to a sterile 15 mL falcon tube.

After reaching 80% confluency, the cells were treated with 100 µL of treatment media according to the treatment map shown in **Figure c.3.2.1** and incubated for 72 hours in the standard cell culture condition. The MTS assay began by replacing the old treatment media with 100 µL DMEM only. 15 µL of MTS reagent was added to each well and the cells were incubated for 3 hours at 37°C in a 5% CO_2 incubator. To measure the cell viability, the absorbance was measured with a 96-well spectrophotometer at 570 nm. The cell viability was calculated with the formula shown below.

$$Cell\ viability\ (\%) = \frac{Absorbance\ of\ samples - absorbance\ of\ blank}{Absorbance\ of\ control - absorbance\ of\ blank}\ x\ 100\%$$

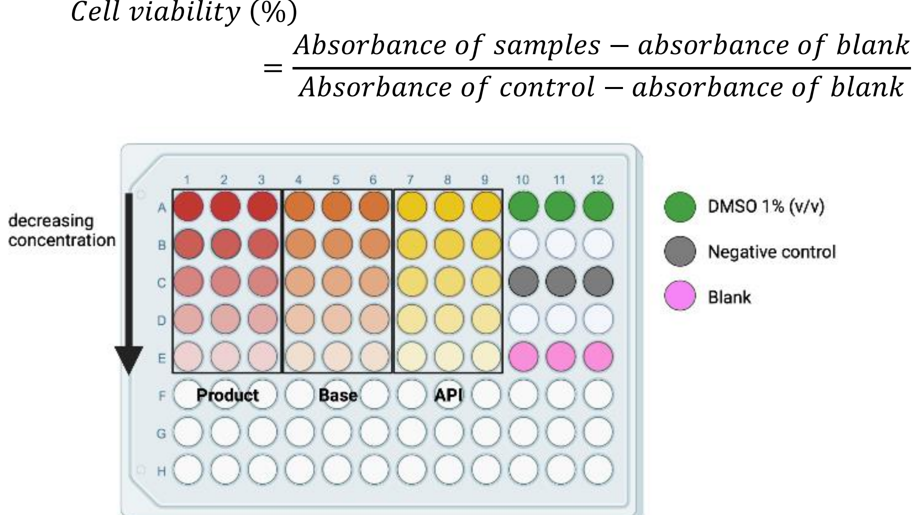

Figure c.3.2.1 MTS treatment layout example for cell viability of one product type (in technical replicates)

c.3.3. ECM expression analysis

The enzyme-linked immunosorbent assay (ELISA) was performed using Human Collagen Type I (COL1), Human Collagen Type III (COL3), Human Elastin (ELN), and Human Hyaluronic Acid (HA) ELISA Kit from MyBioSource, US. The procedures are in accordance with the steps described in the ELISA Kit manual from the vendors.

Prior to ELISA, the HDF cells were seeded into a 24-well plate (50,000 cells/well), treated with serum, essence, and toner using normal usage scaled down for a 24-well plate. The treatment was conducted for 72 h. Following the treatment, the media was collected and subjected to ELISA to measure the ECM level.

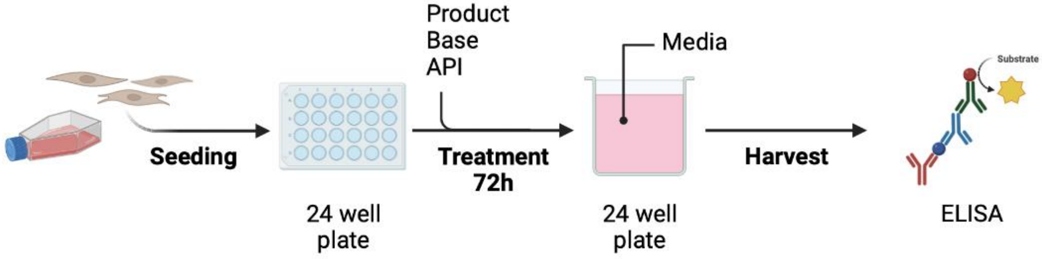

Figure c.3.3.1 ELISA workflow

c.4 Materials and Equipment used

a. Materials
   a. Primary HDF cells obtained from Stem Cell Institute, Kalbe Farma Tbk.
   b. Fibroblast growth medium
   c. Dulbecco's Modified Eagle Medium (DMEM)
      i. Sodium Bicarbonate
      ii. Penicillin-streptomycin (1%)
   d. Complete DMEM (cDMEM): DMEM only with addition of Fetal Bovine Serum (FBS) 10% (v/v), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% (v/v) non-essential amino acid
   e. Product tested
   f. Base/excipient
   g. Active pharmaceutical ingredients (API)
   h. MTS reagent Cell Titer 96® AQueous One Cell Proliferation Assay
   i. Trypan blue
   j. 96-well plate
   k. ELISA Kit
      i. Human type I collagen
      ii. Human type III collagen
      iii. Human elastin
      iv. Human hyaluronic acid

b. Equipments
   a. 37°C CO_2 incubator with 5% humidified atmosphere
   b. Biosafety cabinet (BCS) level 2
   c. Inverted microscope
   d. Light microscope
   e. Haemocytometer
   f. Microplate reader

c. Data Management, Calculation and Statistical Analysis

MTS assays and ELISA assay were all conducted in biological replicates. Graph plotting and statistical analysis were performed using GraphPad Prism 9.2.0. A one-way ANOVA analysis was performed, followed by the Tukey Post-Hoc Test, on both assays with a significance level of 0.05

3. RESULT

3. 1. MTS assay

The treatment of HDF cells with the serum, essence, and toner revealed no significant changes ($p>0.05$) in cell viability in all tested concentrations, and that cell viability for all treatment groups was calculated to be above 70%, suggesting that all three products are safe at all these tested concentrations.

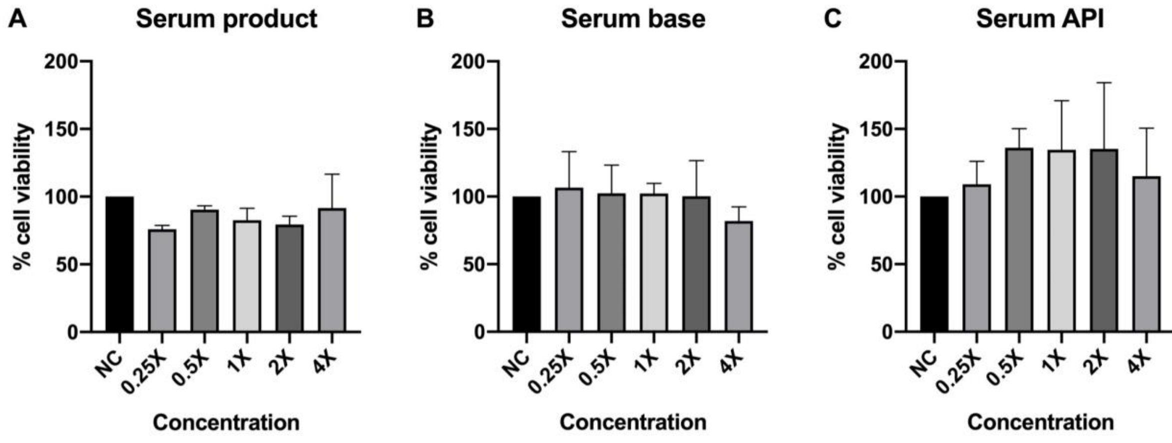

Figure 3.1.1. The MTS assay result for the product, base, and API of the Serum at varying concentrations (n=3).

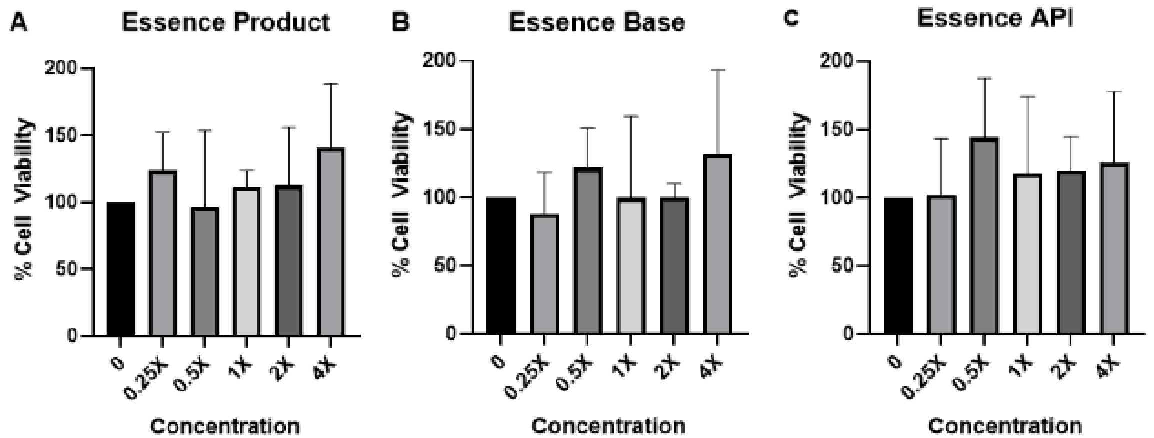

Figure 3.1.2. The MTS assay result for the product, base, and API of the Essence at varying concentrations (n=3).

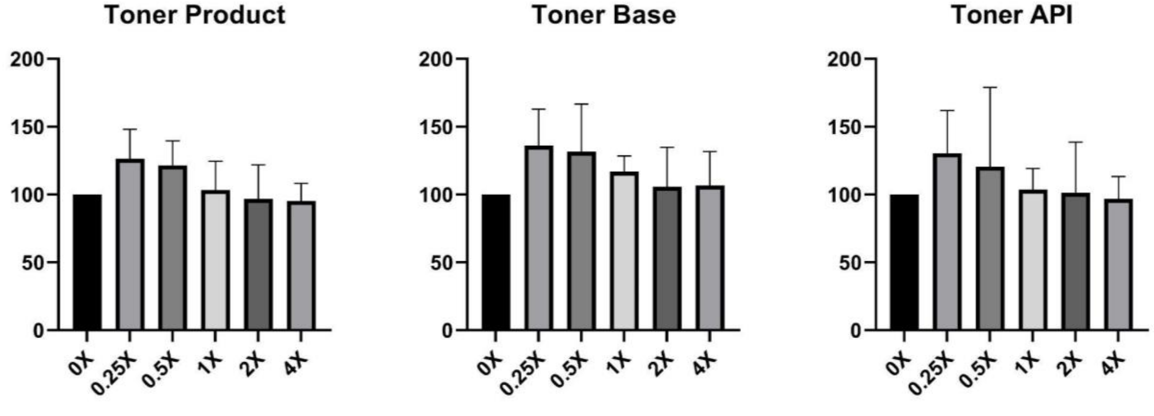

Figure 3.1.3. The MTS assay result for the product, base, and API of the Toner at varying concentrations (n=3).

3. 2. ELISA

The ECM expression of HDF treated with serum was displayed in Figure 3.2.1. The treatment of HDF cells with Serum product revealed significant increase in type I collagen concentration (p = 0.0150). Meanwhile, treatment with serum base and serum API alone did not increase type I collagen level, suggesting that the two components have to be used together to result in desired effect. Neither serum product, serum base, nor serum API increases type III collagen and hyaluronic acid level. The effect of serum on elastin expression is inconclusive as the elastin cannot be detected both in treated and untreated HDF, hence the data is not displayed in the report. Regardless, it can be concluded that serum products contribute to an acceptable anti-aging effect.

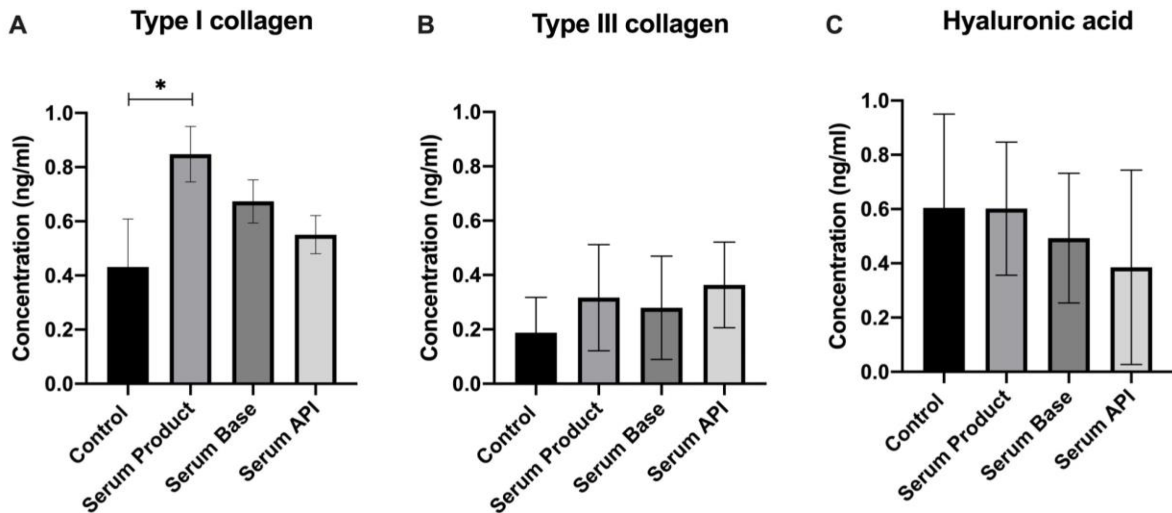

Figure 3.2.1. ELISA displays the concentration of ECM after treatment with serum for 72 h. (A) Type I collagen (B) Type III collagen (C) Hyaluronic acid. *P<0.05

The treatment of HDF cells with the essence, essence base, and essence API revealed no significant changes on the concentration of collagen 3, collagen 1, and hyaluronic acid after 72 hours. Therefore, suggesting that the essence has no significant anti-aging activity towards HDF cells. The effect of essence product essence base, and essence API on elastin concentration was inconclusive as the concentration of ELN was below the detection range (Figure 3.2.2.). The detection range of the ELISA kit used was 0.469-30 ng/mL, while the ELN concentration found was below 0.1 ng/mL; thus the data is not displayed in the report.

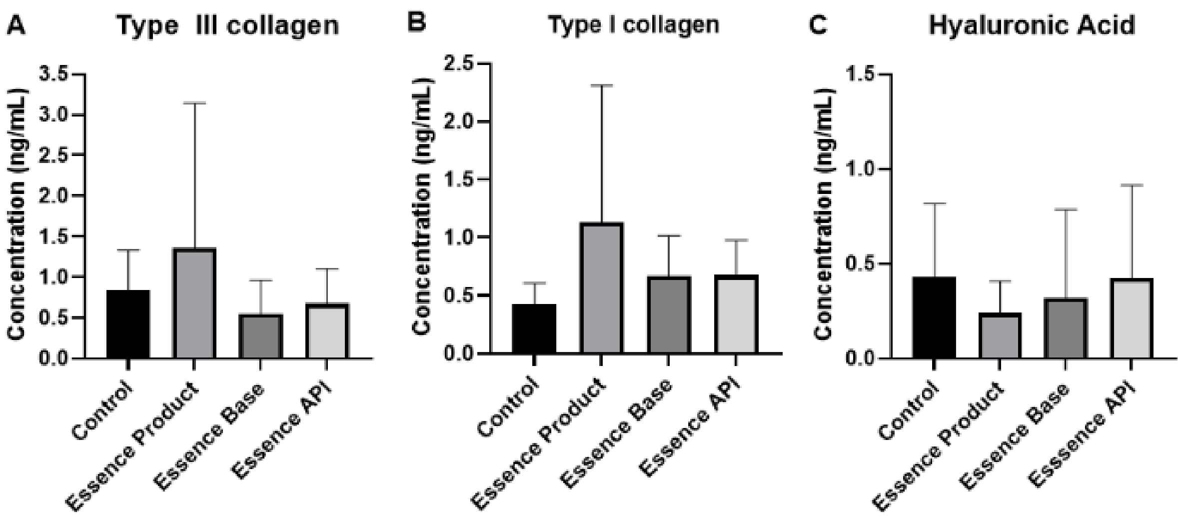

Figure 3.2.2. ELISA displays the concentration of ECM after treatment with essence for 72 h. (A) Type I collagen (B) Type III collagen (C) Hyaluronic acid. *P<0.05

The ECM expression of HDF treated with toner product, toner base, and toner API was displayed in Figure 3.2.3. The treatment of HDF cells with a toner base after 72 hours revealed a significant increase in type III collagen concentration (p = 0.0076). Meanwhile, treatment with toner product and toner API alone did not increase type III collagen level significantly, suggesting that the base itself might have been active in encouraging type III collagen product. Toner product, toner base, and toner API did not significantly increase type III collagen and hyaluronic acid level. The effect of toner on elastin expression is inconclusive as the elastin in both in treated and untreated HDF is outside the detection range of the ELISA kit, hence the data is not displayed in the report.

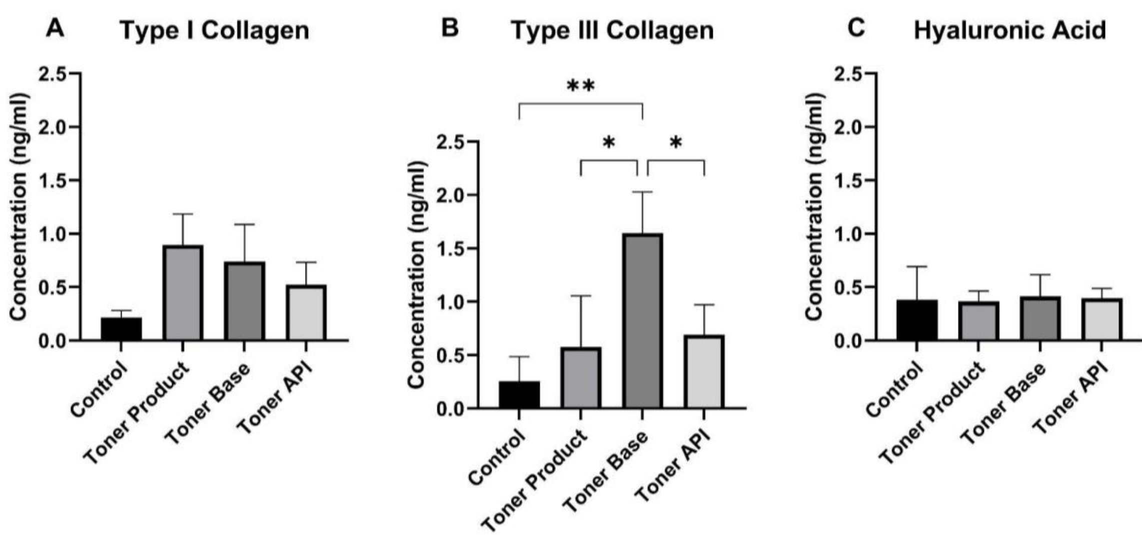

Figure 3.2.3. ELISA displays the concentration of ECM after treatment with toner for 72 h. (A) Type I collagen (B) Type III collagen (C) Hyaluronic acid. *P<.05,**P<.01

The effect of serum, essence, and toner on the concentration of ELN were all inconclusive as the elastin concentration was below the detection range. ELN was first synthesized as a soluble precursor, tropoelastin (TE). TE then formed bundles of elastic fibers in ECM in a process known as elastogenesis. Elastogenesis is a complex process involving the cross-linking of TE monomers with microfibrillar proteins to produce insoluble elastic fibers polymers [13, 14, 15]. In most cell cultures, ELN remains in its soluble and non-cross-linked precursor TE [15], hence the extremely low levels of ELN measured using ELISA. Furthermore, ELN synthesis decreases with age, and as the organ or tissue reaches maturity, ELN synthesis gets repressed by post-transcriptional factors [13, 14]. In addition, MMPs such as collagenases and elastases constantly degrade ECM components. The breakdown of ELN by elastases, in addition to the reduced ELN synthesis due to aging, contributed to the overall low levels of ELN in dermal ECM [13, 14, 16].

4. CONCLUSION

Considering that the serum increased type I collagen production, this product is deemed to show potential for preventing or reversing aging. Toner base increases type III collagen production thus suggesting anti-aging effect. Meanwhile essence was shown to be unable to increase the dermal ECM markers thus suggesting no anti-aging effect related to ECM expression

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