Journal of Medicine and Health (JMH) **e-ISSN: 2442-5257**
Vol. 8 No. 1 February 2026 **https://doi.org/10.28932/jmh.v8i1.13091**

Research Article

Mesenchymal Stem Cell (MSC) Secretome and Vitamin C Combination Additively Enhance Epidermal Keratinocyte Proliferation and Morphology: Evidence from Intrinsic and Extrinsic Rat Skin Aging Models

Kombinasi Sekretom Sel Punca Mesenkimal (SPM) dan Vitamin C Secara Aditif Meningkatkan Proliferasi dan Morfologi Keratinosit Epidermis: Bukti dari Model Tikus Penuaan Kulit secara Intrinsik dan Ekstrinsik

Olivia Belinda[1], Veronika M Sidharta[2*], Komang A Wahyuningsih[2], Reynelda J Sagala[3], Christania Christania[1], Vallery B Setiawan[1], Nadia A Sjoekri[1], Renata Angelia[1], Laurie Marcella[1]

[1]School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia
[2]Department of Histology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia
[3]Department of Pharmacy, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, Jakarta, Indonesia
Jl. Pluit Raya No.2, RT.21/RW.8, Kecamatan Penjaringan, Kota Administratif Jakarta Utara, DKI Jakarta, 14440, Indonesia
**Correspondence author*
Email: veronika.maria@atmajaya.ac.id

Received: August 19, 2025
Accepted: February 11, 2026

Abstract

Skin aging, driven by intrinsic and extrinsic factors, impairs keratinocyte proliferation and morphology. Mesenchymal stem cell (MSC) secretome and vitamin C possess regenerative and anti-aging properties that may counteract these changes. This study aimed to evaluate the additive effects of MSC secretome and vitamin C on keratinocyte proliferation and morphology in intrinsic and extrinsic rat skin aging models. Thirty-two male Sprague-Dawley rats were randomly divided into eight groups: intrinsic aging control (IAC; D-galactose-induced), extrinsic aging control (EAC; UVB-induced), MSC secretome-treated (IS and ES; 0.2 mL MSC secretome), vitamin C-treated (IC and EC; 0.2 mL vitamin C at 75 µg/mL), and combination-treated (ISC and ESC; 0.1 mL of each treatment). Treatments were administered intradermally once weekly for four weeks. Histological analysis assessed keratinocyte count and size in the stratum granulosum and stratum basale. Combination treatment (ISC, ESC) produced the most pronounced and statistically significant increases in keratinocyte counts and reductions in cell size across both strata ($p < 0.01$ to ≤ 0.001), whereas individual treatments showed variable effects, with most failing to reach statistical significance. These findings suggest that combined MSC secretome and vitamin C treatment produces the most pronounced histological effects in keratinocyte counts and size, likely reflecting complementary actions, and supports its potential as a cell-free anti-aging therapy.

Keywords*: skin aging; keratinocytes; mesenchymal stem cell secretome; vitamin C; in vivo*

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How to Cite:
Belinda O, Sidharta VM, Wahyuningsih KA, Sagala RJ, Christania C, Setiawan VB, et al. Mesenchymal stem cell (MSC) secretome and vitamin C combination additively enhance epidermal keratinocyte proliferation and morphology: evidence from intrinsic and extrinsic rat skin aging models. Journal of Medicine and Health. 2026; 8(1): 56-71. DOI: https://doi.org/10.28932/jmh.v8i1.13091.

Abstrak

Penuaan kulit yang dipengaruhi oleh faktor intrinsik dan ekstrinsik menyebabkan penurunan proliferasi dan perubahan morfologi keratinosit. Sekretom sel punca mesenkimal (SPM) dan vitamin C memiliki sifat regeneratif dan anti-penuaan yang berpotensi mengatasi perubahan tersebut. Penelitian ini bertujuan mengevaluasi efek kombinasi sekretom SPM dan vitamin C terhadap proliferasi dan morfologi keratinosit pada model penuaan kulit intrinsik dan ekstrinsik tikus. Sebanyak 32 tikus jantan Sprague-Dawley dibagi secara acak menjadi delapan kelompok: kontrol penuaan intrinsik (IAC; induksi D-galaktosa), kontrol penuaan ekstrinsik (EAC; induksi UVB), kelompok sekretom SPM (IS dan ES; 0,2 mL sekretom SPM), kelompok vitamin C (IC dan EC; 0,2 mL vitamin C konsentrasi 75 µg/mL), serta kelompok kombinasi (ISC dan ESC; 0,1 mL masing-masing terapi). Perlakuan diberikan secara intradermal satu kali seminggu selama empat minggu. Analisis histologis menilai jumlah dan ukuran keratinosit pada stratum granulosum dan stratum basal. Terapi kombinasi (ISC, ESC) menunjukkan peningkatan jumlah keratinosit dan penurunan ukuran sel yang paling bermakna secara statistik pada kedua stratum ($p < 0,01$ hingga $\leq 0,001$), sedangkan terapi tunggal menunjukkan hasil yang bervariasi dan sebagian besar tidak bermakna. Temuan ini menunjukkan bahwa kombinasi sekretom SPM dan vitamin C menghasilkan efek histologis paling nyata pada jumlah dan ukuran keratinosit, kemungkinan melalui efek komplementer, dan berpotensi sebagai strategi anti-penuaan sel bebas.

Kata kunci: penuaan kulit; keratinosit; sekretom sel punca mesenkimal; vitamin C; *in vivo*

Introduction

With rising global life expectancy, skin aging has become a significant concern due to its impact on both appearance and physiological functions of the skin as the body's largest organ.[1,2] Skin aging results from a combination of intrinsic biological processes and extrinsic factors, particularly ultraviolet (UV) radiation, which disrupts keratinocyte proliferation, differentiation, and apoptosis, ultimately leading to epidermal thinning and increased fragility.[1–3] Mesenchymal stem cell (MSC)-based therapies have attracted growing interest in regenerative medicine, with their secretome, the extracellular bioactive factors secreted by MSCs, being primarily responsible for their regenerative effects. The MSC secretome represents a cell-free therapeutic strategy that is safer, less invasive, scalable, and cost-effective, with low immunogenicity and tumorigenicity. Its safety, dosage, and efficacy can also be evaluated using conventional pharmacological approaches, enhancing its clinical practicality.[4–6] Composed with cytokines, chemokines, and

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growth factors with anti-inflammatory and immunomodulatory properties, the secretome promotes keratinocyte proliferation, differentiation, and migration while inhibiting apoptosis.[5–9] By modulating key skin aging pathways, including oxidative stress, DNA damage, chronic inflammation, microRNA dysregulation, and cellular senescence, the MSC secretome holds promise as an anti-aging intervention.[10]

Vitamin C, a potent water-soluble antioxidant, exerts anti-aging effects by neutralizing free radicals and suppressing inflammation.[11,12] It protects the epidermis by neutralizing reactive oxygen species (ROS), suppressing inflammation, reducing DNA damage and apoptosis, and enhancing keratinocyte proliferation and differentiation.[13,14] Age-related ROS accumulation has been shown to impair MSC viability and function.[11] However, vitamin C supplementation may counteract these effects and promote MSC proliferation, potentially enhancing their regenerative capacity.[15,16]

Despite extensive evidence supporting the individual benefits of MSC secretome and vitamin C, their combined effects on aged keratinocytes remain unexplored. To explore their potential additive effect *in vivo*, intrinsic and extrinsic skin aging were induced in rat models using subcutaneous D-galactose injection and chronic UVB exposure, respectively. D-galactose accelerates intrinsic aging through oxidative stress and mitochondrial DNA damage, while UVB induces extrinsic aging through inflammation, ROS overproduction, and matrix metalloproteinase activation.[6,14,17] This study aims to histologically evaluate the additive effects of MSC secretome and vitamin C on keratinocyte proliferation and morphology by quantifying keratinocyte count and size in the stratum granulosum and stratum basale of intrinsic and extrinsic rat skin aging models.

Methods

Study Design and Animal Model

Healthy 2-month-old male *Sprague-Dawley* rats (200-250 g) were used in this *in vivo* experimental study with a randomized post-test only control group design. This study was conducted from January 2022 to July 2022 at the V-Stem Inovasi Prima Laboratory, Bogor, Indonesia, and the School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia. Ethical approval was granted by the Ethical Clearance Commission, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia (16/11/KEP-FKIKUAJ/2021). All rats were obtained from the Indonesian Food and Drug Authority (Badan Pengawas Obat dan Makanan [BPOM] Republik Indonesia) and housed in plastic cages (50 × 30 × 20 cm) with four rats per cage in a well-ventilated room maintained at 26°C and 75% humidity,

under a 12-hour light/dark cycle, with access to food and water *ad libitum.*[18] After 1-2 weeks of acclimatization, thirty-two rats were randomly divided into eight groups (n= 4 per group) using simple randomization as follows: 1. IAC (Intrinsic-Aging Control); 2. EAC (Extrinsic-Aging Control); 3. IS (Intrinsic + MSC Secretome); 4. ES (Extrinsic + MSC Secretome); 5. IC (Intrinsic + Vitamin C); 6. EC (Extrinsic + Vitamin C); 7. ISC (Intrinsic + MSC Secretome + Vitamin C); 8. ESC (Extrinsic + MSC Secretome + Vitamin C).

Establishment of Rat Skin Aging Model

Intrinsic skin aging models were established by daily subcutaneous injections of D-galactose (1000 mg/kg body weight; Sigma-Aldrich, ≥99%) dissolved in 1 mL of normal saline for 8 weeks on the rat dorsal skin.[19,20] Extrinsic skin aging models were created by exposing the rat dorsal skin to UVB radiation (Philips BPL 13W; 290–320 nm) five times a week for 8 weeks, starting with a dose of 60 mJ/cm² for the first two weeks, then increasing to 120 mJ/cm² in week 3, 180 mJ/cm² in week 4, and 240 mJ/cm² for the remaining weeks, resulting in a total dose of 6.9 J/cm².[21]

Treatment Protocol

One week after establishing the rat skin aging models, treatments were administered once weekly for four weeks. Rats in the MSC secretome-treated groups (IS, ES) received 0.2 mL of MSC secretome per session. Vitamin C-treated groups (IC, EC) received 0.2 mL of vitamin C at 75 µg/mL, a concentration previously shown to promote cell proliferation, support antioxidant activity, and enhance collagen synthesis without cytotoxicity.[16] In the combination groups (ISC, ESC), each agent was administered at 0.1 mL while maintaining the same concentration of vitamin C (75 µg/mL), resulting in a total injection volume of 0.2 mL per session. This design allowed evaluation of whether concurrent administration could achieve enhanced effects through complementary mechanisms, while minimizing individual exposure. Aging control groups (IAC, EAC) received 0.2 mL of phosphate-buffered saline (PBS; Gibco). All treatments were intradermally administered at the D-galactose or UVB exposure sites using a 5-pin multi-needle injector. Considering the maximum intradermal injection volume in rats is 0.05–0.1 mL per site, delivering 0.2 mL via five pins resulted in approximately 0.04 mL per needle, minimizing tissue disruption.[22] The MSC secretome used in this study was derived from human umbilical cord mesenchymal stem cells (hUC-MSC) and obtained from the Stem Cell and Cancer Institute (SCI), Jakarta, Indonesia. Doses were standardized by volume (0.2 mL for IS and ES; 0.1 mL for ISC and ESC), and manufacturer assays identified keratinocyte growth factor (KGF) as a major

component (92 pg/mL). The vitamin C (Mesoestetic x.prof 020, 20%) obtained from PT. Herca Cipta Dermal Perdana was diluted in water to achieve 75 µg/mL. Rats were euthanized 24 hours after the final treatment via intraperitoneal injection of ketamine (100 mg/kg BW; KET-A-100 Agrovet, 10%) and xylazine (10 mg/kg BW; XylaHolland, 2%). Following euthanasia, dorsal skin samples (1 × 1 cm, ±2 mm thick) around the injection sites were collected from all groups for analysis.

Skin Histological Analysis

All collected skin samples were fixed in 10% neutral buffered formalin for 48 hours at room temperature, dehydrated through graded ethanol concentrations, cleared with xylene, embedded in paraffin, and sectioned at 5 µm thickness. After deparaffinization, sections were stained with hematoxylin and eosin (H&E) and examined under a binocular microscope (Olympus, Japan) equipped with a digital camera at 400× magnification. Histological analysis of keratinocyte proliferation and morphology was limited to quantitative assessment of keratinocyte count and size, along with qualitative evaluation of cellular appearance on H&E staining, within the stratum granulosum and stratum basale, which are clearly distinguishable in rat epidermis.[23] Keratinocyte counts were performed using standardized counting rules within two fixed 80 × 80 µm areas parallel to the epidermal groove. Cells intersecting the image boundary were included only when more than 50% of the cell body was present within the counting frame. This fixed-area approach was applied to minimize bias related to variations in epidermal thickness and length. Cell size was measured from five randomly selected keratinocytes in the stratum granulosum and ten in the stratum basale per field. All measurements were performed digitally using ImageJ software by an investigator blinded to experimental group allocation.

Statistical Analysis

All results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using IBM SPSS software version 22.0. Data normality was assessed using the Shapiro–Wilk test, and homogeneity of variance was evaluated with Levene's test. Normally distributed data with homogeneous variance were analyzed using one-way Analysis of Variance (ANOVA) test, whereas data with non-normal distribution and/or heterogeneity of variance were analyzed using the Kruskal-Wallis test. A p-value < 0.05 was considered statistically significant. When overall significance was detected, post hoc analyses were conducted to identify specific group differences using Tukey's HSD test for ANOVA and pairwise Mann–Whitney U tests with

Bonferroni correction for Kruskal–Wallis comparisons, with the adjusted significance level set at $p < 0.05$ divided by the number of comparisons.

Results

Treatment Effect on Keratinocyte Count in Intrinsic Skin Aging Model

Epidermal keratinocyte counts were histologically evaluated across all groups of the intrinsic skin aging model (Figure 1). Counts were averaged from five representative fields per slide, showing consistent trends across specimens. Histological examination revealed a thicker epidermis with more densely arranged keratinocytes in treated groups, particularly the combination group (ISC), whereas the control group (IAC) exhibited a thinner epidermis with fewer and more flattened keratinocytes (Figure 1). Keratinocyte counts differed significantly among groups in both the stratum granulosum (one-way ANOVA, $p = 0.001$) and stratum basale (Kruskal–Wallis, $p = 0.016$) (Table 1). In both strata, all treated groups (IS, IC, ISC) demonstrated higher keratinocyte counts compared with the control (IAC), with the combination treatment (ISC) showing the highest numerical values. Statistical significance was observed for all treatments in the stratum granulosum, but only for the ISC group in the stratum basale (Table 1, Figure 2). Post hoc analysis revealed no significant differences between the combination group (ISC) and the individual treatment groups (IS, IC) in either strata (Figure 2).

Table 1 Mean Keratinocyte Count and Size Across All Experimental Groups

Groups	Mean Keratinocyte Count (cells/field)		Mean Keratinocyte Size (µm²)	
	Stratum Granulosum	Stratum Basale	Stratum Granulosum	Stratum Basale
Intrinsic Skin Aging Model				
IAC	5.55 ± 0.62	15.3 ± 1.28	107.45 ± 14.68	92.57 ± 7.09
IS	8.2 ± 1.30	17.95 ± 0.97	95.66 ± 9.48	90.12 ± 1.94
IC	8 ± 1.52	18.45 ± 2.38	82.32 ± 6.27	83.41 ± 5.76
ISC	8.9 ± 0.84	21.05 ± 1.30	69.37 ± 3.88	68.10 ± 3.46
p-value	0,001*	0,016#	0,007#	0,000*
Extrinsic Skin Aging Model				
EAC	4.35 ± 0.60	15.1 ± 1.16	108.95 ± 13.28	84.03 ± 5.17
ES	6.7 ± 2.61	18.1 ± 1.32	84.96 ± 12.15	80.68 ± 6.49
EC	7.5 ± 0.82	19.15 ± 1.90	81.11 ± 6.92	71.24 ± 9.51
ESC	7.8 ± 1.80	20.5 ± 1.47	70.06 ± 5.06	64.63 ± 2.30
p-value	0,042#	0,001*	0,007#	0,003*

Notes: IAC (Intrinsic-Aging Control); IS (Intrinsic + MSC Secretome); IC (Intrinsic + Vitamin C); ISC (Intrinsic + MSC Secretome + Vitamin C); EAC (Extrinsic-Aging Control); ES (Extrinsic + MSC Secretome); EC (Extrinsic + Vitamin C); ESC (Extrinsic + MSC Secretome + Vitamin C). For each slide, five fields were analyzed; keratinocyte counts were quantified in two 80 × 80 µm areas, and cell size was measured from five keratinocytes in the stratum granulosum and ten in the stratum basale per field using ImageJ software. Data are presented as mean ± SD (n = 4 per group).*: $p < 0.05$ by one-way ANOVA test; #: $p < 0.05$ by Kruskal–Wallis test.

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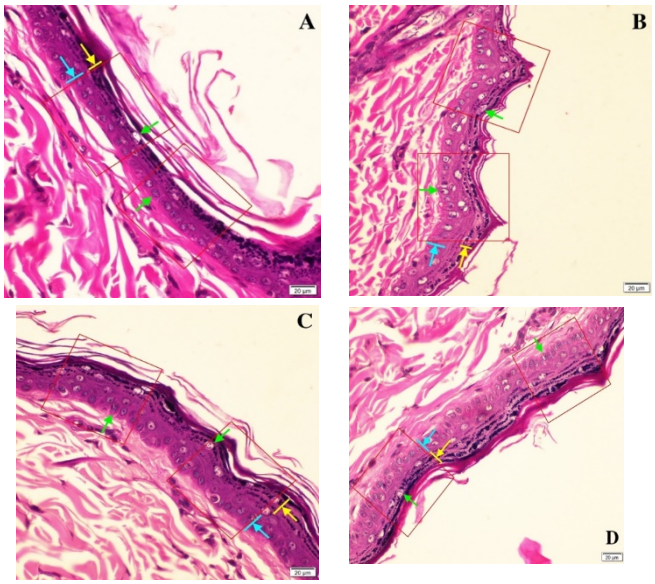

Figure 1 Histological appearance of rat skin epidermis from the intrinsic skin aging model

(**A)** IAC (Intrinsic-Aging Control) Group; **(B)** IS (Intrinsic + MSC Secretome) Group ; **(C)** IC (Intrinsic + Vitamin C) Group; **(D)** ISC (Intrinsic + MSC Secretome + Vitamin C) Group. Representative images of sections stained with H&E were presented at 400x magnification, showing the number of keratinocytes (green arrow) in the stratum granulosum (yellow arrow) and basale (blue arrow); scale bar = 20 µm.

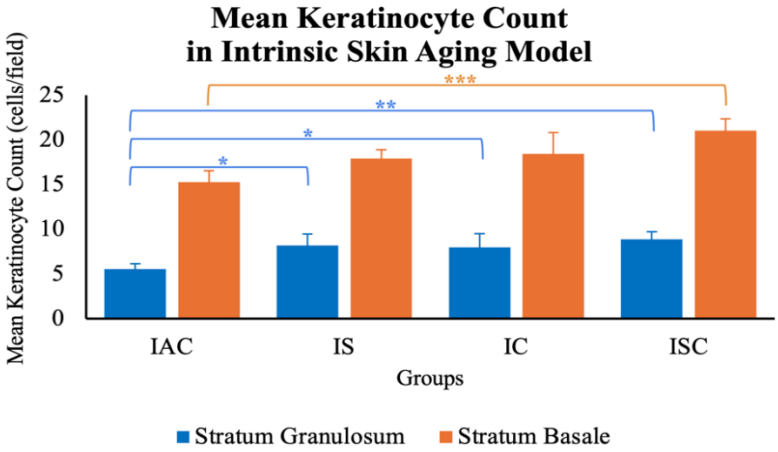

Figure 2 Effects of Treatments on Keratinocyte Counts in Intrinsic Skin Aging Model

Notes: IAC (Intrinsic-Aging Control); IS (Intrinsic + MSC Secretome); IC (Intrinsic + Vitamin C); ISC (Intrinsic + MSC Secretome + Vitamin C).

Data are presented as mean ± SD (n = 4 per group). Statistical significance was determined using one-way ANOVA followed by Tukey's HSD test for the Stratum Granulosum, and Kruskal-Wallis followed by pairwise Mann-Whitney U test for the Stratum Basale. *: $p < 0.05$, **: $p < 0.01$, ***: $p \leq 0.001$ versus control group (IAC).

Treatment Effect on Keratinocyte Size in Intrinsic Skin Aging Model

Epidermal keratinocyte size was histologically evaluated across all groups in the intrinsic skin aging model (Figure 3). Measurements averaged from five representative fields per slide showed consistent trends across specimens. Histological examination demonstrated smaller and more regularly shaped keratinocytes in the treated groups compared with larger, more irregular keratinocytes in the control group (Figure 3). Keratinocyte size differed significantly among

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groups in both the stratum granulosum (Kruskal–Wallis, p = 0.007) and stratum basale (one-way ANOVA, p = 0.000) (Table 1). In the stratum granulosum, significant reductions in keratinocyte size were observed in the IC and combination (ISC) groups compared with the control (IAC), whereas the IS group showed a non-significant reduction. In the stratum basale, a significant size reduction was observed only in the ISC group (Table 1, Figure 4). Post hoc analysis further showed that the combination group (ISC) exhibited significantly smaller keratinocytes than individual treatments (IS, IC) in both strata (Figure 4).

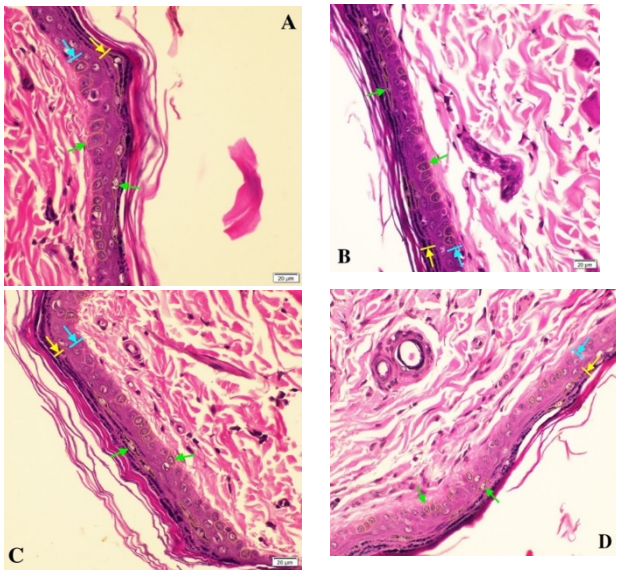

Figure 3 Histological appearance of rat skin epidermis from the intrinsic skin aging model
(A) IAC (Intrinsic-Aging Control) Group; **(B)** IS (Intrinsic + MSC Secretome) Group; **(C)** IC (Intrinsic + Vitamin C) Group; **(D)** ISC (Intrinsic + MSC Secretome + Vitamin C) Group
Notes: Representative images of sections stained with H&E were presented at 400x magnification, showing the size of keratinocytes (green arrow) in the stratum granulosum (yellow arrow) and basale (blue arrow); scale bar = 20 µm.

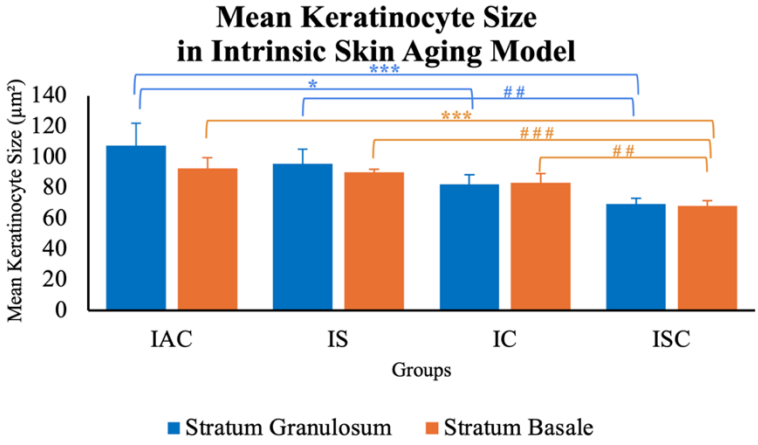

Figure 4 Effects of Treatments on Keratinocyte Size in Intrinsic Skin Aging Model
Notes: IAC (Intrinsic-Aging Control); IS (Intrinsic + MSC Secretome); IC (Intrinsic + Vitamin C); ISC (Intrinsic + MSC Secretome + Vitamin C).
Data are presented as mean ± SD (n = 4 per group). Statistical significance was determined using Kruskal-Wallis followed by pairwise Mann-Whitney U test for the Stratum Granulosum, and one-way ANOVA followed by Tukey's HSD test for the Stratum Basale. *: $p < 0.05$, **: $p < 0.01$, ***: $p \leq 0.001$ versus control group (IAC); # #: $p < 0.01$, # # #: $p \leq 0.001$ for comparison among treatment groups.

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Treatment Effect on Keratinocyte Count in Extrinsic Skin Aging Model

Epidermal keratinocyte counts were histologically evaluated across all groups of the extrinsic skin aging model (Figure 5). Counts averaged from five representative fields per slide showed consistent patterns across samples. Histological examination revealed a thicker epidermis with more densely packed keratinocytes in the treated groups, whereas the control group (EAC) exhibited a thinner epidermis with fewer and more flattened cells (Figure 5). Keratinocyte counts differed significantly among groups in both the stratum granulosum (Kruskal–Wallis, $p = 0.042$) and stratum basale (one-way ANOVA, $p = 0.001$) (Table 1). In both strata, treated groups showed higher keratinocyte counts than controls, with the combination (ESC) group displaying the highest numerical values. In the stratum granulosum, significant increases were observed in the EC and ESC groups, whereas in the stratum basale, statistical significance was observed only in the combination group (Table 1, Figure 6). Post hoc analysis revealed no significant differences between ESC and the individual treatments (ES and EC) in either stratum (Figure 6).

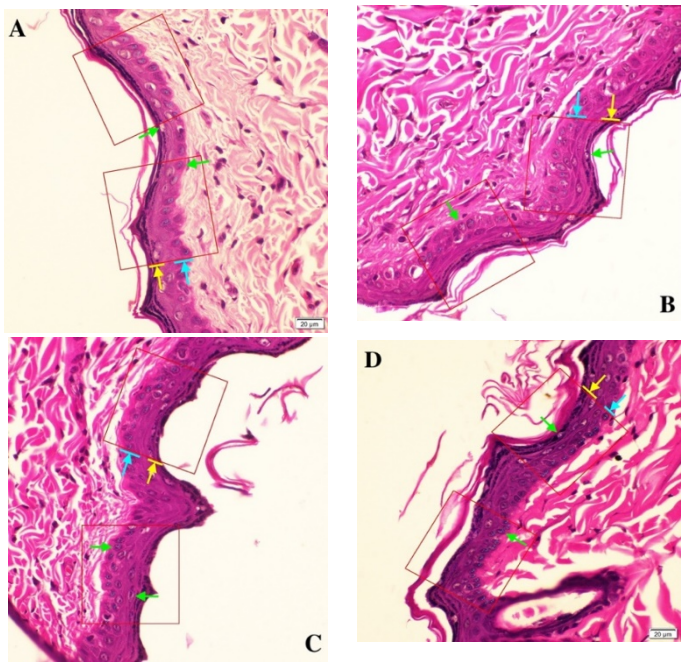

Figure 5 Histological appearance of rat skin epidermis from the extrinsic skin aging model
(A) EAC (Extrinsic-Aging Control) Group; **(B)** ES (Extrinsic + MSC Secretome) Group ; **(C)** EC (Extrinsic + Vitamin C) Group; **(D)** ESC (Extrinsic + MSC Secretome + Vitamin C) Group
Notes: Representative images of sections stained with H&E were presented at 400x magnification, showing the number of keratinocytes (green arrow) in the stratum granulosum (yellow arrow) and basale (blue arrow); scale bar = 20 µm.

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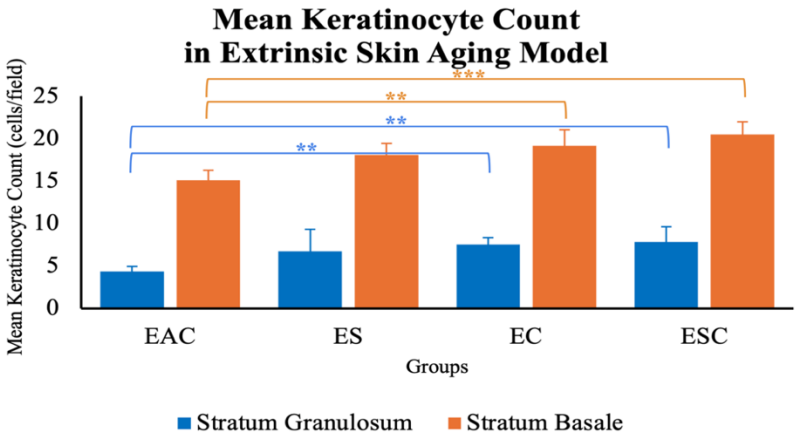

Figure 6 Effects of Treatments on Keratinocyte Counts in Extrinsic Skin Aging Model

Notes: EAC (Extrinsic-Aging Control); ES (Extrinsic + MSC Secretome); EC (Extrinsic + Vitamin C); ESC (Extrinsic + MSC Secretome + Vitamin C)

Data are presented as mean ± SD (n = 4 per group). Statistical significance was determined using Kruskal-Wallis followed by pairwise Mann-Whitney U test for the Stratum Granulosum, and one-way ANOVA followed by Tukey's HSD test for the Stratum Basale. *: $p < 0.05$, **: $p < 0.01$, ***: $p \leq 0.001$ versus control group (EAC).

Treatment Effect on Keratinocyte Size in Extrinsic Skin Aging Model

Epidermal keratinocyte size was histologically evaluated across all groups of the extrinsic skin aging model (Figure 7). Measurements averaged from five representative fields per slide showed consistent trends across samples. Histological examination revealed smaller and more uniformly shaped keratinocytes in the treated groups compared with larger and more irregular cells in the control group (EAC) (Figure 7). Keratinocyte size differed significantly among groups in both the stratum granulosum (Kruskal–Wallis, $p = 0.007$) and stratum basale (one-way ANOVA, $p = 0.003$) (Table 1). In the stratum granulosum, significant size reductions were observed in the EC and combination (ESC) groups compared with the control (EAC), whereas the ES group showed a non-significant reduction. In the stratum basale, a significant decrease in keratinocyte size was observed only in the ESC group (Table 1, Figure 8). Post hoc analysis indicated that keratinocytes in the ESC group were significantly smaller than those in the ES group in the stratum basale, with no other significant differences between the combination and individual treatment groups in either stratum (Figure 8).

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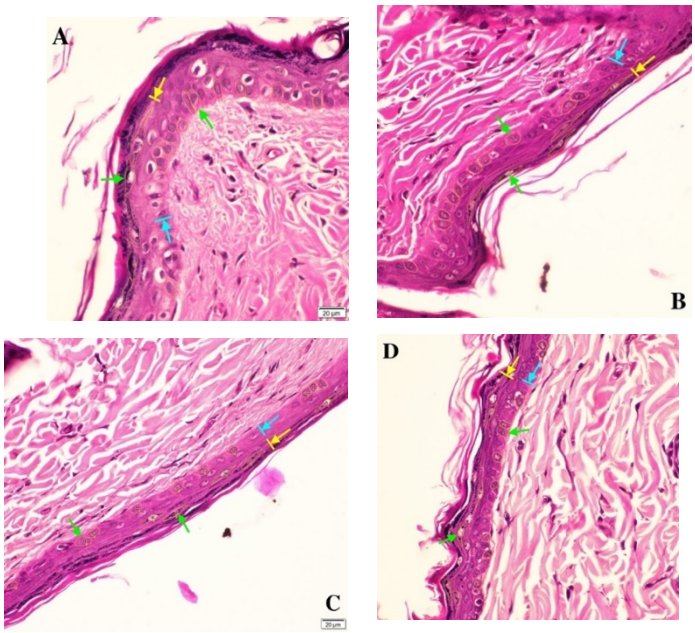

Figure 7 Histological appearance of rat skin epidermis from the extrinsic skin aging model
(**A)** EAC (Extrinsic-Aging Control) Group; **(B)** ES (Extrinsic + MSC Secretome) Group ; **(C)** EC (**Extrinsic** + Vitamin C) Group; **(D)** ESC (Extrinsic + MSC Secretome + Vitamin C) Group
Notes: Representative images of sections stained with H&E were presented at 400x magnification, showing the size of keratinocytes (green arrow) in the stratum granulosum (yellow arrow) and basale (blue arrow); scale bar = 20 µm.

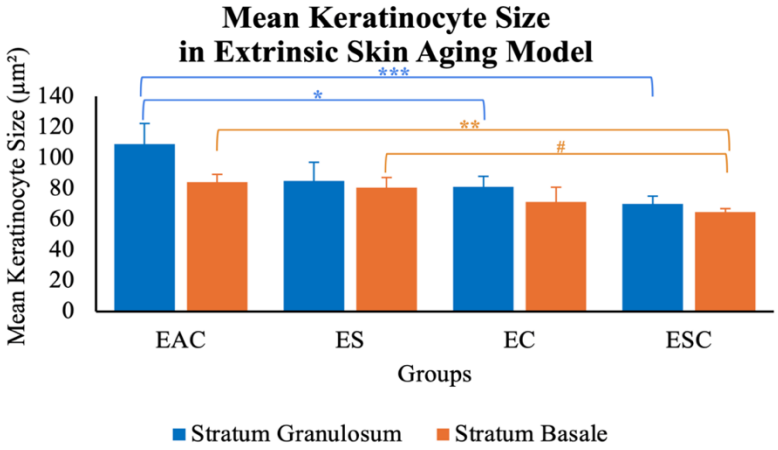

Figure 8 Effects of Treatments on Keratinocyte Size in Extrinsic Skin Aging Model
Notes: EAC (Extrinsic-Aging Control); ES (Extrinsic + MSC Secretome); EC (Extrinsic + Vitamin C); ESC (Extrinsic + MSC Secretome + Vitamin C)
Data are presented as mean ± SD (n = 4 per group). Statistical significance was determined using Kruskal-Wallis followed by pairwise Mann-Whitney U test for the Stratum Granulosum, and one-way ANOVA followed by Tukey's HSD test for the Stratum Basale. *: $p < 0.05$, **: $p < 0.01$, ***: $p \leq 0.001$ versus control group (EAC); #: $p < 0.05$ for comparison among treatment groups.

Discussion

One promising strategy to prevent skin aging is maintaining the epidermal integrity, as it plays a vital role as a skin barrier. Skin aging is a complex process driven by intrinsic and extrinsic factors. Intrinsic aging, mediated by cellular senescence, oxidative stress, and mitochondrial damage, reduces keratinocyte proliferation, increases apoptosis, and impairs epidermal turnover,

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resulting in fewer but enlarged cells, while disrupted cytokinesis and accumulated intracellular debris further enlarge keratinocytes.[1,24,25] Extrinsic aging, primarily from chronic UV exposure, induces DNA damage, elevates ROS, and alters cytokines and growth factors, leading to keratinocyte loss, impaired differentiation, and compensatory epidermal hypertrophy.[1,26–28] In this study, intrinsic and extrinsic aging were modeled in 32 healthy male Sprague-Dawley rats using subcutaneous D-galactose injections and UVB exposure, respectively, both previously validated as reliable aging models.[19–21] Prior studies using the same D-galactose regimen reported increased malondialdehyde (MDA) levels and decreased superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), indicative of enhanced oxidative stress and impaired antioxidant defense, accompanied by histopathological features of aged skin, including loosely arranged epidermal layers and accumulation of advanced glycation end products (AGEs).[19,20] Similarly, the applied UVB protocol has been shown to induce a significant 4.23-fold increase in epidermal thickness, upregulate matrix metalloproteinase (MMP)-1 and MMP-3, promote keratinocyte enlargement and irregular morphology, and significantly increase senescence-associated β-galactosidase (SA-β-gal)-positive, a recognized biomarker of cellular senescence.[21] Consistent with these established mechanisms, the intrinsic (IAC) and extrinsic (EAC) aging control groups in the present study exhibited the lowest keratinocyte counts and the largest cell size relative to normal controls, findings that align with the expected morphological features of aged skin (Table 1; Figures 2, 4, 6, and 8).

MSC secretome and vitamin C have been extensively studied for their skin anti-aging effects. As a bioactive molecule of MSC, the MSC secretome is rich in anti-inflammatory cytokines (TGF-β, HGF, IDO-1, IL-1, IL-6, IL-10) and growth factors (FGF-6, FGF-7, HGF-1, PDGF, EGF, IGF), supporting regenerative potential in skin aging.[5,6] Vitamin C is a potent antioxidant that mitigates oxidative stress, inhibiting MMP-1 expression and promoting TIMP synthesis, particularly relevant in extrinsic aging.[12] In this study, we investigated their individual and combined effects on keratinocyte count and size in intrinsic and extrinsic skin aging models. In the intrinsic skin aging model, combination therapy (ISC) significantly increased keratinocyte counts in both the stratum granulosum ($p < 0.01$) and stratum basale ($p \leq 0.001$), whereas individual treatments (IS and IC) reached significance only in the stratum granulosum (Figure 2). Similarly, in the extrinsic skin aging model, ESC significantly enhanced keratinocyte counts in both strata (stratum granulosum, $p < 0.01$; stratum basale, $p \leq 0.001$), while ES and EC individually showed trends toward improvement, with EC significant only in the stratum granulosum (Figure 6). Regarding keratinocyte size, the intrinsic skin aging model demonstrated that combination therapy (ISC) significantly reduced keratinocyte size in both the stratum

granulosum and stratum basale ($p \leq 0.001$ each), whereas only individual treatments with vitamin C (IC) reached significance in the stratum granulosum (Figure 4). In the extrinsic skin aging model, ESC significantly decreased keratinocyte size in both strata (stratum granulosum, $p \leq 0.001$; stratum basale, $p < 0.01$), while individual treatments (ES and EC) showed trends toward reduction, with EC significant only in the stratum granulosum (Figure 8). These findings align with prior studies showing the MSC secretome protects keratinocytes from oxidative stress, inflammation, and DNA damage, and promotes proliferation, differentiation, and migration via growth factor stimulation and PI3K/Akt pathway activation.[7–9] Vitamin C similarly protects keratinocytes from apoptosis and oxidative damage while promoting keratinocyte proliferation and differentiation through antioxidant-mediated pathways.[13,14]

Our results indicate that MSC secretome and vitamin C independently improve keratinocyte proliferation and reduce cell size. However, the limited significance of individual treatments may reflect suboptimal dosing. The MSC secretome was administered at an experimentally optimized dose, which may have been insufficient for robust *in vivo* effects, whereas excessive concentrations could potentially induce adverse biomolecular outcomes.[13,14,29] The vitamin C concentration used for *in vivo* administration (75 µg/mL) was extrapolated from *in vitro* studies identifying this dose as optimal for promoting stem cell proliferation, antioxidant capacity, and collagen and VEGF expression without cytotoxic effects.[30] Nevertheless, because this concentration was derived from *in vitro* conditions, its stability and biological effectiveness *in vivo* remain uncertain, particularly given the known instability and potential dose-dependent toxicity of vitamin C.[31,32] Across both intrinsic and extrinsic aging models, the stratum basale exhibited a more limited treatment response than the stratum granulosum, likely reflecting suboptimal bioactive penetration and intrinsic layer-specific biology.[33,34] Suprabasal keratinocytes are more responsive due to their active roles in differentiation and barrier remodeling, whereas basal keratinocytes constitute a tightly regulated stem and progenitor compartment with slower turnover and limited short-term morphological change.[34,35] Moreover, H&E-based histomorphometry primarily captures structural alterations and may be less sensitive to early functional or proliferative changes in the basal layer. Given its key role in epidermal homeostasis and heightened susceptibility to aging,[1] future strategies should prioritize targeting deeper epidermal layers.

On the other hand, combination therapy (ISC, ESC) consistently yielded the most significant improvements, suggesting that vitamin C may potentiate the regenerative capacity of MSC secretome. Notably, each component in the combination was administered at a half-dose regimen, yet additive effects were still observed, consistent with previous *in vitro* evidence that

optimal vitamin C supplementation can optimize secretome biological activity,[16] suggesting its potential to promote keratinocyte proliferation and differentiation for epidermal regeneration.

Post hoc analyses provided further insight into the comparative effects of combination versus individual treatments, revealing that combination therapy produced the largest increases in keratinocyte counts, but differences from individual treatments were not significant in either intrinsic (ISC vs. IS, IC) or extrinsic (ESC vs. ES, EC) skin aging models (Figures 2 and 6). In contrast, combination therapy significantly reduced keratinocyte size compared with individual treatments in the stratum granulosum (ISC vs. IS, $p < 0.01$) and stratum basale (ISC vs. IS, $p \leq 0.001$; ISC vs. IC, $p < 0.01$) of the intrinsic skin aging model, and in the stratum basale (ESC vs. ES, $p < 0.05$) of the extrinsic skin aging model (Figures 4 and 8). Although combination treatment did not significantly increase keratinocyte counts beyond those achieved by individual treatments, it produced a pronounced reduction in cell size, suggesting that its primary effect is on keratinocyte morphology rather than proliferation. The decrease in cell size reflects additive complementary benefits, promoting keratinocyte maturation, improved epidermal organization, and functional optimization, rather than cellular stress or premature senescence, providing a biologically plausible explanation for this paradoxical effect. Vitamin C and MSC secretome appear to act through complementary mechanisms, with vitamin C enhancing proliferation and antioxidant defense, and MSC secretome supporting growth factor–mediated tissue regeneration.

To our knowledge, this is the first study to evaluate the combined effects of MSC secretome and vitamin C on epidermal keratinocytes in aged rat skin. These findings demonstrate that combination therapy yields superior outcomes by selectively improving keratinocyte morphology while maintaining cell counts, highlighting its potential as a targeted strategy to mitigate epidermal aging. Nevertheless, several limitations should be noted: the rat model cannot fully replicate human skin physiology; The limited group size ($n = 4$) may constrain sensitivity to subtle effects and inadequately capture biological variability; and dosing regimens were based on preliminary or *in vitro* data, which may not represent optimal therapeutic concentrations *in vivo*. Furthermore, the mechanisms underlying the combination's effects remain unclear, as this study was limited to histological assessments. Future research should involve larger cohorts, optimized dosing, and complementary *in vitro* and clinical models to validate the safety, efficacy, and underlying additive mechanisms in human skin, utilizing functional readouts, assessment of ROS levels, and key growth factors to confirm the additive effects on epidermal keratinocytes.

Research Article

Conclusion

This study demonstrates that MSC secretome and vitamin C, administered individually or in combination, exert model-specific effects on keratinocytes in intrinsic and extrinsic rat skin aging models. Vitamin C consistently increased keratinocyte counts and reduced cell size, whereas the MSC secretome selectively promoted keratinocyte proliferation in the intrinsic skin aging model. Combination therapy produced the most pronounced histological improvements in keratinocyte counts and size, likely reflecting complementary actions. The MSC secretome, with keratinocyte growth factor (KGF) as a major component, likely contributed to these effects, although its full biomolecular profile remains to be characterized. These findings highlight the potential of MSC secretome and vitamin C, particularly in combination, as a cell-free strategy to improve aged epidermal structure and function. Future studies are warranted to validate safety, efficacy, and underlying additive mechanisms in human skin through assessment of functional readouts, oxidative stress markers, and key molecular mediators relevant to skin aging.

Acknowledgement

The authors thank V-Stem Inovasi Prima Laboratory, Bogor, and the Laboratory of Anatomical Pathology, School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, for providing research facilities, and gratefully acknowledge the School of Medicine and Health Sciences, Atma Jaya Catholic University of Indonesia, for funding support.

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