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SPACE-Peptide succesfully improves dermis penetration of amniotic membrane stem cell metabolite product as antiaging

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ABSTRACT: Amniotic Membrane Stem Cell Metabolite Product (AMSCMP) is a totipotent stem cell derived metabolite with contains protein size of 75.33 kDa so it is difficult to penetrate the dermis. Skin Penetrating and Cell Entering (SPACE) proven to facilitate protein molecule penetration. The advantage of SPACE its reversible and non irritating. This study was to assess the parameters of successful penetration and the level of skin irritation after AMSCMP was added with SPACE Peptide. The method used is in vivo research using test animals, then penetration analysis is seen from measuring the depth of staining, while the penetration test looks at the changes that occur in the skin after the sample is applied in the form of edema, PMN and degenerative. Penetration tests showed differences in color fluorescence before and after the addition of SPACE peptide. This indicates that the parts of the mouse skin that exhibited color fluorescence had better penetration (F2, F3, F4) compared to those that did not fluoresce (F1). The F1 measurement results did not have a penetration value, while F2, F3, and F4 had values of more than 1200 micrometers. The irritation test showed that F1 and F2 had no irritation index (zero) with edema, polymorphonuclear, and degenerative parameters, while F3 and F4 had a value of 1.3 from the average results with a very mild irritation category. The study results demonstrated that the addition of SPACE significantly increased the penetration of the formulation. Based on statistical analysis using SPSS, the penetration parameter differed significantly among the formulas ($p<0.05$), indicating that the addition of SPACE had a significant effect on enhancing the penetration of the active ingredient. In contrast, the irritation test results showed that the addition of SPACE caused irritation in the very mild category. Nevertheless, statistical analysis revealed no significant difference in irritation scores among the formulas ($p > 0.05$), suggesting that the addition of SPACE did not significantly affect the degree of irritation caused.

KEYWORDS: AMSCMP; irritation; penetration; SPACE-Peptide.

INTRODUCTION

Amniotic Membrane Stem Cell Metabolite Product (AMSCMP) is a metabolite derived from totipotent stem cell cultured in a conditioned medium. This process produces a bioactive metabolite rich ini various growth factors and cytokines. One of the majör bioactive components of AMSCMP is Transforming Growth Factor- β (TGF- β) whic plays a crucial role in skin rejuvenation. TGF- β stimulates dermal fibroblast proliferation and enhances the synthesis of collagen, particularly types I and III, thereby improving the structural integrity and protective function of the skin 1,2. Futhermore TGF-β promotes the synthesis of laminin V, collagen IV, and collagen VII while ihibiting the activity of Matrix Metalloproteinases (MMPs), thereby reducing extracellulaer matrix degradation. Previous studies have demonstrated that TGF- β contributes to maining healthy and youthful skin by preserving fibroblast density within the dermiş. Despite these therapeutic benefits, the clinical application of AMSCMP remains callenging because it contains protein with a molecular weight aof approximately 75.33 kDa, which limits their ability to penetrate the dermal layer aeffectively 3, 4,5, 6. Achieving optimal therapeutic outcomes required efficient transdermal delivery of active compounds across the skin barrier.to address this challenge, considerable effort have been

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directed toward improving the penetration of active ingredients, thereby enhancing the bioavaibility and therapeutic efficacy of topical formulations. However, successful transdermal drug delivery depends on several critical factors, including formulation optimizaation to accommodate regional variation in skin properties, the complex structure and physiology of the skin, physiological changed induced by environmental exposure or pathological conditions, and interindividual variability. These factors collectively influence the penetration efficiency an overall therapeutic performance of transdermal delivery systems 7

Skin Penetrating and Cell Entering (SPACE) is known to increase the penetration of protein molecules into the skin, inducing changes in the alpha helix and beta sheet folds of these structure 8,9. The advantage of SPACE it is reversible its reversible and non irritating nature ti the skin. With the addition of SPACE, the AMSCMP mechanism can by pass the route of directing its effects on the intracellular pathway by loosening the secondary structure through polypeptide bonds stabilized by C-O and N-H groups 8. After passing through the stratum corneum, AMSCMP interacts with specific receptors on keratinocytes and triggers a cytokine signaling cascade that affects fibroblasts and other cells in the dermis, thereby impacting collagen density 10. This study carried out there stages of evaluation, penetration and irritation testing as safety standards.

Improvements in skin texture, appearance, and psychological well-being have contributed to the growing use of cosmetic and personal care products across both genders. However, increased usage has led to a higher incidence of unwanted effects, mainly mild to moderate skin irritation, with severe reactions occurring in sensitive populations such as children. Although cosmetic ingredients undergo skin irritation testing before marketing, testing of final formulations is not mandatory for manufacturers 11

Amniotic membrane stem cell metabolite product (AMSC-MP) contains a variety of growth factors, cytokines, and other bioactive biomolecules with potential antiaging benefits, particularly in promoting skin rejuvenation and improving age-related skin changes. Previous clinical and topical studies have reported the beneficial effects of AMSC-MP or conditioned medium on photoaging-associated parameters. Nevertheless, the topical delivery of AMSC-MP remains challenging because many of its active constituents are hydrophilic macromolecules with high molecular weights, which hinder their penetration through the stratum corneum. Consequently, despite its promising biological potential, the effective translation of AMSC-MP into topical dosage forms is still limited by poor skin penetration[12]. This is the latest research to increase penetration of the active ingredients AMSCMP which is added with a SPACE Peptide enhancer to deliver therapeutic target as antiaging.

▪ MATERIALS AND METHODS

Materials

AMSCMP was collected from the tissue bank of Dr.Soetomo Hospital, Surabaya, Indonesia. Etanol 96% (Merck, Sumber ilmiah persada), Skin Penetrarting and Cell entering/SPACE (Sigma aldrich, USA), Phenoxyethanol (Pt multiverse anugerah chemindo), Mice (Mus Musculus) atrain balb/c, 6-8 week, weight 20-25 grams, no skin disease, no open wounds and no wounds after the mices hair was shaved.

Tools

Olympus FX-100 Fluorosence microscope and confocal miscroscope.

Methods

Animals conditioning

Male BALB/c mice aged 6–8 weeks were used in this study and were sourced from the Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia. The animals were maintained under supervised conditions in a controlled holding facility, with a 24 hour light cycle (lights on from 8:00 a.m. to 8:00 p.m.), ambient temperature of 28 ± 2°C, and relative humidity of 75±5%. The mice were housed in transparent plexiglass cages and given one week to acclimate to the cage environment before the experiment began. They received standard food and air without restriction. All measures to ensure animal comfort were kept to a minimum throughout the procedure.

All experiments complied with guidelines for the care and use of laboratory animals and received approval from the Ethics Committee of the Faculty of Veterinary Medicine, Airlangga University, Surabaya, Indonesia. In this study, we attempted to use the lowest possible number of animals and irritants with ethical clearence No.2.KE.088.04.2019.

Formula of AMSCMP

As much 25 mg of SPACE-Peptide in powder form was dissolved in 1000 ml of Phosphate Buffer Saline (PBS). AMSCMP was added to PBS containing the desired amount of SPACE in the formula, then Phenoxyethanol was added as a preservative, then shaken.

Table 1. Formula of AMSCMP with SPACE Peptide.

Material	Function	Formula (%)			
		1	2	3	4
AMSCMP	Active ingredients	0.008	0.008	0.008	0.008
SPACE	Enhancer	0	0.008	0.016	0.024
Phenoxyethanol	Preservatif	0.5	0.5	0.5	0.5
PBS	solvent	100	100	100	100

*AMSCMP (freeze dried form)
*PBS (Phosphat Buffer Saline)

In vivo penetration test

Using an Olympus FX-100 microscope, 1.25 mg ketamine anesthetic solution was injected intraperitorially to render the mice unconscious. The mice's hair was then shaved on their backs, measuring 2 x 2 cm, and then the sample was applied. Each test was carried out at 1, 3, and 5 hours, then the mice were sacrificed by dislocation. The sample-applied area was cut, the tissue was washed using absolute alcohol, and embedded into a 1 mm2 holder block with a thickness of 5 µm. A total of twelve male mice were used in this study. The animals were randomly divided into four experimental groups, with three mice in each group. The penetration depth was then visualized using the rhodamine B marker and observed with a fluorescent microscope.

In vitro irritation test

Mice were anesthetized with ketamine intraperitoneally 1 (one) hour before treatment. After the mice were unconscious, the hair on their backs was shaved, then the sample was applied and left for 24 hours. Each mouse was given a separate cage so that they did not touch each other. Then the mice were sacrificed and the skin on their backs was taken and soaked in formalin solution. After that, it was cut using a microtome and made into a block preparation with Hematoxylin Eosin (HE) staining. Then the preparation was observed under a light microscope with histopathological scoring parameters, the indicators of which were edema, polymorpho nuclear (PMN), and degenerative.

▪ RESULTS

Penetration test

Penetration testing was conducted to evaluate the ability of the formulation to deliver the active substance across the membrane and to assess the effect of adding a penetration enhancer on the amount of active substance successfully penetrated. The use of a penetration enhancer in topical formulations is intended to increase the diffusion of the active substance through the barrier layer, thereby improving the effectiveness of active substance delivery to the target site. Therefore, the results of the penetration test constitute an important parameter in determining the success of the developed formulation. The test was carried out under a microscope where the uppermost skin structures were the epidermis, dermis, and hypodermis. The lines in Figure 1 (a, b, and c) indicate the penetration depth, where the numbers can be seen in Table 2. The results of statistical analysis using IBM 25 showed a sig value <0.05 at all times (1, 3 and 5 hours).

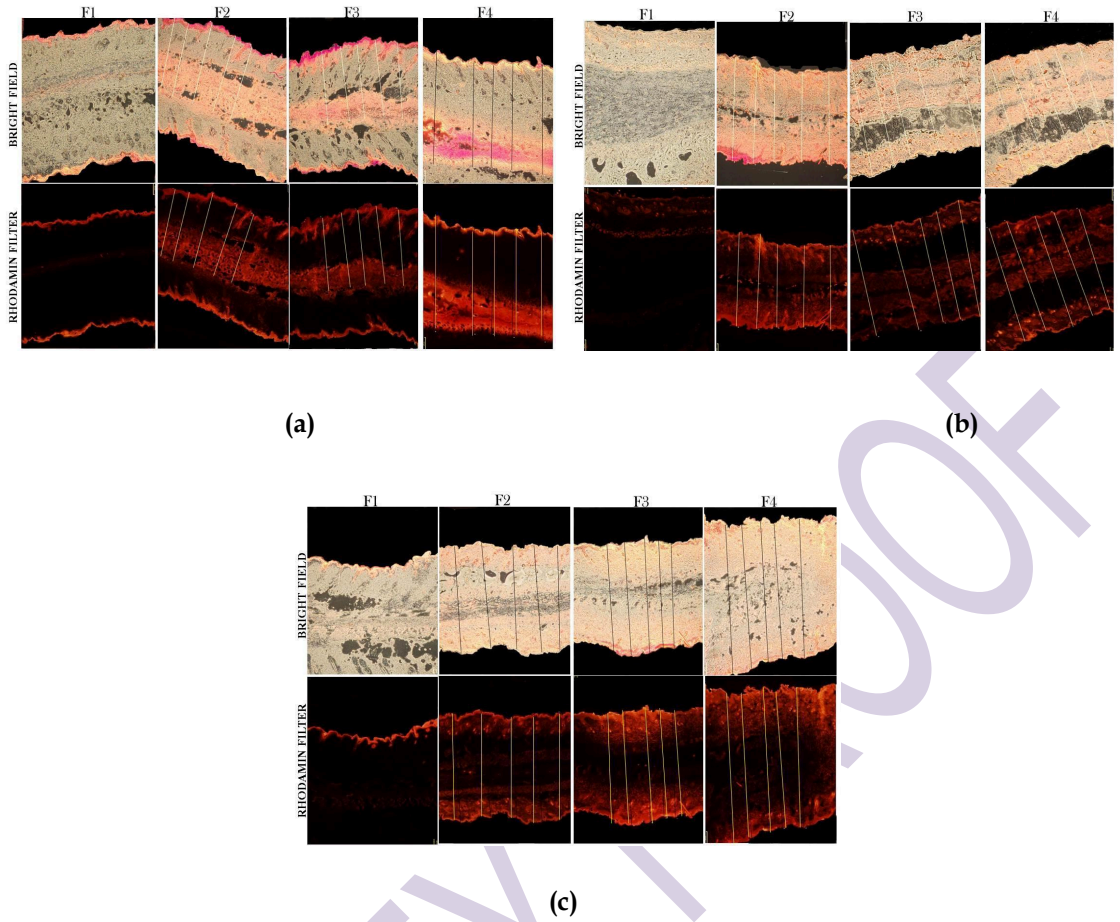

Figure 1. (a) Penetration test results on mice dorsal skin at 1 hour; **(b)** Penetration test results on mice dorsal skin at 3 hours **(c)** Penetration test results on mice dorsal skin at 5 hours.

Table 2. In vivo penetration depth test of AMSCMP formula on the back skin of mice at 1, 3, and 5 hours.

Formula	Average skin penetration (µm) ±SD*	
F1	1 hr	0
	3 hr	0
	5 hr	0
F2	1 hr	1250±17.55
	3 hr	1435±9.07
	5 hr	1326±17.77
F3	1 hr	1361±59.53
	3 hr	1540±45.96
	5 hr	1670±36.82
F4	1 hr	1714±101.83
	3 hr	1948±3.78
	5 hr	1796±52.84

Irritation test

In this study, AMSCMP was formulated as a solution containing SPACE peptide in PBS and only phenoxyethanol as a preservative. The solution was then applied to the backs of mice, and the results were observed at 24 hours. The results showed that F3 and F4 had edema, PMN, and degenerative changes. Furthermore, the irritation score was averaged, with a score of 1.3, categorized as very mild irritation.

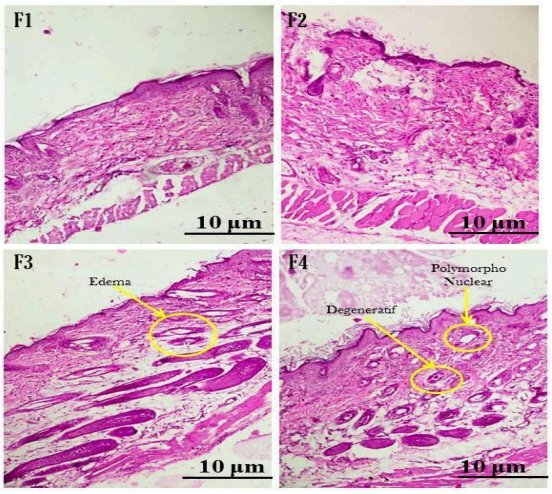

Figure 1. irritation test result on the back skin of mice after 24 hrs.

Table 3. In vivo Irritation test of AMSCMP formula on the back skin of mice at 24 hours

Parameters	F1			F2			F3			F4		
	1	2	3	1	2	3	1	2	3	1	2	3
Edema	0	0	0	0	0	0	0	1	1	0	1	0
Polymorphonuclear	0	0	0	0	0	0	0	0	1	1	1	0
Degenerative	0	0	0	0	0	0	0	0	1	0	1	0
	0	0	0	0	0	0	0	1	3	1	3	0
Total	**0**			**0**			**1.3**			**1.3**		

DISCUSSION

Penetration data was obtained at 1, 3, and 5 hours for each sample, and differences were observed. The results are in Table 2 and Figures 1 (a and b). The deeper the penetration value into the tissue, the longer it takes for the skin structure to be loosened by the C-O and N-H groups, resulting in deeper penetration of AMSCMP. SPACE delivery can permeate through the skin barrier, extending from teh epiderrmis to the hypodermis. The depth and extent of SPACE permeation into the skin depend on the concentration applied 12. In vivo studies by Wender et al. (2002) demonstrated that peptide enhancers were able to penetrate the entire stratum corneum and traverse all skin layers following topical application (Wender et al., 2002)13. This effect results from hydrogen bonds and van der Waals forces that stabilize the skin's rigid secondary structure. One-way ANOVA analysis showed a significant difference in penetration depth at 1 hour (sig. [0.000] < 0.05); a significant difference in penetration depth at hour 3 (sig. [0.000] < 0.05); and a significant difference in penetration depth at hour 5 (sig. [0.000] < 0.05). A post hoc test was then performed.

The penetration test was performed to evaluate the ability of each formulation to deliver the active substance across the membrane. The results demonstrated that the cumulative amount of active substance penetrating the membrane increased progressively throughout the observation period in all formulations. This indicates that the active substance was released from the formulation and diffused across the membrane over time. Among the tested formulations, the formulation containing SPACE exhibited the highest cumulative penetration compared with the other formulations. This finding suggests that the incorporation of SPACE enhanced the penetration of the active substance through the membrane. In contrast, the formulation without SPACE showed a lower penetration value, indicating that the absence of a penetration enhancer limited the diffusion of the active substance across the membrane barrier. The improved penetration observed in the SPACE-containing formulation may be attributed to the role of SPACE as a penetration enhancer, which facilitates the transport of the active substance by altering the barrier characteristics of the membrane. Therefore, the addition of SPACE contributed positively to the penetration performance of the developed topical formulation. [15]

Skin irritation risk is a key factor in cosmeceutical SPACE peptide design. Lim et al. reported that increased lipophilicity of Argireline did not significantly influence skin irritation, while structural modification improved skin permeation 14. The enhanced skin permeation observed after structural modification of Argireline may be explained by alterations in the peptide's physicochemical properties, particularly the reduced zwitterionic character, increased affinity for the stratum corneum, and improved partitioning into the lipid matrix of the skin. Native Argireline is highly hydrophilic, charged, and relatively large in molecular size, all of which limit its ability to penetrate the lipophilic barrier of the stratum corneum. When the peptide structure is modified to increase lipophilicity while reducing its tendency to exist in a zwitterionic form, the molecule can more readily partition from the vehicle into the lipid domains of the stratum corneum. As a result, diffusion across the skin barrier becomes more efficient, leading to a greater amount of peptide permeating the membrane. In line with this explanation, Lim et al. reported that lipophilic analogs of Argireline exhibited higher skin permeation than the parent peptide. [15]

Nevertheless, skin irritation is not determined solely by increased lipophilicity or by the greater amount of compound penetrating the skin. Rather, irritation is more closely associated with the extent to which a substance disrupts skin barrier integrity, extracts intercellular lipids, denatures proteins, induces keratinocyte stress, or triggers local inflammatory responses. In cosmetic peptides such as Argireline and SPACE, the increase in permeation appears to result primarily from improved molecular interactions with skin components rather than from substantial disruption of the barrier structure. In other words, structural modification may enhance the ability of the molecule to enter the skin without necessarily causing severe disturbance to the intercellular lipid organization that commonly contributes to irritation. This interpretation is consistent with reports showing that several skin-penetrating peptides, including SPACE, can enhance dermal delivery while exerting minimal effects on barrier integrity and maintaining a relatively low irritation potential [17] The histopathology scoring results were semi-quantitative. No significant differences were found. The results are shown in Table 3 and Figure 2. SPACE has a very low irritation value, so it is still permitted for use within certain limits. This is supported by in vitro studies showing that the peptide has very low levels of toxicity and is well tolerated. [15],[16].

SPACE peptide can potentially cause irritation, but the mechanism is usually indirect and barrier-related, not because the peptide is inherently a classic "corrosive irritant". Increased interaction with the stratum corneum can disturb barrier homeostasis. SPACE peptide is designed as a skin-penetrating peptide, meaning that it enhances the transport of molecules across the skin barrier. To do this, the peptide must interact with components of the stratum corneum, especially intercellular lipids and possibly keratin-rich corneocytes [18]. If this interaction becomes too strong particularly at higher concentrations or after repeated application it may partially disturb the highly organized lipid structure of the stratum corneum. Even a mild disruption of this barrier can increase transepidermal water loss (TEWL), reduce water retention, and make the skin more susceptible to burning, stinging, erythema, or dryness[20]. Skin penetrating peptides often work by improving molecular interaction with biological membranes or skin proteins. Although this can enhance delivery, excessive interaction with cell membranes may alter membrane fluidity or cellular homeostasis. In keratinocytes, such disturbance may trigger subclinical cellular stress, followed by the release of inflammatory mediators such as IL-1α, TNF-α, or other cytokines. Once this occurs, the skin may show signs of irritation, including redness, discomfort, or mild inflammation. Thus, the irritation potential of SPACE peptide is likely dose-dependent and formulation-dependent rather than an unavoidable intrinsic property of the peptide itself [21], [22]. In practice, irritation is rarely caused by the peptide alone. It is usually influenced by SPACE concentration, duration and frequency of application, vehicle composition (gel, cream, ethanol-containing system, ethosomes, surfactants, etc.), co-administered active ingredients, skin condition (intact, damaged, dry, or sensitive skin). For example, if SPACE is incorporated into a formulation containing surfactants, alcohol, or other penetration-promoting excipients, the overall barrier-disrupting effect may become greater than that of SPACE alone. In that situation, the observed irritation may reflect the combined effect of the delivery system, not solely the peptide [23], [24].

Contact dermatitis is an inflammatory skin condition that occurs due to exposure to irritating substances. Based on how they act on the body, there are two main categories of this condition: both irritant and allergic contact dermatitis. The mechanisms of these two types differ, particularly in the initial phase of inflammation. In allergic contact dermatitis, upon exposure to an irritant, an initial sensitization process occurs, and from the second contact onward, the acquired immune system begins to react. Unlike allergic contact dermatitis, in irritant contact dermatitis, upon initial exposure to the substance, the pre-existing immune system is activated and triggers an inflammatory process, although it eventually also activates the

acquired immune system after multiple exposures [25]. The subsequent processes leading to the formation of eczema lesions are quite similar in allergic contact dermatitis and irritant contact dermatitis, as both exhibit similar patterns of cytokine and chemokine production, cell infiltration, apoptosis, and necrosis. This causes lesions caused by allergic contact dermatitis and irritant contact dermatitis to appear clinically similar and are often difficult to distinguish from one another.

With the numerous irritants present in the workplace, irritant contact dermatitis is one of the most common disorders in today's work context. Therefore, it is a significant issue from both a health and economic perspective. However, there is currently no truly effective therapy for irritant contact dermatitis. Given that current treatments focus solely on symptom management, considerable research is underway to understand and elucidate the complex pathophysiological mechanisms of irritant contact dermatitis and develop potential product of pharmacy especially cosmetics.

OECD Test Guideline No. 439 (2015) [17]. According to the literature, the irritation index produced by the SPACE peptide is negligible at 4.8% out of 100%. Calculating this figure, the irritation index in this study was 1.3% out of 100% [18] .Normality test was then performed for the irritation scores, and the results showed that the data were non-parametric, so the Kruskal-Wallis statistical test was performed. Kruskal–Wallis analysis indicated no significant difference in irritation test scores among the formulations ($p = 0.392 > 0.05$). Therefore, no significant variation in irritation responses was observed between the formulas.

▪ CONCLUSION

The study results demonstrated that the addition of SPACE significantly increased the penetration of the formulation. Based on statistical analysis using SPSS, the penetration parameter differed significantly among the formulas ($p < 0.05$), indicating that the addition of SPACE had a significant effect on enhancing the penetration of the active ingredient. In contrast, the irritation test results showed that the addition of SPACE caused irritation in the very mild category. Nevertheless, statistical analysis revealed no significant difference in irritation scores among the formulas ($p > 0.05$), suggesting that the addition of SPACE did not significantly affect the degree of irritation caused. Irritation tests revealed that the addition of SPACE can cause irritation, but only in the very mild category.

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Conflict of interest statement: The authors declare that there is no conflict of interest.

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