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TITLE

In Vitro Study of Type I and III Collagen Expression by Active AAG1-AAI on With 2D Human Dermal Fibroblast Culture Using ELISA

Study Sponsor : PT. Paragon Innovation and Technology
Laboratory : Skinovation Centre, Indonesia International Institute for Life Sciences
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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.

Date : 6 June 2022

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1. Introduction

Aging is an essential and inevitable part of the biological process in living organisms. As a slow and chronic process, aging causes the gradual deterioration of physiological functions necessary for survival and fertility, such as deoxyribonucleic acid (DNA) repair and immune responses. The skin exhibits the most visible signs of aging due to its large volume and location on the body surface; creating an issue for most of the population which idealizes young and healthy skin. Hence, considerable efforts have been exerted in cosmetic and pharmaceutical sectors as a way to find remedies to delay or reverse aging (Kazanci, Kurus, & Atasever, 2016).

Most anti-aging products target extracellular matrix (ECM) component production by fibroblasts, as its reduced production is one of the main mechanisms of dermal atrophy, a condition tightly related to aging. One of said ECM components, type I collagen, is an anti-aging marker as it constitutes the majority of collagens in the skin. Type I collagen typically constitutes 80% of the total collagen content in young skin, this is followed by type III collagen which constitutes 12%. With its role in providing tensile strength and elasticity to the skin, changes in collagen content would have visible results; as observed in skin wrinkling. It has been observed that the ratio between type I and type III collagen decreases with age, further emphasizing the significance of both collagen types in aging (Reilly & Lozano, 2021). With such findings, the anti-aging efficacy of a product can be measured from its ability to induce collagen production.

To test cosmetic products, it is essential to have appropriate test systems to properly simulate the complexity of human skin; a property which is not represented in two-dimensional (2D) cell cultures. Hence, artificial skin equivalents such as the EpiDerm™ Skin Model can be used as a solution. The ability of skin equivalents to simulate processes involved in the skin, such as penetration, makes it ideal for usage in this study (Neupane et al., 2020).

This study aims to evaluate the effect of active AAG1-AAI on type I and III collagen in human dermal fibroblasts (HDF) with the EpiDerm™ Skin Model artificial skin equivalent as a skin barrier model by using enzyme-linked immunosorbent assay (ELISA).

2. Material and Methods

2.1. In vitro model

- Model : Human dermal fibroblast primary cell line
- Culture conditions : 37°C, 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)

2.2. Test Compound (Groups of study or experimental design)

Sponsor	Product Reference	Reception Date of The Study	Aspect	Product Storage	Study Date
PT Paragon Technology and Innovation	Anti-aging Serum A (Product - AAG1-AAI)	30/12/21	Liquid	In a transparent bottle, protected from light	27/01/2022 to 29/05/2022
	Anti-aging Serum B (Base)		Liquid	In a transparent bottle, protected from light	

	Anti-aging Serum C (Comparator - AAG3-AAD)		Liquid	In a transparent bottle, protected from light	
	Anti-aging Serum D (Combination - AAG1-AAI, AAG3-AAD)		Liquid	In a transparent bottle, protected from light	

2.3. Method

2.3.1. Method Origin

The effect of active AAG1-AAI was analyzed through an ELISA protein expression analysis by observing the expression of type I and III collagen following a cell cytotoxicity study. These analyses were performed based on a previous study done by Dooley and colleagues in 2018. In addition, the method on EpiDerm™ Skin Model usage was done in accordance with the protocol developed by MatTek. In this study, the aforementioned methods were modified to fulfill the study objectives.

2.3.2. Method Principle

The study utilized monolayer primary HDF cell culture for the analysis of protein expression upon cell treatment with AAG1-AAI. The experiment was conducted in three technical replicates. Prior to protein expression analysis, a cytotoxicity test was performed to determine the most suitable concentration of product containing AAG1-AAI, base, comparator, and combination. The concentration was determined based on the standard of ISO-10993-5 for *in vitro* cytotoxicity test, where cell viability of <70% is considered cytotoxic; along with a significance test through statistical analysis. After sample concentrations were set, treatment was done on HDF cells where the products were applied through a skin model (EpiDerm™) to simulate skin barrier and determine the product penetration. The amount of products that penetrated to the skin was expected to interact and affect the skin cells and then the type I and III collagen expressions were analyzed through an ELISA study to determine the effect of product treatment on extracellular matrices (ECM production. The method measures the relative expression of proteins upon cell treatment in comparison to the untreated control. By the end of this study, it is expected that the ability of the product to penetrate the skin and then induce the collagen production would be able to be conducted

2.3.3. Description of The Method

2.3.3.1. HDF Cell Culture

The primary HDF cells used throughout this study were obtained from the Stem Cell and Cancer Institute (SCI) of Kalbe Farma, 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, pyridoxine hydrochloride, and sodium pyruvate; supplemented with 3.7 g/L sodium bicarbonate, 1% penicillin-streptomycin, 10% fetal bovine serum, 2 mM L-glutamine, and 1% (v/v) non-essential amino acids (Moay et al., 2021). The media was further supplemented by adding FGM at a 1:1 ratio. The cells were maintained in a humidified 5% CO_2 incubator at 37°C.

2.3.3.2. Cytotoxicity Test

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 0.01 x 10^6 and grown until they reached approximately 80% confluence.

Along with the cell seeding process, treatment media were made by preparing product, base, comparator, and combination in four different concentrations of 2%; 1%, 0.5%, and 0.25%.

The stock media was prepared with the highest treatment media concentration. The samples were weighed and added with DMEM only, then the treatment media was

sterilized through a filtration process with a 0.22 µm polyethersulfone (PES) syringe filter and the sterilized media were transferred to a sterile 15 mL centrifuge tube.

After reaching 80% confluency (~24 hours), the cells were treated with 200 µL of treatment media and incubated for 72 hours in the standard cell culture condition. The MTS assay was performed by replacing the old treatment media with 100 µL DMEM only, adding 15 µL of MTS reagent to each well, then incubating for 3 hours in standard cell culture condition. To determine the cell viability, the absorbance was measured with a 96-well spectrophotometer at 490 nm. The cell viability was calculated with the formula shown in **Figure 2.3.3.2.2**.

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

Figure 2.3.3.2.2. The formula used to calculate the cell viability percentage based on the absorbance measurement.

Table 2.3.3.2.2. The layout of the 96-well plate for the MTS assay of the moisturizer. C1-C4: is the range of concentration listed in previously (2% to 0.25%); **(-)** Control: Cells + DMEM; Blank: DMEM

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C2	C3	C4		C1	C2	C3	C4			
B	C1	C2	C3	C4		C1	C2	C3	C4			
C	C1	C2	C3	C4		C1	C2	C3	C4			
D												
E												
F	C1	C2	C3	C4		C1	C2	C3	C4			
G	C1	C2	C3	C4		C1	C2	C3	C4			
H	C1	C2	C3	C4		C1	C2	C3	C4			

Product
Base
Comparator
Combination
(-) Control
Blank

2.3.3.3. EpiDerm Skin Model (EPI-200-X) Preparation

Prior to cell treatment, the EPI-200-X was given a pre-treatment to allow its full recovery from shipment stress. This was done by transferring the EPI-200-X cell inserts into 6-well plates containing 900 µL pre-warmed MatTek assay medium under sterile conditions, then incubating in a humidified 5% CO_2 incubator at 37°C overnight prior to treatment.

2.3.3.4. ELISA Protein Expression Analysis

Prior to the cell treatment media preparation, the HDF cells were seeded to a 6-well plate with a cell density of 0.2 x 10^6 cells per well with 2.5 mL complete media. The cells were cultured in a standard cell culture condition until it reached approximately 80% cell confluency.

Based on the MTS assay results, the treatment concentration of 0.5% was used for the protein expression analysis as it maintained cell viability of >70% and created no significant difference in cell viability compared to the control group. The treatment media was prepared by weighing the necessary amount of samples and mixed with DMEM only,

sterilizing through a filtration process with a 0.22 µm PES syringe filter, and transferring to a new sterile 15 mL centrifuge tube.

Prior to administering the treatment solutions, MatTek Permeation Fixtures (EPI-200-FIX) were prepared, as shown in **Figure 2.3.3.3.1.**, to hold the EPI-200-X and ensure its stability within the wells. Each EPI-200-FIX consists of a top and bottom piece which are connected by 3 screws locked into place by corresponding nuts. To use the EPI-200-FIX, the 3 screws were removed to take out the top piece, then the EPI-200-X insert was placed into the bottom piece. Once the insert was in place, the top part was replaced and the screws were tightened properly.

The cell treatment process was then conducted by adding 500 µL of each treatment media through the top part of EPI-200-FIX to penetrate through EPI-200-X into the HDF layer, then adding 2 mL DMEM only as the receiver solution. The cells were incubated for 72 hours, while the treatment media was refilled daily to reach a total treatment volume of 2.5 mL.

To harvest samples for ELISA, the EPI-200-FIX were removed from the plate, then the supernatant in each well was pipetted into microcentrifuge tubes and centrifuged using a 5417R centrifuge at 2,000 rpm for 10 minutes. The supernatant was transferred to new microcentrifuge tubes to ensure that the samples are free of any debris.

The ELISA protein expression analysis was performed using the Human Collagen Type I ELISA Kit and Human Collagen Type III ELISA Kit by MyBioSource, and done according to the manufacturer's protocol. The absorbance of each sample was read using Infinite® M200 NanoQuant plate reader at 450 nm to determine the protein expression. Once absorbance readings were obtained, the relative OD value of samples was calculated by using the formula shown in **Figure 2.3.3.3.2.**

A standard curve was then plotted with standard concentrations on the X axis and relative OD450 of each standard as the Y axis. Through this standard, a trendline was generated for the relative OD450 of standard which can then be used to calculate the collagen concentration in each sample.

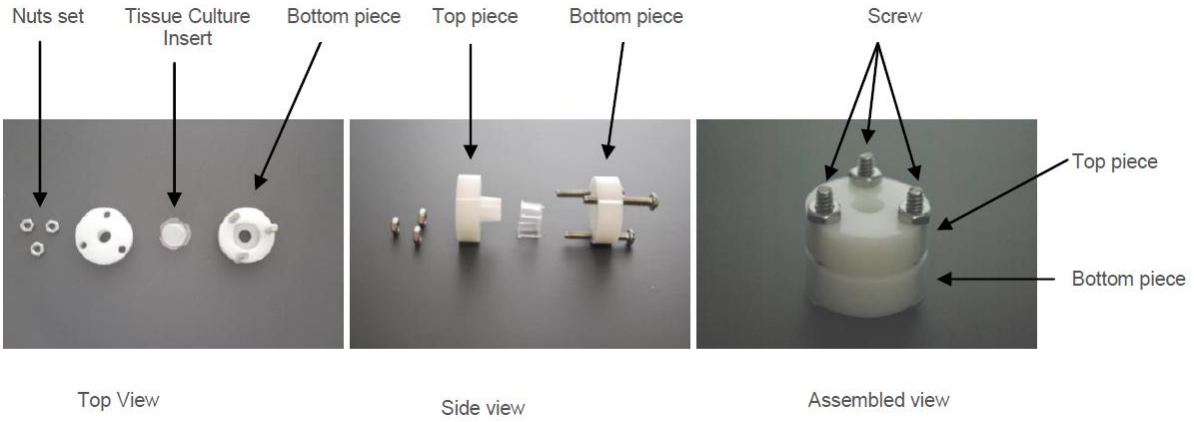
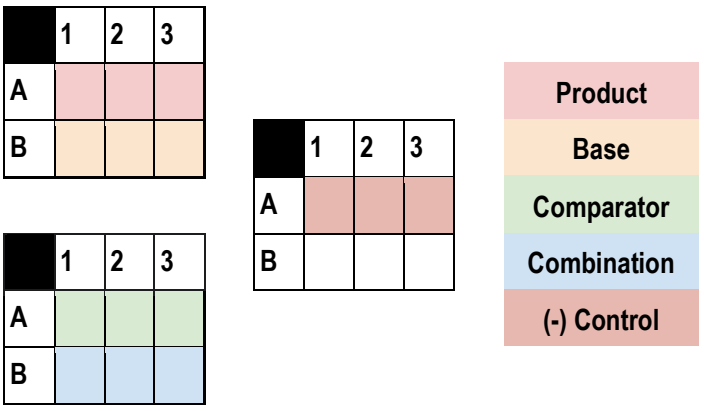


Figure 2.3.3.3.1. MatTek Permeation Fixtures (EPI-200-FIX) Parts and Assembly.

$$Relative\ OD450 = OD450\ sample - OD450\ blank$$

Figure 2.3.3.3.2. The formula used to calculate the relative absorbance value for determination of protein expression.

Table 2.3.3.3.1. The layout of the 6-well plate for the ELISA. **(-)** Control: Cells + DMEM



2.3.4. Materials and Equipment

The materials and equipment used in this study were listed in **table 2.3.4.1**.

Table 2.3.4.1. The list of materials and equipment utilized and incorporated in the study.

No.	Materials	Equipment
1	T25 flask	Biosafety Cabinet
2	6 well plate	Pipette Gun
3	96 well plate	Micropipette
4	1000 µL micropipette tips	Inverted microscope
5	200 µL micropipette tips	Tally Counter
6	10 µL micropipette tips	Hemocytometer
7	25 mL serological pipette	Cell incubator
8	10 mL serological pipette	Centrifuge
9	5 mL serological pipette	Ice Box
10	50 mL centrifuge tube	Analytical Balance
11	15 mL centrifuge tube	Infinite® M200 NanoQuant plate reader
12	1.5 mL microcentrifuge tube	Forceps
13	10 mL syringe	5417R centrifuge

14	3 mL syringe	
15	1 mL syringe	
16	0.22 µm polyethersulfone syringe filter	
17	Dulbecco's Modified Eagle Medium	
18	Fetal bovine serum	
19	Sodium bicarbonate	
20	Penicillin-Streptomycin	
21	L-Glutamine	
22	Non-essential amino acids	
23	Trypsin-EDTA	
24	EpiDerm Skin Model (EPI-200-X)	
25	MatTek Permeation Fixtures (EPI-200-FIX)	
26	CellTiter 96® AQueous One Solution Reagent (Promega)	
27	Human Collagen Type I ELISA Kit (MyBioSource)	
28	Human Collagen Type III ELISA Kit (MyBioSource)	
29	Anti-aging Serum A (AAG1-AAI)	
30	Anti-aging Serum B (Base)	
31	Anti-aging Serum C (AAG3-AAD)	
32	Anti-aging Serum D (AAG1-AAI, AAG3-AAD)	

2.4. Data Management, Calculation, and Statistical Analysis

The results were processed with GraphPad Prism 9.3.1. (GraphPad Software, San Diego, California USA), in which the results are described as mean ± standard error of mean (SEM). For the significance test, the normally distributed data was processed with one-way ANOVA. Data showing a p value of below 0.05 was considered as statistically significant.

3. Result

3.1. MTS Assay

The MTS cytotoxicity assay done by treating HDF cells using the Product, Base, Comparator, and Combination with concentrations of 2%, 1%, 0.5%, and 0.25% showed the cell viability of each sample, as presented in **Figure 3.1.1.** The results showed a cell viability of >70% on every sample, with ANOVA analysis revealing a statistically non-significant difference between groups for all treatments. It was observed that the 0.5% concentration of Product along with Comparator treatments showed an increased cell viability in particular, though the difference is statistically non-significant (P>0.05). Based on these results, 0.5% was chosen as the concentration to be tested in the protein expression analysis.

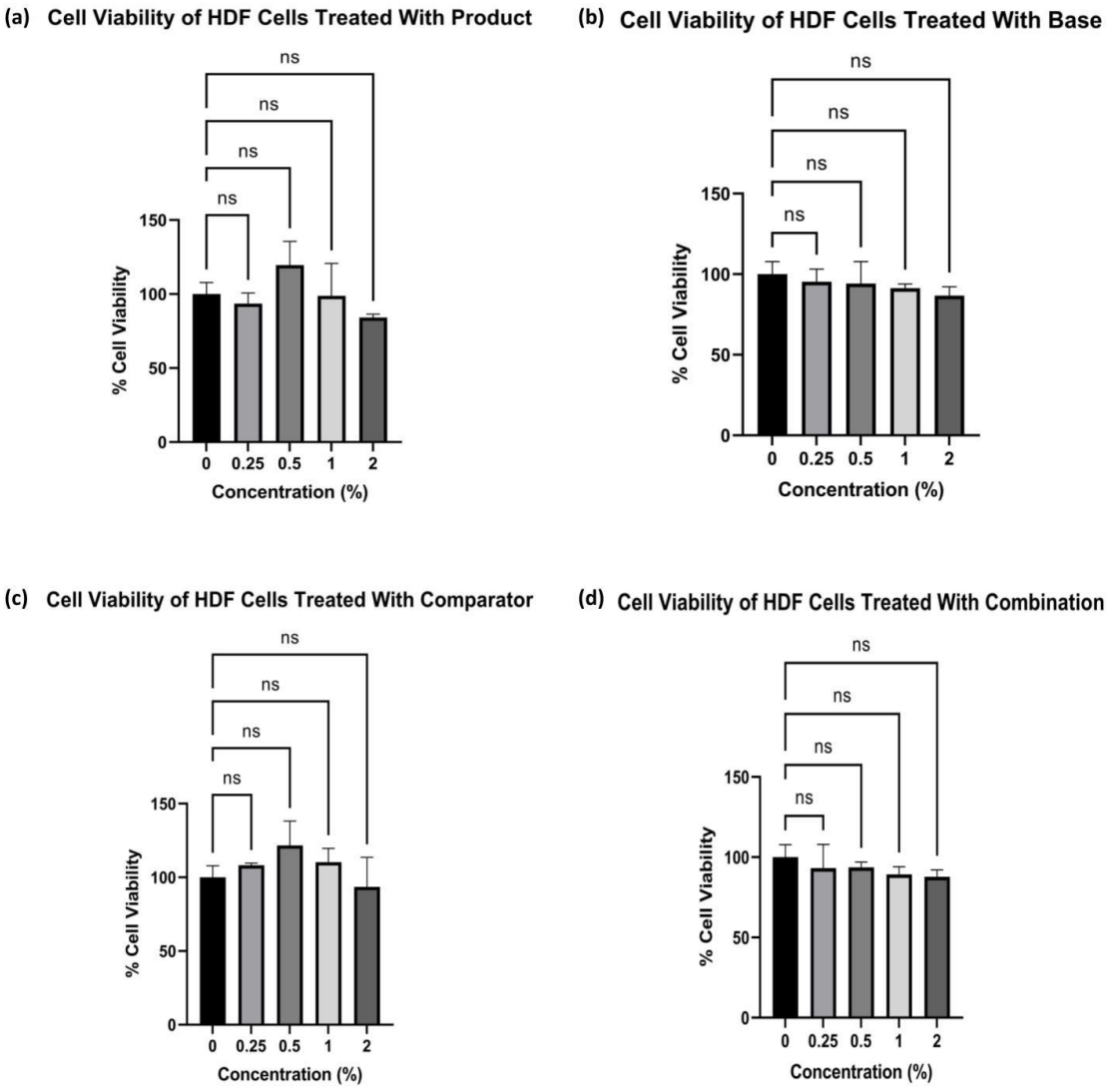


Figure 3.1.1. Effect of Product Treatment in Various Concentrations on HDF Cell Viability. **(a)** Product. **(b)** Base. **(c)** Comparator. **(d)** Combination. Value of ns P>0.05 indicates non-significant difference to Control. (n=3, ANOVA).

3.2. ELISA

3.2.1. Type I Collagen Expression

The treatment of HDF cells with Product, Base, Comparator, and Combination at a concentration of 0.5% through an EPI-200-X skin model were shown to have increased type I collagen in comparison to the untreated Control group (**Figure 3.2.1.1.**). ANOVA analysis indicated that the Comparator group induced a statistically significant increase in concentration, followed by the Base group. Both the Product and Combination groups showed increased concentrations as well, though not statistically significant (P>0.05).

This increase in comparison to the untreated Control indicates that the treatments were able to sufficiently penetrate through the EPI-200-X skin model and stimulate type I collagen production; hence suggesting an anti-aging effect.

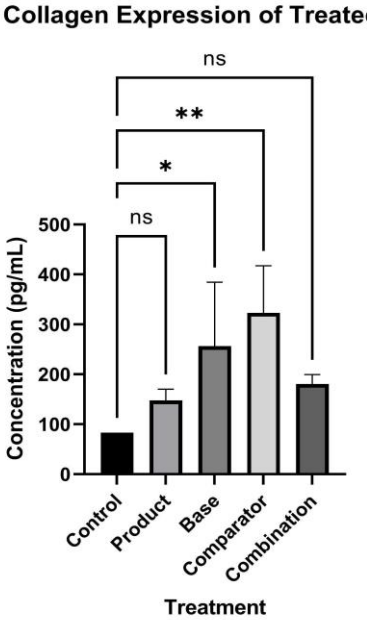


Figure 3.2.1.1. Type I Collagen Expression of Treated HDF Through EPI-200-X Skin Model After Treatment With Product, Base, Comparator and Combination. Value of ns P>0.05 indicates a non-significant difference to Control, while values of *P<0.05 and **P<0.01 indicate significant difference to Control. (n=3, ANOVA).

3.2.2. Type III Collagen Expression

As with the type I collagen expression, the treatment of HDF cells with Product; Base; Comparator; and Combination at a concentration of 0.5% through EPI-200-X Skin Model were shown to have increased type III collagen expression in comparison to the untreated Control group (**Figure 3.2.2.1.**). ANOVA analysis validates that the increased concentrations are statistically significant for the Product, Comparator, and Combination groups; though not statistically significant for the Base group.

This increase in comparison to the untreated Control indicates that the treatments were able to sufficiently penetrate through the EPI-200-X skin model and stimulate type I collagen production; hence suggesting an anti-aging effect.

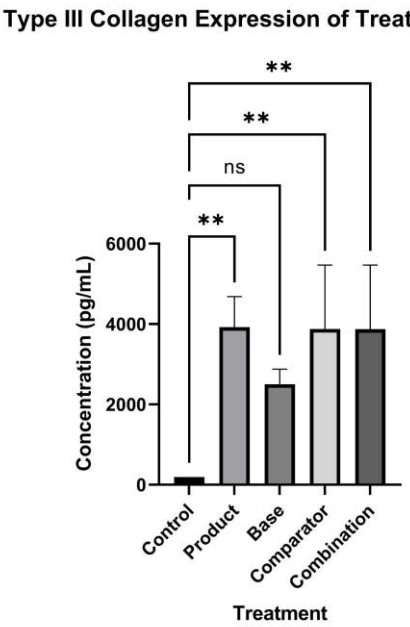


Figure 3.2.2.1. Type III Collagen Expression of HDF Through EPI-200-X Skin Model After Treatment With Product, Base, Comparator and Combination. Value of ns P>0.05 indicates a non-significant difference to Control, while value of **P<0.01 indicate significant difference to Control. (n=3, ANOVA).

4. Conclusion

In conclusion, this study has shown that all treatment groups were non-cytotoxic with >70% cell viability, and the 0.5% concentration showed the highest cell proliferation and lowest cell death; thus it was selected for ELISA treatment. The results of ELISA analysis **showed a significant increase in type III collagen for the product, while the increase in type I collagen was not significant for this study.** Such results suggest that the treatments were able to sufficiently penetrate through the EPI-200-X Skin Model and induce type I (for base and comparator) as well as type III collagen production (for product, comparator and combination) within the dermal layer, which would potentially be reflected during application on human skin.

5. References

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