Observational study

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Wound healing capacityof using mesenchymal stem cell-derived exosomes originated from human adipose tissue and cold atmospheric plasma

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Abstract

Background. Recently, cold atmospheric plasma (CAP), as well as adipose mesenchymal stem cells derived exosomes (ADMSCs-EX), have been applied separately to wound healing treatment. However, no study has investigated the additive effect on the healing mechanism of these two methods in the same skin lesion treatment model.

Aim. We conduct this study to describe the results of using CAP and human ADMSCs-EX on in vitro wound healing.

Methods. Exosomes were isolated from donor adipose tissue samples by ultracentrifugation method, characterized by transmission electron microscopy (TEM) and Western blot. Assessment in vitro wound healing on proliferation and migration evaluation experiments on human fibroblasts with culture medium supplemented with 10µg total exosomal proteins/1 mL and irradiated with CAP with an intensity of 30 seconds/cm^2.

Results. Experimental results to evaluate the ability to stimulate fibroblast migration, showed that cell migration speed in the group supplemented with ADMSCs-EX was equivalent to the group with a combination of CAP and ADMSCs-EX and had the highest rate with 87.8 ± 4.2 % and 84.4 ± 5.3 % while in the control group it was the lowest with 61.9 ± 11.4% ($p<0.05$). The group supplemented with CAP gave fibroblast proliferation and migration results similar to the control group ($p>0.05$), showing the safety of CAP with the growth of the cells.

Conclusions. Therefore, in animal models, we intend to use a combination of these two therapies by using ADMSCs-EX injection therapy into the dermis at the wound edge to avoid the impact of CAP affecting the cell proliferation. ***Clin Ter 2024; 175 (2):135-143 doi: 10.7417/CT.2024.5046***

Keywords: *CAP, exosomes, mesenchymal stem cells, ADMSC-derived exosomes, scratch wound healing assay*

Introduction

The skin is a structure covering the human body that has an essential function in maintaining the balance between the internal and external environment of the body. In cases where the skin's structural and functional integrity is compromised, various physiological and pathological mechanisms involved in wound healing are initiated to protect the organism. The wound healing mechanism is a complex process involving a wide range of cells through interacting cell signals in the extracellular matrix (1). This process mainly consists of several interwoven stages such as hemostasis, inflammation, proliferation, migration, and tissue repair (2,3). If the wound is not treated or intervened properly, the prolonged healing process can cause a pathological spiral that causes complications such as inflammatory infiltration, ischemia, or necrosis (2,3). Many methods of supporting wound healing to improve the speed and quality of wound healing such as low-level laser methods, improved wound dressings, and skin grafts have been applied (1). In particular, two methods that are receiving much attention in wound healing are cold atmospheric plasma (CAP) and the use of exosomes extracellular secretions, especially secretions from isolated mesenchymal stem cells from adipose tissue (ADMSCs-EX) (1,4). Although thermal plasmas have long been used in various medical fields (e.g. sterilization of medical instruments). Recent experimental studies have shown that cold plasma jets at ambient conditions atmospheric pressure allow effective sterilization of various pathogens such as gram-positive and gram-negative bacteria, fungi, viruses, spores and parasites while precisely removing damaged tissue thereby creating conditions that stimulate wound healing (4). Cell culture

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studies demonstrate that plasma treatment beneficially affects wound healing primarily through reducing bacterial colonization, thereby protecting and enhancing cell growth and epidermal cells (5,6). Meanwhile, studies have also shown that adipose mesenchymal stem cells can secrete exosomes, promoting re-epithelialization of skin wounds by inducing epithelial cell proliferation, tissue and angiogenesis, activating collagen and elastin secretion by fibroblasts and preventing fibroblast formation thereby reducing scar formation in the injured area (1,7, 8-11).

In recently, CAP and ADMSCs-EX have been applied separately *in vitro*, *in vivo* wound healing models and some cases, especially those with chronic skin lesions (1,5,6,8-11). However, no study has investigated the additive effect on the healing mechanism of these two methods in the same skin lesion treatment model. Therefore, we conducted this study to optimize combination of these two therapies on *in vitro* wound healing before treating wounds on animal models.

Materials and methods

Research materials

Human adipose tissue-derived mesenchymal stem cell isolation and culture

After being gathered, minced, and digested for one hour at 37^0C using a 0.1% human albumin solution and 200 U/mL collagenase type I solution (Gibco, Massachusetts, USA), adipose tissues (AD) were used. Cells were centrifuged at 500 × g for 10 minutes, after which the supernatant was removed. Following a resuspension of the pellets in the mesenchymal stem cell (MSC) culture media (StemMACSTM MSC Expansion Media, Miltenyi Biotec, Bergisch Gladbach, Germany), the combination was centrifuged at 300×g for five minutes in order to extract the MSCs. The collected MSCs were then planted onto cell culture flasks (Nunc, Thermo Scientific, Massachusetts, USA) coated with solution CTSTM CELLstartTM substrate (diluted in PBS at ratio 1: 300) (Gibco, Massachusetts, USA) after being first concentrated at a density of 3.000–5.000 cells/cm^2 in the MSC culture mix. After that, the flasks were cultivated at 37^0C with 5% CO2. Every two days, culture replaced the media. Once the cells reached 80% confluency, they were split with 0.05% trypsin for the subsequent passage.

Human primary MSCs marker analysis

Using the Human MSC Analysis Kit (BD Biosciences, California, US), human primary MSCs isolated from AD were analyzed for MSC markers at the third passage when the supernatant was collected for exosomes extraction. The kit contains the negative MSC cocktail (PE CD45, PE CD34, PE CD11b, PE CD19, and PE HLA-DR) as well as the positive MSC cocktail (FITC CD90, PerCP-CyTM5.5 CD105, and APC CD73). A Beckman Coulter flow cytometer and Navious software were used for the flow cytometry procedure.

Exosomes production and isolation

For EV generation, the ADMSCs at the third passage were kept operational. ADMSCs were grown in StemMACSTM MSC Expansion Media (Miltenyi Biotec, Bergisch Gladbach, Germany). In order to produce exosomes, the cells from the second passage were divided and seeded onto the fresh flasks that had been coated with CTSTM CELLstartTM substrate solution (diluted in PBS at a ratio of 1: 300) for passage 3. After that, the cells were cultured for approximately 4–5 days at 37^0C and 5% CO2 to achieve 80% confluency. In order for the cells to produce exosomes into the supernatant, the cell culture medium were not changed during the incubation period. When the cells reached 80% confluency, the supernatant, which contained exosomes, was collected. Apoptotic bodies were removed by centrifuging the mixture at 2.500 × g for 10 minutes at 4^0C, and microvesicles were removed by centrifuging the mixture at 16.500 × g for 30 minutes at 4^0C. The exosomes were obtained using an Optima XPN-100 Ultracentrifuge (Beckman Coulter, California, USA) centrifuged at 100.000 × g for 90 minutes at 40C. To extract cleaned exosomes, the exosomes pellets were resuspended, cleaned in PBS, and concentrated again at 100.000 × g/90 minutes at 4^0C. After cleaning, the exosomes were again suspended in 100 µL PBS and kept for later use at -80^0C.

CAP device

We used PlasmaMed in the study, which was patented by the Scientific Council of the Institute of Physics, Vietnam Academy of Science and Technology in 2014. PlasmaMed is a low-temperature plasma jet detector at gas pressure. atmosphere is based on the sliding arc principle. This machine version for treatment and wound healing is a complete system including a high-quality plasma generator, crankshaft, power source, control pedal and gas supply system including two tanks. Argon Med gas. PlasmaMed is easily controlled in two modes: auto-pulse and custom via the touch screen (Fig. 1).

Research methods

Protein extraction

In Protein Lo-Bind tubes (Eppendorf, Hamburg, Germany), a volume of ADMSCs-EX was combined with an equal volume of RIPA extraction buffer, and the mixture was agitated for 15 minutes at room temperature. Once the mixtures were combined, they were centrifuged at 14.000 × g for 15 minutes at 4°C. The protein supernatant was then removed and kept at -20°C until needed.

Western blot

Total exosomes protein (10 μg/lane) was separated by 4–12% SDS-PAGE gels (Invitrogen. USA) at 200 V for 35 minutes at 4^0C. Proteins were then transferred to PVDF membrane (Amersham TM, GE Healthcare Life Sciences, Illinois, US) at 200 mA for 2 h at 4^0C before being blocked

(A: PlasmaMed device; B: Researcher manipulating CAP radiation in a biological safety cabinet; C: CAP emitter beam is projected onto the cell culture medium before being added to the culture plates)

Fig. 1. Cold atmospheric plasma device in the study.

with 5% skimmed milk in TBST buffer for 1 hour. The membrane was probed with diluted primary antibodies against CD9, CD63 (Santa Cruz Biotechnology, Texas, US), AGO2 (Abcam, Cambridge, UK) and Tubulin (Thermo Scientific, Massachusetts, US) overnight at 4^0C and then incubated with secondary antibodies (Amersham ECL Mouse IgG, HRP-linked whole Ab, GE Healthcare Life Sciences, Pittsburgh, USA). Antibody binding was detected with ECL chemiluminescence substrate (Sigma-Aldrich, Singapore) and imaged on ImageQuant LAS 500 (GE Healthcare Life Sciences, Illinois, US).

Transmission electron microscopy (TEM)

Exosomes samples were fixed with 4% paraformaldehyde and then deposited onto Formvar-carbon coated grids (Ted Pella Inc, California, USA). Samples were washed eight times with PBS before being stained with uranyl-oxalate. The grids were dried at room temperature. Imaging was performed using a JEOL 1.100 transmission electron microscope (TEM, JEOL Ltd, Tokyo, Japan) at 80 kV.

In *vitro* design setting

We used fibroblasts for experiments on *in vitro* model, culture wells were divided into 4 groups: the control group used medium containing exosomes removed by centrifugation at 100,000 x g for 27 hours (Control group), the group removed the old medium and added new medium containing 10 µg total exosomes protein/mL 5% FBS DMEM medium (FBS removed EVs by centrifugation at 100,000 x g for 27 h) (100 µL/1 well) from samples of ADMSCs incubated for 48 h (EX group), the group was removed the old medium and added the new medium irradiated with CAP with an intensity of 30 seconds/cm^2 (CAP group) and the combination group (EX-CAP group) supplemented with ADMSCs-EX and CAP with the same above dose as the CAP and EX group.

Proliferation assay

Human dermal fibroblasts were seeded into a 96-well plate with culture medium (5% FBS and 1% Pen/Strep in DMEM/F12) containing exosomes with doses of 10μg total exosomal proteins/1 mL depleted media. Depleted medium was used as the control group which fetal bovine serum (FBS) was centrifuged at 100.000 × g for 27 hours to removed FBS vesicles Cells were incubated at 37^0C and 5% CO_2 overnight for attachment before proliferation analysis using 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit (Abcam, Cambridge, UK). Step by step was performed following instructions by the manufacturer. The proliferation of cells was measured at time points of 0 hours (as control) and 24 hours, 48 hours and 72 hours.

Cell migration assay

Human dermal fibroblasts were seeded into a 24-well plate with a culture medium (5% FBS and 1% Pen/Strep in DMEM/F12) with the density of 2×10^5 fibroblasts to obtain 100% confluency. Cells were incubated at 37^0C and 5% CO_2 to attach to the bottom of the cell culture plate (24-well). Then, the cells were incubated with Mitomycin C (10 μg/mL) for 2 h to inhibit cell proliferation. The cells were washed with DMEM twice before creating a scratch. Detached cells were removed by washing the wells with DMEM for twice. The cells were added fibroblast culture medium containing exosomes with doses of 10 μg total exosomal protein/mL. A depleted medium was used for the control group which FBS was centrifuged at 100.000 × g/27 h to remove FBS vesicles. Cell migration was observed and captured by inverse microscope (Canon, Tokyo, Japan) with 4X magnification for different time points 4h, 24h and 48 h. The rate of cell migration to close the wounded area was analyzed using ImageJ software (version 1.48). Procedure was detailed at Fig. 2.

Statistical Analysis

The statistically significant differences between groups were assessed by Mann-Whitney U tests between two independent variables, Kruskal-Wallis test between more two independent variables; p-value < 0.05 was considered statistical significance. All data were shown as means ± SD.

All statistical analyses were performed using IBM SPSS statistics 22 software.

Ethics statement

The project was approved by the Ethics Committee in Biomedical Research of Hanoi Medical University (Certificate of Approval of Ethical Aspects No. 488/GCN-

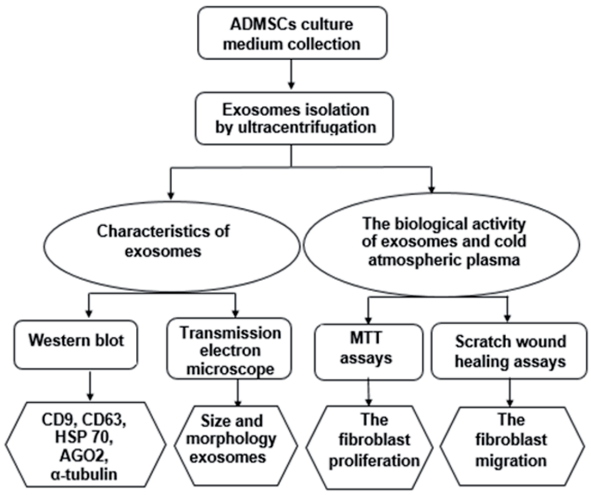


Fig. 2. The procedure of the study

HDDDNCYSH-TDHYHN, 2021) before implementing the collection of donor adipose tissue samples.

Results

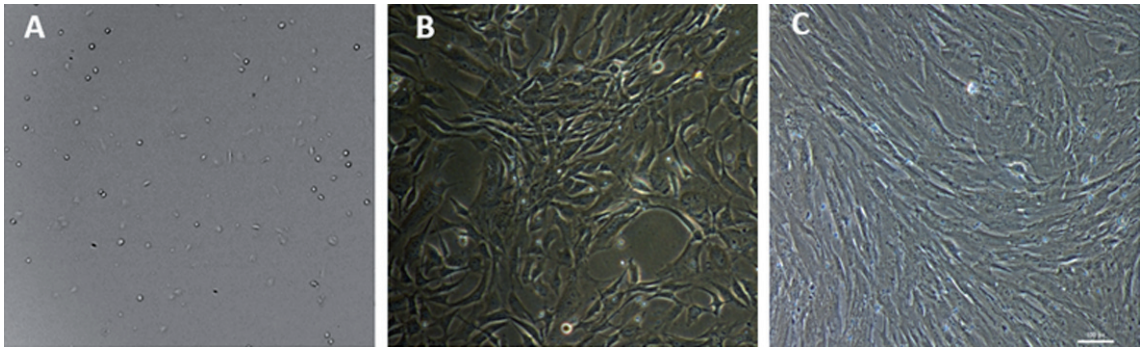
ADMSC cell culture and cellular senescence

We evaluated the morphology and cell surface markers of passage three human primary MSCs originated from adipose tissue at the time point of supernatant collection by microscopy and Flow Cytometry. We observed similar morphology in all three evaluated ADMSCs (Fig. 3).

In addition, all samples ADMSCs expressed highly MSC positive markers of CD90, CD105, and CD73 (>95%) and very low MSC negative markers of CD45, CD34, CD11b, CD19 and HLA-DR (<1%) (Table 1 and Fig. 4).

Table 1. Results of identification of mesenchymal stem cells by flow cytometer

Sample	% Positive Markers			% Negative Markers
	CD90	CD105	CD73	
1	99.88	98.36	99.95	0.31
2	99.97	99.76	99.94	0.62
3	99.95	99.09	99.95	0.34
4	99.99	99.73	99.99	0.88
5	99.90	99.17	99.87	0.51
6	99.89	98.86	99.86	0.07
7	99.97	99.69	99.95	0.03
8	99.91	99.74	99.87	0.28
x ± SD	99.93 ± 0.04	99.3 ± 0.32	99.92 ± 0.05	0.38 ± 0.28



(A: Mononuclear cells isolated from adipose tissue when just sown into culture flask; B: The MSCs at passage 0 (P0) after 7 days cultivation; C: The MSCs at passage 3 (P3) after 6 days cultivation)

Fig. 3. Human primary mesenchymal stem cell isolated from adipose tissue

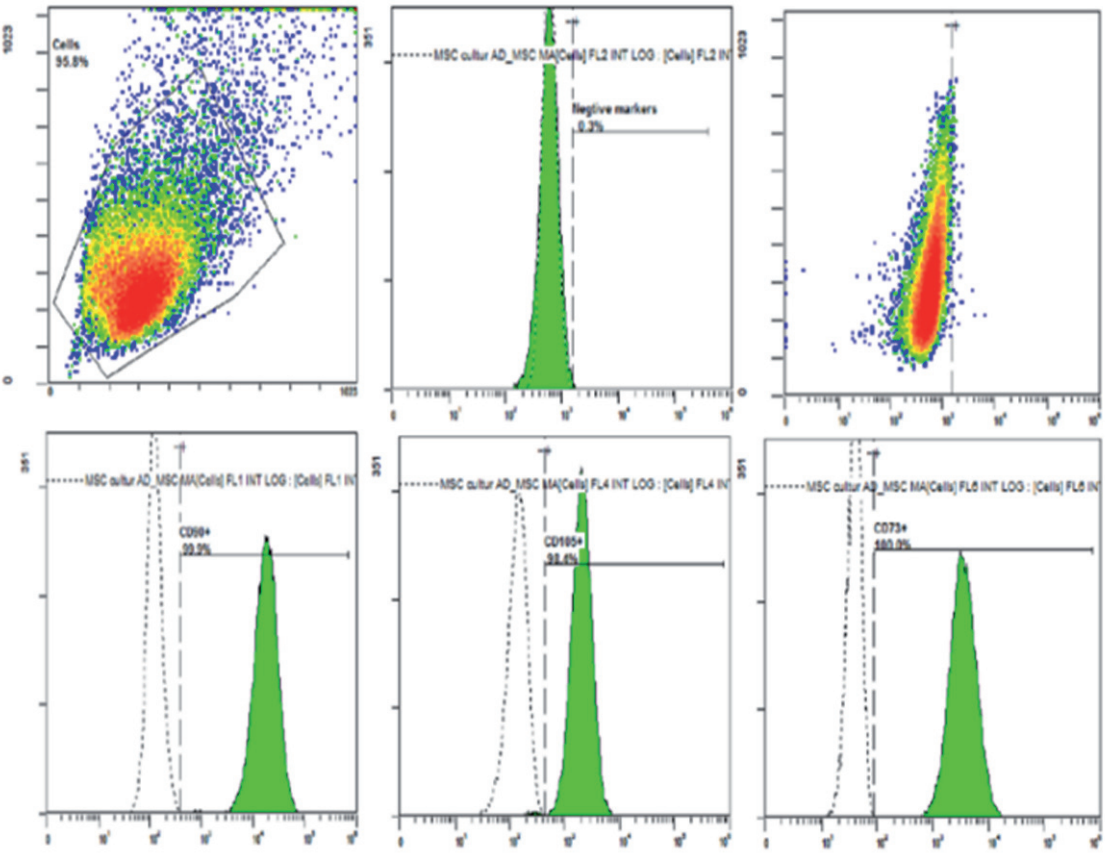


Fig. 4. Data illustrating the results of adipose me

Characterization of exosomes originated from ADMSCs

Analyzing the morphology of exosomes generated from ADMSCs source using transmission electron microscope (TEM), we found that all sample ADMSCs-EX had a cup-shaped morphology and were less than 250 nm in size (Fig. 5). As an essential criterion for the evaluation of exosomes, the study examined Western blot's expression of specific proteins such as transmembrane proteins (CD9 and CD63) and intracellular proteins (AGO2). The results showed that exosomes from ADMSCs samples all expressed CD9, CD63, AGO2 and Tubulin (Fig. 5)

The capacity of human primary MSC-derived exosomes with cold atmospheric plasma in inducing cell proliferation

To investigate the capacity of statistical significance and CAP to induce cell proliferation, we performed a colorimetric MTT assay to evaluate the effect of ADMSCs-EX and CAP in dermal fibroblast. The absorbance of the solution after the MTT reaction, was quantified by measuring the optical absorbance with a spectrophotometer, detailed in Table 4.

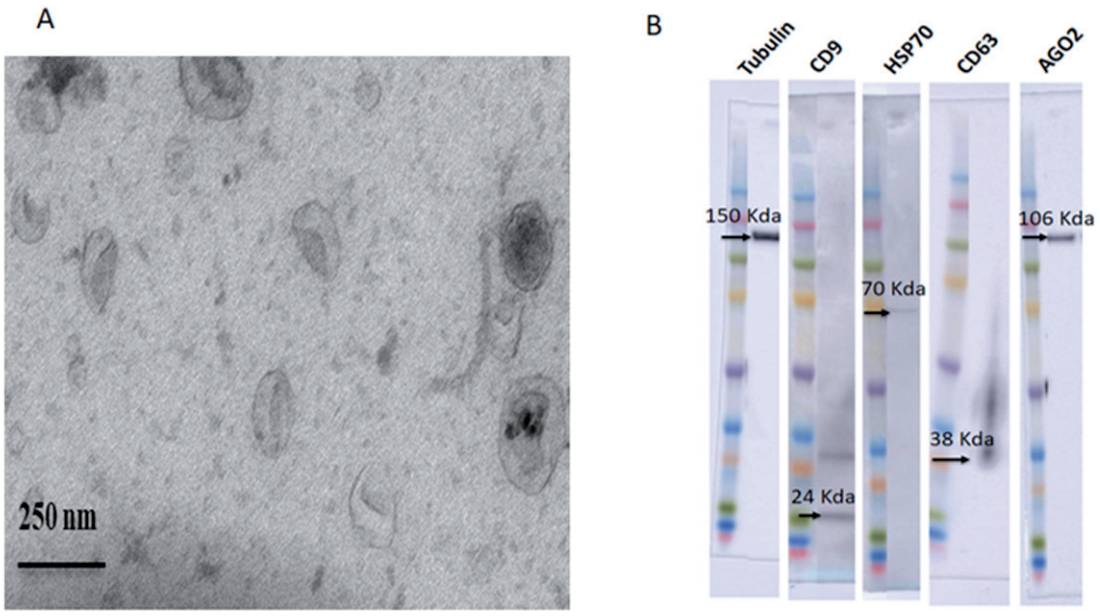
Analysis of results showed that the group of wells supplemented with ADMSCs-EX in all time points of follow-up: 24 hours, 48 hours and 72 hours had the highest fibroblast proliferation rate compared with the rest of the groups ($p<0.05$). The well group supplemented with ADMSCs-EX and CAP revealed better fibroblast proliferation efficiency than the control group at 24 hours and 72 hours. The wells supplemented with CAP gave better fibroblast proliferation results than the control group at 24 hours, but after 48 hours and 72 hours follow-up, the proliferation result was lower than that of the control group ($p <0.05$).

The capacity of human adipose MSC-derived exosomes in cell migration and cold atmospheric plasma

We cultured human fibroblast cells in a 24-well plate and examined their migration in a medium containing ADMSCs-EX at a concentration of 10 µg total EX protein/mL and CAP with dose for 30 seconds in the culture medium added to wells. Fibroblasts were incubated at 5% CO_2 and 37^0C and cell migration was photographed at time points: 0 hour, 24 hours and 48 hours.

Observing Fig. 6, we found that the incisions were made equivalently with an incision device with a silicon tip at 0 hours (Start point time). After 24h and 48h, the movement speed in 2 groups supplemented with ADMSCs-EX was the best compared to the other groups.

Based on the images obtained at the time points, we calculated the incision area on the groups using Image J software. the detailed analysis results are shown in table 5 and illustrated in Fig. 7 below.



Morphology analysis of exosomes observed under TEM showed all exosomes have a cup-shape morphology (Scale bar: 250 nm) (B) Exosomal proteins. 10 µg of total protein was loaded in each lane including CD9, CD63, AGO2 and Tubulin, enriched in ADMSCs-EX.

Fig. 5. The morphology and marker analysis of exosomes derived from human primary ADMSCs

Table 4. The optical absorbance of fibroblast proliferation experiment with follow up time points

Group	Control (G0)	EX (G1)	CAP (G2)	EX-CAP (G3)	p
24 hours	0.066 ± 0.003	*0.074 ± 0.004	0.071 ± 0.007	*0.075 ± 0.002	0.007
48 hours	0.102 ± 0.005	*0.119 ± 0.004	*0.091 ± 0.002	0.095 ± 0.009	0.001
72 hours	0.077 ± 0.002	*0.094 ± 0.014	*0.066 ± 0.0027	*0.088 ± 0.0043	0.0001

p: Kruskal-Wallis test; * Mann-whitney U test compared with G0 p<0.05

Table 5. Results of fibroblast migration rate (%) on in vitro scratch wound healing

Group	Control (G0)	Exo (G1)	CAP (G2)	Exo-CAP (G3)	p
4h	6.9 ± 1.5	8.9 ± 1.4	8.3 ± 3.0	7.4 ± 1.9	0.311
24h	42.1 ± 7.2	48.6 ± 6.3	41.3 ± 4.4	45.3 ± 4.0	0.175
48h	61.9 ±11.4	*87.8 ± 4.2	76.9 ± 6.4	*84.4 ± 5.3	0.008

p: Kruskal-Wallis test; * Mann-whitney U test compared with G0 p<0.05

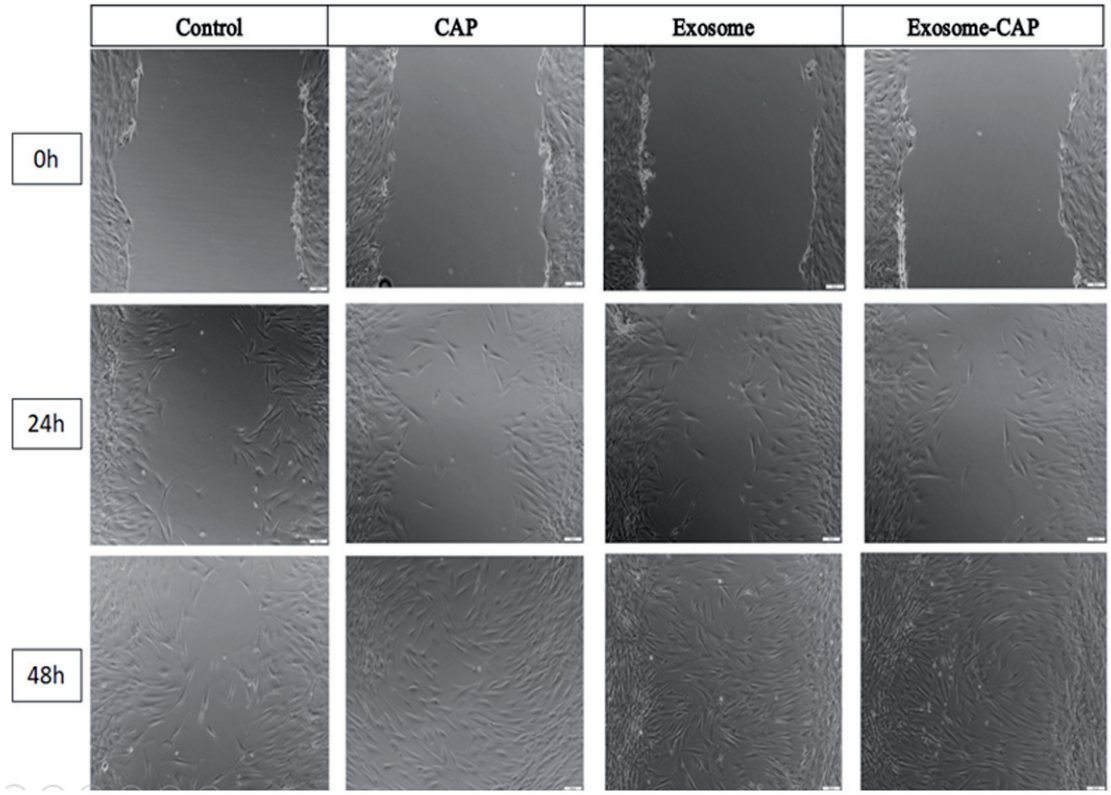


Fig. 6. The capacity to stimulate fibroblast migration on in vitro scratch wound healing

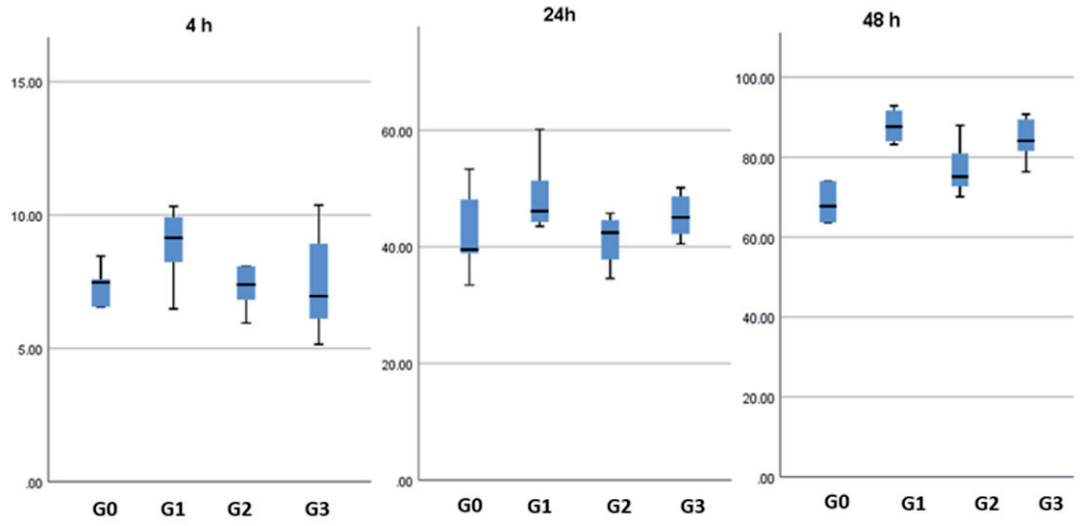


Fig. 7. The fibroblast migration rate on in vitro scratch wound healing

Analyzing of the results showed that after 4 hours after making an incision on the fibroblast culture plate, the cell migration rate in the group supplemented with ADMSCs-EX was the highest. with 8.9 ± 1.4 (%) but we have not recorded statistical significance (p> 0.05). After 48 hours, the cell migration rate in the group supplement with ADMSCs-EX was the highest with 87.8 ± 4.2 % respectively, followed by the EX-CAP group with 84.4 ± 5.3%, while the control group was the lowest with 61.9 ± 11.4% (p<0.05). The addition of CAP after 48 hours helped the fibroblasts migrated at 76.9 ± 6.4 %, but this result was not significantly different from the control group (p> 0.05).

Discussion

In developing the procedure for isolating ADMSCs-EX, we considered the cost, performance and quality criteria of exosomes. The study selected the ultracentrifugation that is the most common exosomes isolation method currently. The ultracentrifugation method has a lower yield of exosomes isolation than commercial separation kits, but the ratio of exosomes secretion/total protein concentration and exosomes marker expression using this method is higher in the same total protein concentration resulting in higher exosomes purity compared with commercial separation kits (12). Notably, all isolates from MSCs culture medium obtained exosomes with uniform quality. To demonstrate whether the exact population of exosomes is obtained, the study evaluates the characteristics of exosomes based on the expression of specific proteins and characteristic morphological characteristics. The images taken under transmission electron microscopy showed that the exosomes isolated in this study had a typical morphology of exosomes which is cup-shaped with a diameter in the range of 40 - 250 nm (Figure 5). In addition, exosomes derived from adipose tissue MSCs in this study exhibit transmembrane and intracellular protein expression, including CD9, CD63, and AGO2 which are considered specific markers of exosomes (Figure 5). This result shows that our study has successfully isolated exosome secreted by MSCs derived from adipose tissue.

To evaluate the biological effects of ADMSCs-EX with CAP on wound healing on *in vitro* model, we selected either scratch wound healing assay and the cell proliferation assay. We perform experiments on fibroblasts in the above two experiments because these cells play an important role in the wound healing process in skin tissue. The results of our follow-up on both experimental models showed that the wells supplemented with ADMSCs-EX had the best fibroblast migration and proliferation results compared to the other groups. This result is consistent with previous studies on the ability to stimulate the migration and proliferation of fibroblasts. The study of Li Hu et al (2016) showed that in the *in vitro* incision closure experiment, at 12 h and 24 h, in the group supplemented with ADMSCs-EX, there was an increase in fibroblast migration compared with the control group (p<0.001) (13). In this study, the authors also demonstrated that the addition of ADMSCs-EX to fibroblast cultures increased the mRNA expression of N-cadherin, Cyclin-1, and PCNA (p < 0.001) which play important roles in cell migration and proliferation in tissues (13). The study of Arsalan Shabbir et al. (2015) also showed that the addition of exosomes from MSCs at a concentration of 10 μg/mL in the *in vitro* incision experiment also increased the migration rate of human fibroblasts compared with the control group (p<0.05) (14). Thus, consistent with published results, we also demonstrate that ADMSCs-EX in this study maintain fibroblast proliferation and migration stimulation at 10 μg/mL total protein concentration. In addition to the evaluation results on the morphology, size and characteristics of the specific proteins of exosomes, this result contributes to the biological effects of ADMSCs-EX culture medium by ultracentrifugation has stable quality, suitable for use in *in vivo* experiments and promising as a premise for future clinical trials.

Our study used the PlasmaMed device manufactured and licensed by the Vietnam Heath Ministry, to generate a cold plasma beam source from an inert Argon gas supply, to evaluate the safety and interaction of CAP with exosomes isolated from ADMSCs. Due to the design of *in vitro* experiments using fibroblast as adhesion cell lines on the surface of culture flasks, in order to avoid the effect of plasma beam when irradiating on cells to affect adhesion ability, cell adhesion, we used the method of irradiating cold plasma to the culture medium and then adding it to the wells on the cell culture plates. Experimental results showed that CAP did not affect the cell migration-stimulating bioactivity of ADMSCs-EX when added to the same cell culture medium. In the study, CAP still ensures fibroblast proliferation during the cell development period from 0-48 hours. These results suggest that it is possible that the simultaneous activity of CAP and ADMSCs-EX can reduce the biological activity of ADMSCs-EX, so we aim to use a combination of these two therapies in future animal experimental models by using ADMSCs-EX injection into the dermis at the wound edge skin without using exosomes coating on the wound surface, to avoid the impact of CAP when projecting on the wound surface, helps ADMSCs-EX best support cell proliferation.

Due to limited resources, we only tested the synergistic effects of ADMSCs-EX and CAP on the cell line human fibroblasts, which play an important role in remodeling matrix extracellular and forming granulation tissue at wound. However, the wound healing process is a complex process with the participation of many other cell types such as keratinocytes or endothelial cells. Therefore, it is better to evaluate the more comprehensive mechanism of impact when using ADMSCs-EX and CAP on wound healing with these cell types in the future.

One of the wound healing effects of CAP is its ability to kill and inhibit the growth of bacteria, which has been demonstrated by many research groups (4). However, due to the characteristics of the cell culture room in the *in vitro* model, we have not been able to evaluate this effect of CAP. Therefore, in the direction of further research on *in vivo* models, we hope to be able to conduct experiments on the interaction between these two treatments with skin tissue and the ability to affect bacteria, on the skin in a realistic animal wound model, to more accurately simulate the interaction between the two treatments and the wound healing process, especially the group of wounds at risk of chronic progression.

Conclusions

Our study shows that exosomes isolated from ADMSCs have the ability to stimulate fibroblast cell migration and proliferation and using CAP safety for cells. In the future, it is recommended that using CAP to support bactericidal combined with the method of injecting ADMSCs-EX into the dermis on animal models to support the wound healing process.

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Conflicts of interest

None to declare

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