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Conditioned Medium Adipose-Derived Stem Cell Inhibited Tyrosinase Expression and Melanin More Effective Than Hydroquinone in Guinea Pig Skin

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Abstract: ***Hydroquinone 4% (HQ) is the gold standard for protecting skin from hyperpigmentation. However, using this regime for the long-term causes side effects such as erythema and ochronosis. Conditioned Medium Adipose-Derived Stem cells (ADSC-CM) contain cytokines and growth factors reported to have a whitening effect and safer than HQ. This study aimed to evaluate ADSC-CM's effect in inhibiting the increase of tyrosinase enzyme expression and melanin amount compared to 4% HQ in guinea pig skin exposed UVB. Post-test only control group study design was performed in 30 male guinea pigs (Cavia porcellus), 3-4 months old, weight 300-350 grams. The subjects were divided randomly into three even groups. All three groups were exposed to UVB and given base cream (group 1), 4% HQ cream (group 2), and ADSC-CM (group 3). The total dose of UVB was 390 mJ/cm^2 for two weeks. Histopathological examination of the skin was performed to examine the tyrosinase expression and the amount of melanin. The data were analysed using one-way ANOVA. Histopathological examination of the skin was performed to analyze the tyrosinase expression of the amount of melanin. The mean of tyrosinase expression in group 3 (10.1±1.88%) was significantly lower than group 2 (12.26±2.12%;p=0.039) and group 1 (27.37±2.64%; p<0.001). Furthermore, the mean amount of melanin cells in group 3 (1.29±0.53%) was also statistically lower than group 2 (1.74±0.50%; p=0.04)and group 1(4.4±0.32%; p<0.001). ADSC-CM inhibited the increase of tyrosinase expression and melanin more effectively than 4% HQ cream and base cream.***

Keywords: Adipose-Derived Stem Cell, Liposomes, Melanin, Tyrosinase

1. Introduction

Tyrosinase is an enzyme that plays a vital role in melanin synthesis.[1,2] Inhibiting the synthesis of tyrosinase is the first-line principle in hyperpigmentation treatment and HQ in 4% concentration remains the gold standard. HQ inhibits tyrosinase action by preventing tyrosine's conversion to DOPA and causing melanosome degradation and melanocyte destruction by inhibiting DNA and RNA synthesis. HQ has a cytotoxic effect on melanocytes through free radical oxidation mechanism.[3] However, the formation of free radicals damages not only melanocytes, but nearby cells, and cells' physical structure. Irritation and ochronosis were reported after using 4% HQ, thus needed close supervision in their use.[4,5] It is necessary to naturally look for other anti-hyperpigmentation ingredients with fewer side effects.Several studies were previously conducted by utilizing natural ingredients from plants with antioxidant effects and acting as tyrosinase inhibitors.[6]

Another treatment option currently being developed is regenerative medicine, which uses stem cells and growth factors. ADSC is a stem cell that has plasticity and multi-lineage developmental secretions such as Vascular Endothelial Growth Factor (VEGF), Insulin Growth Factor (IGF), Hepatocyte Growth Factor (HGF) and Transforming Growth Factor beta1 (TGF-β1), and protein. Growth factors and other proteins are also found in ADSC's grown medium. This medium is in the future called the conditioned medium of ADSC (ADSC-CM). The production and secretion of growth factors contained therein have essential functions to stimulating collagen synthesis, migration of dermal fibroblasts during the wound healing process[7], and inhibiting melanogenesis through downregulating Tyrosinase and protein-1 expression (TRP-1) in B16 melanoma cells via the mechanism of TGF β-1.[8]

Besides, ADSCs and their secretory factors act as antioxidants by protecting skin fibroblasts from oxidative stress caused by chemicals and UVB irradiation.[9] Antioxidants inhibit the chemical reactions leading to melanin formation, change the type of melanin formed, and interfere with pigment distribution and melanosome transfer. After using ADSC-CM, there have been no side effects or toxic effects in previous studies and may present an excellent natural ingredient therapy option for anti-hyperpigmentation.[10]

The skin as the primary target at the same time can act as a significant barrier in topical delivery, especially the content of ADSC-CM, which contains protein and growth factors that are known to have large molecular weights. Several methods have been developed to increase the permeation of

the drug into the skin. One of them is drug formulation with liposomes. With liposomes, ADSC-CM can permeate through the lamella lipid region of the stratum corneum. The addition of surfactants allows the liposomes to change shape again after passing through a narrow gap without losing shape and size. Liposomes can also act as carriers for low or high molecular weight drugs.[9] This study was conducted to evaluate liposome ADSC-CM applied topically in inhibiting the increase in tyrosinase expression and the amount of melanin compared to 4% HQ in guinea pig skin exposed to UVB.

2. Materials and Method

A post-test only control group experimental design was conducted in 30 male guinea pigs (*Cavia porcellus*), 3-4 months old, weigh 300-350 grams. The subjects have shaved their back hair in area 4 cm2. Group 1 (control) was received base cream, Group 2 was received 4% HQ cream, and Group 3 was received 0.2 ml of liposome ADSC-CM. All three groups were exposed to UVB 20 minutes after the cream was applied. The cream was reapplied four hours after being exposed to UVB.UVB exposure was administrated three times a week with a dose of 65 mg/cm2 for 65 seconds on each guinea pig, for 14 days at a 15 cm distance from the light source. The total cumulative UVB exposure dose was 390 mg/cm^2.

ADSC was taken from fat tissue obtained and stored from the Stem Cell and Cancer Institute Laboratory of Kalbe Farma Company. ADSC was isolated and catalyzed, and the conditioned medium from ADSC (ADSC-CM) was implemented. The base cream was obtained from the Pharmacy Division of Immortal Cosmeceutical and Pharmaceutical Laboratories Indonesia Company. The 4% hydroquinone (Nygrox®) cream was applied. A punch biopsy was performed on the subjects' shaved backs to performed histopathological preparations to measure the tyrosinase expression and melanin amount in the epidermal layer. The data were analysed using one-way ANOVA and ended by the Least Significance Difference (LSD). The Ethical Committee of the Faculty of Veterinary Medicine, Udayana University, has approved the study protocol with the ethical review number #24/UNI4.2.9/PT.01.04/2020.

3. Results

The comparative analysis of tyrosinase expression are presented in Figure 1 and the amount of melanin cells in Figure 2. The results showed a significant difference in the average expression of tyrosinase and melanin between the three groups ($p < 0.05$). From the results of LSD test, it was found that the mean tyrosinase expression were lower in the ADSC-CM group compared to 4% HQ (10.1±1.88% vs. 12.26±2.12%; p=0.04) and base cream (control) (10.1±1.88% vs. 27.37±2.64%; p=0.000).

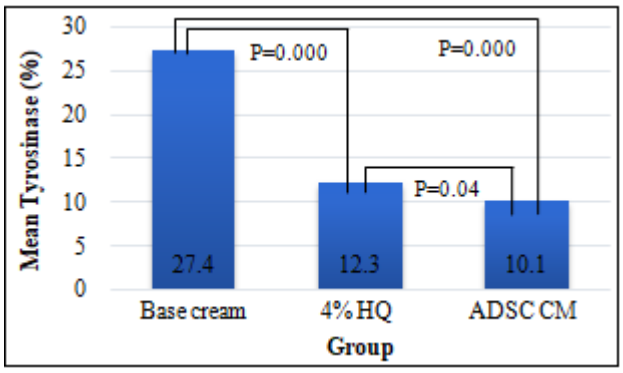

Figure 1: The comparison between base cream group, 4% HQ and ADSC-CM group. The figure shows that the tyrosinase expression is lower in the ADSC-CM group than 4%HQ and Base cream group.

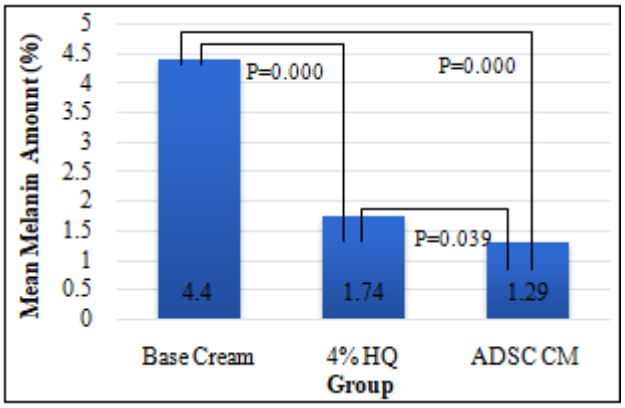

Figure 2: The comparison between base cream group, 4% HQ and ADSC-CM group. The figure shows that the melanin amount is lower in the ADSC-CM group than 4%HQ and Base cream group.

Moreover, the amount of melanin in the ADSC-CM group were also lower compared to the 4% HQ group (1.29±0.53% vs. 1.74±0.50%; p=0.039) and base cream (control) (1.29±0.53% vs. 4.4±0.32%; p=0.000). The linear correlation result showing positive strong correlation between tyrosinase expression and melanin amount (r=0.987; p=0.012) (Figure.3).

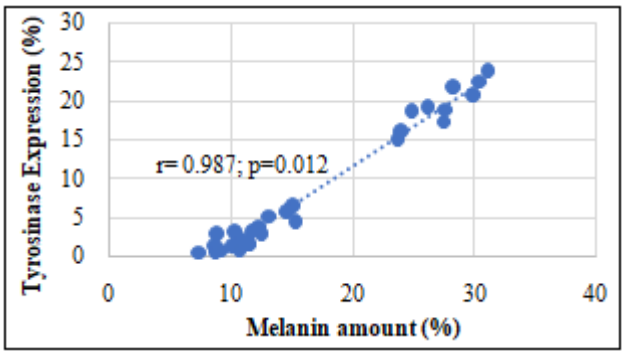

Figure 3: Correlation Between Tyrosinase Expression and Melanin Amount(r=0.987;p=0.012)

The clinical features at days 0 (before treatment), 7th and 14th for the three groups are shown in Figure 4. Pigmentation and irritation of the guinea pig skin were minimal in the ADSC-CM group than 4% HQ and control (base cream). The cross-sectional histology of the guinea pig skin is shown in Figure 5where the tyrosinase expression

and melanin were more prominent in the control group and least in the ADSC-CM group.

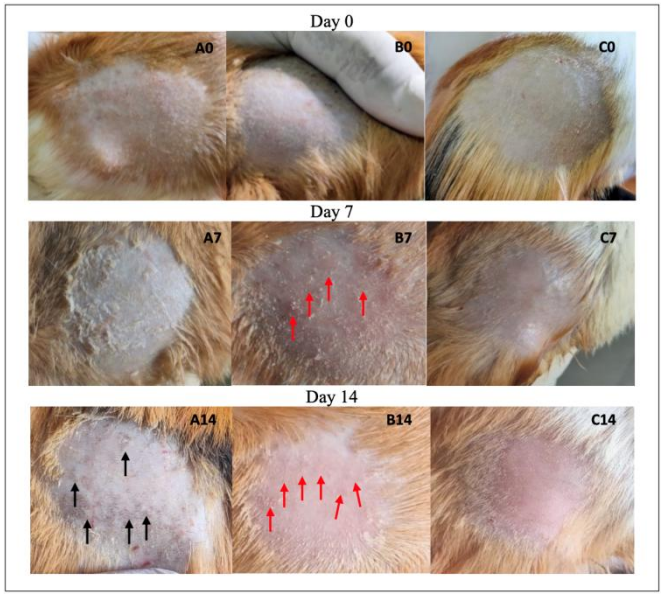

Figure 4: A. Control Group; B. 4% HQ group; C. ADSC-CM group.

The black arrows in the image show a hyperpigmented lesion based on group and time of intervention. The red arrow in the image indicates a reddish irritation. In the control group, hyperpigmentation lesions are more prominent on day 14 compared to the other two groups. In the 4% HQ group, hyperpigmentation lesion is not visible, but reddish irritation appears on day 7 and even more visible on day 14. In the ADSC-CM group, hyperpigmentation lesions and irritation are not visible on day 7 nor day 14.

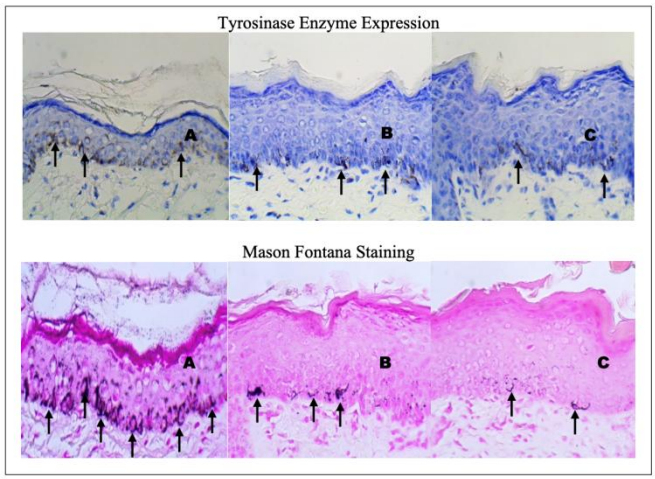

Figure 5: A. Control Group; B. 4% HQ group; C. ADSC-CM group. Black arrow depicting tyrosinase enzyme expression (above) in immunohistochemical stain and is shown by the nucleus melanocyte cells in blue with brown cytoplasm. In the Mason Fontana staining (below), the black arrows indicate the black image of melanin. The tyrosinase expression and melanin amount were the most noticeable in the control group compared to the other two groups. In the 4% HQ group, tyrosinase expression and melanin amount were less visible than the control group but more than the ADSC-CM group. In the ADSC-CM group, tyrosinase expression and melanin amount are the least seen among the three groups.

4. Discussion

The tyrosinase levels and the melanin amount in the ADSC-CM group had a lower mean than the 4%HQ group and the control group. This shows that ADSC-CM administration inhibit the increase in tyrosinase expression and melanin better than 4% HQ and base cream. Hydroquinone inhibits tyrosine and phenoloxidase's enzymatic oxidation and inhibits melanin production by inhibiting sulfhydryl groups and acting as a substrate for tyrosinase. This activity does not 'whiten the skin' but gradually suppresses melanin pigment production.[11]

Hydroquinone is useful in the treatment of hyperpigmentation and has been used for more than 30 years. Hydroquinone is generally well tolerated. However, several side effects have been documented, including erythema, mild contact irritants.[12] Long-term use of hydroquinone and stimulation of sunlight can cause the melanocytes to descend into the papillary dermis and be taken up by fibroblasts, causing elastic changes in fiber production and excretion of abnormal material into new fiber bundles, causing histological brownish yellow-brown fibers of exogenous ochronosis.[13]

The first case reported in Chinese was published in 2008. The biopsy results of two patients with exogenous ochronosis were reported in Singapore after applying 4% HQ for one year.[14] In the same year, four exogenous ochronosis cases in middle-aged women were reported in Chile after using 2-6% HQ for 10-25 years.[15] In India, exogenous ochronosis cases have been reported in Indian women aged 50 years after prolonged use of 2% topical hydroquinone, a rare complication with commonly used over-the-counter drugs.[16]Other side effects that have been reported include erythema, mild irritant contact dermatitis, dryness, and pruritus.[12]

Unlike hydroquinone, using ADSC-CM in previous studies, there were no side effects such as erythema, dryness, desquamation, burning, itching, stinging, or in this study.[17] This is related to the nature of stem cell-based therapy, which is known to be a regenerative treatment, which can increase skin regeneration ability through molecular pathways, and does not cause oxidation reactions. The role of growth factor through paracrine or autocrine effects, in this case, TGF β-1 inhibits melanin synthesis through the signaling pathway of melanogenesis so that it suppresses tyrosinase formation through suppression of MITF[7]and the capacity of ADSC-CM as a potent antioxidant, suppresses the formation of melanosome transfer.[9]

Topical use of ADSC-CM has been studied. Various ways are used to increase the penetration of protein and growth factors in it. Kim et al. have conducted a study on the effectiveness of topical administration of polymersome-ADSC-CM compared to undiluted ADSC-CM solution and base cream.[18] Subjects were divided into three groups, and each contained 20 subjects. The control group (C), experimental group 1 (ADSC-CM group 5%, E1), and experimental group 2 (containing ADSC-CM polymer 5% group, E2) were applying the cream (3 ml) twice daily to the

entire face using an airbrush, morning and evening, for 28 days. The results showed that there were significant differences between groups ($p<0.001$), and the decrease in melanin was significantly lower in the polymersome-ADSC-CM group compared to the undiluted solution ADSC-CM group and the control group.[18]

The effectiveness of ADSC-CM has been observed, and the results were higher for ADSC-CM samples containing polymersome than undiluted solution samples of ADSC-CM.[19] This effectiveness is thought to be related to the polymersome formulation's role in increasing percutaneous absorption. The use of liposomes with the type of SUV (small unilamellar vesicle) measuring <20nm in this study has the same objective as the previous study, namely to increase the penetration of ADSC-CM, which contains protein and growth factor even though it has a considerable molecular weight, which is known to have difficulty penetrating the skin barrier.[20]

There are few limitations in this study include the absorption efficiency test of ADSC-CM in the liposome carrier material was not carried out, the mixing technique of ADSC-CM with liposomes, not being analyzed for other content in ADSC-CM which also plays a role in inhibiting melanogenesis, such as Interleukin-6, TNF- α, procollagen 1-α, other proteins.

5. Conclusion

The study results have proven a significant reduction in tyrosinase expression and the amount of melanin in male guinea pig skin treated with ADSC-CM after exposure to UVB, and the effect is morethan 4% HQ.

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