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Investigation of exosomes in aged human fibroblasts cultured in serum-free medium

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Abstract

Introduction: Exosomes are small vesicles (30 to 100 nm) crucial for intercellular communication and influence various biological and pathological processes. This study examined exosome secretion in human skin fibroblasts in vitro.

Materials and methods: Supernatants from young fibroblasts (passage 3) and aged fibroblasts (passage 12) cultured in DMEM medium, with or without 10% fetal bovine serum (FBS), were collected for analysis. After confirmation of exosome presence by scanning electron microscopy, the number of exosomes was measured using flow cytometry with magnetic beads coated with a specific antibody (anti-CD81). Additionally, the protein profile of these exosomes was examined using SDS-PAGE.

Results: Electron microscopy revealed exosomes with diameters from 33 to 93 nm in fibroblast culture supernatants. Aged fibroblasts showed a significantly reduced abundance of exosomes in serum-starved conditions at 16 and 72 hours compared to controls ($P<0.05$). However, there was no significant difference in exosome abundance between young and aged fibroblasts in serum conditions. Young fibroblasts exhibited no significant differences in exosome levels across serum-starved and control groups at various time points. The concentration and mean fluorescent intensity (MFI) supported these findings. Electrophoresis showed exosome proteins ranging from 14 to 116 kDa, with no significant differences between age groups. Protein band density in serum-starved exosomes was lower than in controls, with only one exception noted.

Conclusions: In aged fibroblasts, there were no significant changes in exosome quantity or protein patterns, but under serum-starved conditions, notable differences arose. The overall number of exosomes decreases under serum deprivation, though not significantly, while protein band density significantly decreases. Aged cells continue to secrete exosomes in serum deprivation, but their protein content diminishes. This finding improves our understanding of wound healing, cancer, and cell therapy.

Keywords: Exosomes, Cellular Senescence, Fibroblasts, Serum Deprivation

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Introduction

Cellular senescence refers to a permanent halt in the cell cycle, which arises from the limited capacity for cell replication, particularly in normal human fibroblasts (1). This phenomenon is believed to contribute to the aging process of fibroblasts. Various factors influence the aging process, including genetic background, genomic instability, free radical production, changes in telomerase enzyme activity, dietary restrictions, waste product accumulation, DNA methylation, stress, mitochondrial mutations, DNA damage and repair, DNA-protein interactions, histone acetylases, and histone deacetylases (2, 3). Senescent cells can be differentiated from other non-proliferative cells through specific markers and morphological changes. These changes include the absence of the proliferative activity marker SAβGAL, the expression of pro-inflammatory factors like IL-6 and IL-8, the presence of chemokines, cell cycle inhibitors, tumor suppressors, and signs of DNA damage. The theory of cellular senescence, also known as the Hayflick limit, was first proposed by Leonard Hayflick and Paul Moorhead (4). It is important to note that aging manifests at multiple levels: organ, tissue, cell, and molecular. Senescent fibroblasts build up in older organisms, especially in certain tissues. The highest concentrations of these fibroblasts have been observed in the skin, liver, lungs, and spleen. Fibroblasts are among the most abundant cell types in the body, especially within connective tissues, and play a crucial role in wound healing and the aging process (5). As organisms age, these fibroblasts build up in the lower layer of the skin and secrete substances typically released only during wound healing. These substances include collagenase and elastase, which are matrix metalloproteinases that degrade elastin and collagen in the skin (6). In addition to the above functions, senescent fibroblasts secrete enzymes that can degrade the basement membrane, which is vital for the proper organization and function of epithelial cells. Other substances secreted by fibroblasts include TGFβ, insulin-like growth factor-binding protein 1 (IGF1), PAI1, inflammatory cytokines, and decreased levels of lamin B1, VEGF, and matrix metalloproteinases. These changes allow senescent fibroblasts to communicate with each other and their surrounding environment (7). They also release high levels of exosomes, which can alter the local microenvironment and promote the growth and spread of nearby tumor cells (8). Some research suggests that these changes may inhibit the spread of cancer cells, reduce cell motility, and limit oncogenic transformations in cancer cells during early stages. Thus, aging serves as a double-edged sword concerning cancer development (9). Our study has shown that when fibroblasts are subjected to serum deprivation— a form of cellular stress— they secrete substances into the surrounding medium that encourage fibroblast migration, as observed in scratch tests. Unpublished findings indicate that these fibroblast secretions also enhance wound healing in vivo (10). Given the crucial role of exosomes in various diseases, especially cancer and autoimmune conditions, this study aims to investigate the secretion of exosomes and compare the proteins they contain under senescence conditions and serum deprivation in fibroblast supernatants (11). Exosomes secreted from cells infected with pathogens express specific antigens that interact with the histocompatibility complex, presenting these antigens to immune cells. Meanwhile, exosomes released from cancer cells can carry antigens that act as both tumor-promoting and immunosuppressive agents. Besides their regulatory role in the immune system, exosomes can stimulate tumor progression through mechanisms such as angiogenesis.

Materials and methods

Sampling

Nine foreskin samples were collected from newborns with an average age of 2 months during circumcision at Babol Clinic in Babol City. The procedures were carried out under completely sterile conditions. The samples were placed in a culture medium consisting of 80% DMEM (PAA, Austria), 10% Penicillin/Streptomycin (PAA, Austria), and 10% FBS (PAA, Austria), and then transferred to the culture room. All steps for cell isolation were conducted under a laminar flow hood using sterile materials and equipment.

Isolation of fibroblast cell lines from foreskin by enzymatic method

In this method, the tissue was washed 2 to 3 times with ethanol to reduce the risk of contamination. The

samples were also washed 2 to 3 times with PBS to remove blood cells. Next, the samples were cut into small pieces using a surgical blade, and the slimy, bloody layer was separated. This layer was then transferred to a Falcon tube containing the enzyme dispase at a concentration of 5 mg/ml, maintained at 37 degrees Celsius in a water bath for 3 hours. During this process, the epidermis layer was separated from the dermis layer by the enzyme disease. Afterward, the dermis layer was divided into very small pieces and transferred to a Falcon tube containing the enzyme collagenase at a concentration of 1 mg/ml, also maintained at 37 degrees Celsius in a water bath for 20 minutes. Collagenase breaks down collagen proteins, allowing the cells to separate from the tissue. The supernatant containing the separated cells was then removed and placed in a Falcon tube with a culture medium, and collagenase was added to the remaining tissue again. This step was repeated multiple times until all the tissue was dissolved in the collagen solution. Subsequently, the culture medium containing the cells was passed through a cell separation filter to create a uniform cell suspension. The cells were then centrifuged at 1500 rpm for 7 minutes at 4°C to sediment them. The supernatant was discarded, and the cells were homogenized in 1 ml of culture medium. Finally, after counting the cells and checking the percentage of viable cells, the cells were cultured in specialized cell culture flasks.

Cell culture

Cells isolated from the skin were placed in a 25 cm^2 flask, with a density of 100,000 cells per flask. They were grown in a medium made up of 89% DMEM, 10% FBS, and 1% Penicillin/Streptomycin. This setup was maintained in an incubator under standard conditions (temperature: 37°C, 5% carbon dioxide, and 95% humidity). After 24 hours, the culture medium was discarded, the adherent cells were rinsed with PBS, and a fresh culture medium was introduced. The culture medium was then changed every two days until the cells covered the entire surface of the flask continuously.

Culturing fibroblasts in serum-free medium

Ten^5 cells (from passages 8, 4, and 12) obtained from three skin samples were placed in a 25 cm^2 flask containing a culture medium composed of 89% DMEM, 10% FBS, and 1% Streptomycin/Penicillin. The flask was incubated under standard conditions (37°C, 5% carbon dioxide, and 95% humidity) for 48 hours until cell attachment reached approximately 70 to 80%. After this period, the complete culture medium was removed, and the cells were washed several times with PBS. Subsequently, a serum-free DMEM culture medium was added to the flasks and incubated for 16, 48, and 72 hours. Additionally, a complete culture medium containing 10% FBS was added to one flask as a serum-containing positive control.

Cell culture supernatant storage

After the specified incubation periods, the supernatant, which consisted of a DMEM culture medium and other cellular secretions, was carefully removed. It was then stored in 500 µl microtubes at -20°C for subsequent experiments, including exosome extraction using the desired kit, electron microscopy, flow cytometry, and SDS-PAGE.

Analysis of supernatant of fibroblasts cultured in serum-free and serum-free medium (SFS)

To obtain a general view of the protein composition of the supernatant from fibroblasts cultured in serum-free media (maintained at -20°C) and serum-containing media, the electrophoretic migration pattern of proteins was examined using sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE).

Reducing polyacrylamide gel electrophoresis

In this study, a discontinuous electrophoresis system was employed following the method established by Laemmli (1970). First, a separating gel with a pH of 8.8 (see Table 2-1) was poured between two electrophoresis vials. After the gel polymerized, a concentrating gel with a pH of 8.6 (see Table 2-2) was added on top, and wells were formed by placing a comb in the gel. Once the gel was completely polymerized, samples were prepared that included exosomes extracted from both old fibroblasts (passage 12) and young fibroblasts (passage 3) at various time intervals of serum deprivation. Additionally, exosomes from positive control samples, which consisted of fibroblasts cultured in DMEM containing FBS and previously concentrated using the exosome extraction kit, were

included. Each sample was mixed with a 4x sample buffer (refer to Table 2-3) in a ratio of 1:3 (sample buffer to sample), and then heated for 2 to 5 minutes. The prepared samples were loaded into the wells created in the gel. Freshly prepared tank buffer (see Table 2-4) was used for the migration process. Bromophenol blue served as a migration control in this technique. Initially, a voltage of 80 V was applied while the samples were in the concentrating gel. When the samples entered the separating gel, the voltage was increased to 120 V and subsequently to 150 V.

Staining polyacrylamide gel with Coomassie blue

Coomassie blue is the most commonly used dye for staining proteins. Its advantages include ease of use, color stability, and relatively high sensitivity, with the ability to detect proteins in amounts ranging from 0.2 to 0.5 µg per band. In this method, the steps of fixing and staining the proteins are conducted simultaneously.

Coomassie Blue staining method

A sufficient volume of dye solution was added to the gel, and then the container was closed and placed on a shaker for 1-2 hours. Afterward, the dye solution was drained, and the gel was thoroughly washed with plain water. Next, a dye remover solution was added, and the container was placed on the shaker again. This process was repeated several times until the gel background became transparent and the protein bands were visible. In the final step, the gel was placed in a 7% acetic acid solution, which allows for long-term storage.

Exosome extraction

Sample preparation

The supernatant from fibroblasts cultured in young (3-5 passages) and old (12-15 passages) is collected and then centrifuged at 2000 g for 30 minutes. This process removes dead cells and other debris. After the centrifugation, the clear supernatant is carefully transferred to a new, clean microtube, making sure not to mix it with the sediment in the original microtubes.

To extract exosomes from the fibroblast supernatant, we utilized an exosome extraction kit (TEI). First, we took 1 ml of the centrifuged fibroblast supernatant prepared in the previous step and added 0.5 ml of the extraction buffer provided in the kit, following the protocol. Next, we mixed the supernatant and extraction buffer thoroughly by vortexing or using a pipette to create a homogeneous solution. The microtubes containing this mixture were then incubated at a temperature between 2-8°C for 24 hours. After the incubation period, the samples were centrifuged at 10,000 g for 1 hour, also at 2-8°C. Subsequently, we discarded the supernatant from the microtubes, leaving behind a precipitate that contains the exosomes. To prepare a uniform solution, we added 25-100 µL of PBS x1 buffer to the exosomal precipitate. The extracted exosomes can be stored for one week at 2-8°C or for an extended period at -20°C.

Extraction of CD81-positive exosomes

Preparation of Assay Buffer

The measurement buffer consists of 0.1% BSA + PBS, which has been passed through a 0.2 µm filter.

Preparation of beads that detect exosomes with CD81 marker

Dynabeads were utilized in this study. These beads are magnetic polystyrene beads measuring 2.7 µm in size. They are coated with a primary monoclonal antibody against the membrane molecule CD81, which is expressed by most human exosomes. To prepare the Exosome-Human CD81 Flow Detection beads (Thermo Fisher Scientific, USA), the vial was vortexed for 30 seconds to ensure a homogeneous solution. Then, 20 µl of the bead solution was removed and added to a microtube containing 1 µl of buffer. The buffer-bead mixture was placed on a magnet for 1-2 minutes. While the microtubes were still attached to the magnet, the supernatant buffer was carefully removed, and 90 µl of fresh buffer was added. After that, 10 µl of the sample containing exosomes was introduced to the buffer-bead mixture. The resulting mixture was then placed on a rotator and incubated at 2-8°C for 24 hours, allowing the beads and exosomes to mix and bind to each other. After incubation, the samples were briefly centrifuged for 1-2 seconds. Subsequently, 300 µL of Assay Buffer was added, and the mixture was placed on the magnet for another 1-2 minutes. Afterward, the supernatant was removed before separating the samples from the magnet. This process was repeated after adding another 300 µL of buffer to wash the beads attached to the exosomes for

purification. In the final step, an additional 300 µL of buffer was added to the samples, resulting in a solution that was used for subsequent experiments, including electron microscopy and flow cytometry.

Observation of exosomes by scanning electron microscopy (SEM)

To observe exosomes by electron microscopy, 10 µl of the solution prepared in the previous step was spread on a slide and allowed to dry. This sample was then analyzed by electron microscopy (SEM).

Flow cytometry

In this step, 100 µL of the exosome sample bound to the beads is added to a microtube, followed by the addition of 20 µL of monoclonal antibody CD81 (Mouse anti-human CD81-PE, BD Bioscience, USA). The samples are subsequently placed on a shaker set at 1000 rpm and incubated at room temperature for 45 minutes. During this incubation, it is important to keep the samples away from light and in a dark environment. After 45 minutes, 300 µL of buffer is added to each sample, which is then placed on a magnet for 1-2 minutes. The samples are washed by first removing the supernatant before separating them from the magnet. Afterward, 300 µL of buffer is added again, and this process is repeated for a thorough wash. In the final step, an additional 300 µL of buffer is added. The volume of buffer in the final step may vary depending on the specific flow cytometry device used, and it can be adjusted up to 1 mL. Once prepared, the samples are analyzed using a BD FACSCalibur device.

Data analysis

The obtained data were analyzed and interpreted using SPSS 16 statistical software, Microsoft Excel, and Flowjo software, and $P<0.05$ was considered a significant difference.

Results

Results from exosome extraction

To investigate the secretion of exosomes by fibroblasts isolated from human skin under conditions of cellular stress, we collected equal volumes of supernatants from fibroblasts cultured in both serum-free and serum-containing media. The exosomes from these supernatants were then concentrated and extracted using the Total Exosome Isolation Kit (Thermo Fisher Scientific, USA). The figure below **(Figure 1)** shows that exosomes are present in the fibroblast supernatants, even under serum-deprived conditions.

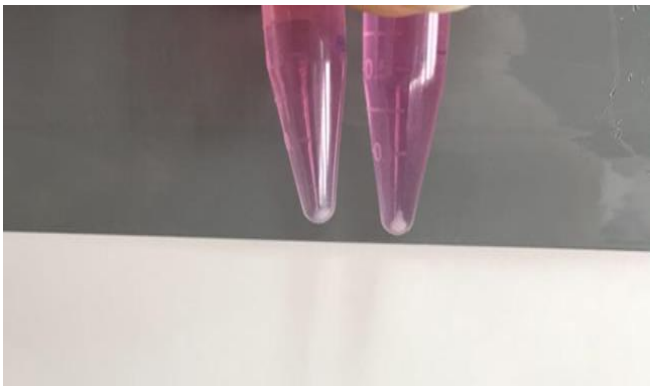
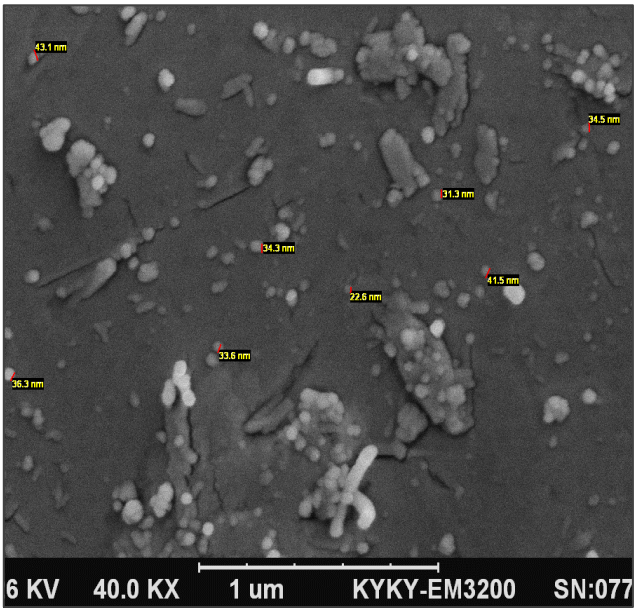


Figure 1. Pellet obtained by centrifugation of equal volumes of control (right) and serum-starved (left) fibroblast supernatants.

Results from scanning electron microscopy (SEM)

To investigate the morphology of exosomes and to prepare a positive control sample for flow cytometry, we prepared exosome sediment from the supernatant of fibroblasts cultured in a serum-containing medium. This sample was sent to the Rezaei Electron Microscopy Laboratory in Tehran for scanning electron microscopy (SEM) imaging. **Figure 2** illustrates the presence of exosomes in the fibroblast supernatant, with sizes ranging from 33 to 92 nm.



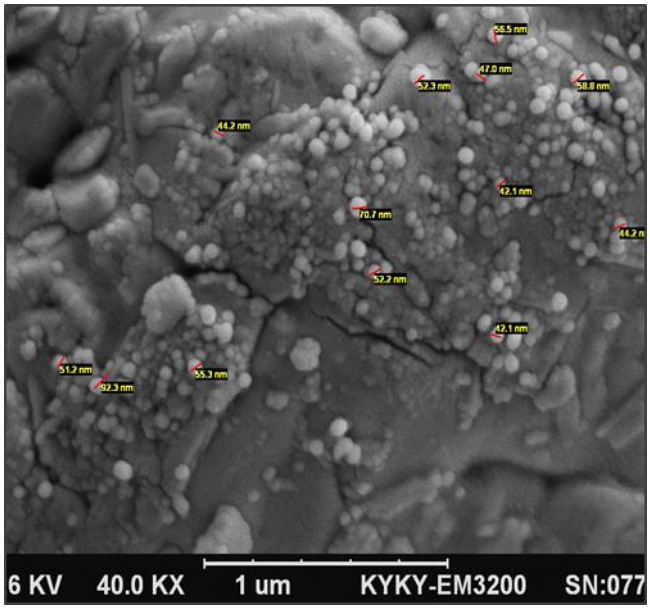
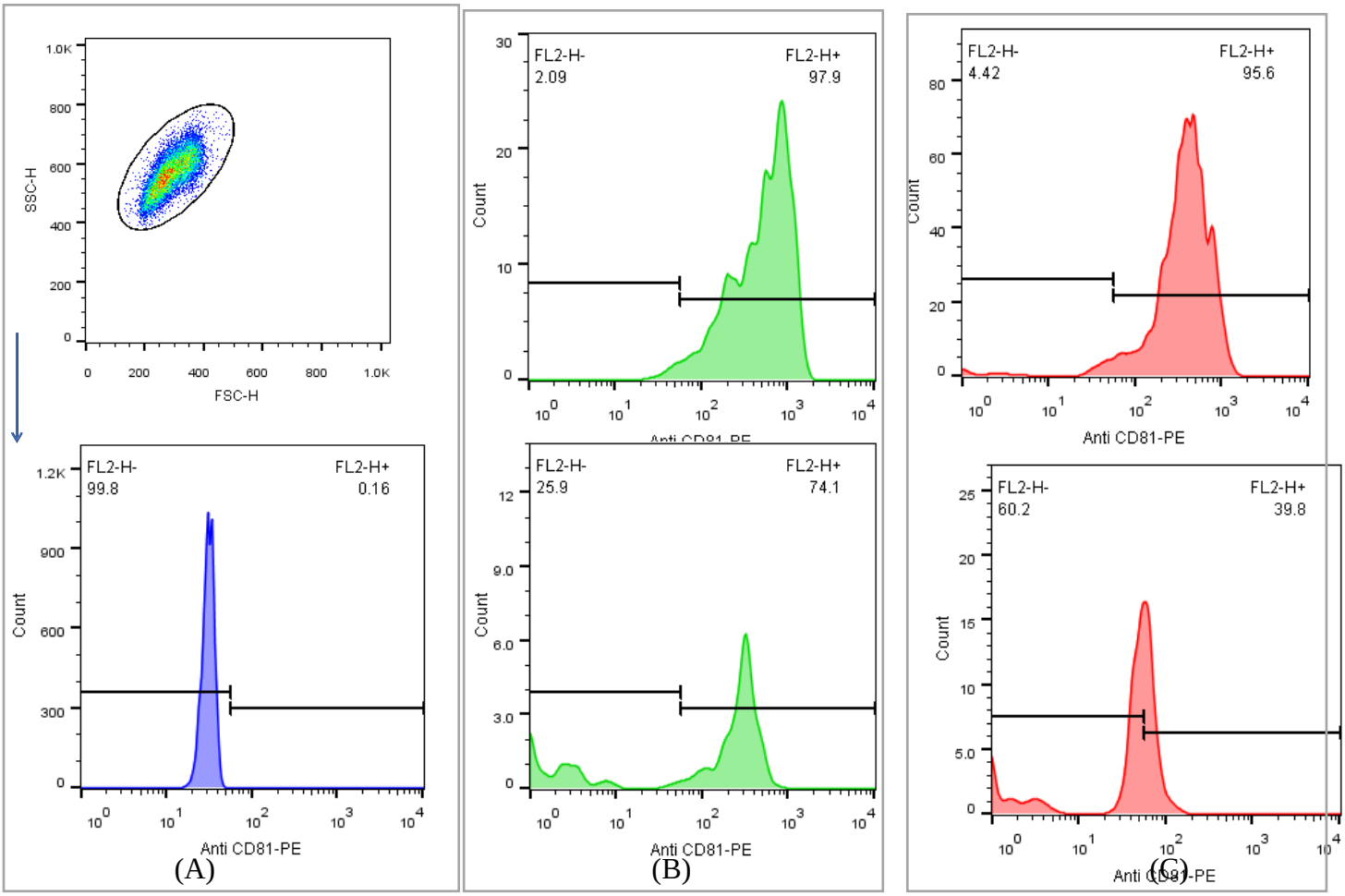


Figure 2. Results from scanning electron microscopy of exosomes.

Flow cytometry results

For semiquantitative measurement of exosomes, the supernatant from fibroblasts was purified using magnetic beads that contain antibodies specific to the CD81 marker. This was followed by labeling with a secondary antibody conjugated to a PE fluorescent marker. The analysis was conducted using a Becton-Dickinson instrument, focusing on the FL2 channel. The figure below illustrates the presence of CD81-positive exosomes in the supernatant of fibroblasts under both serum-containing and serum-free culture conditions. As shown in the image, the quantity of exosome production varies and is notably reduced in serum-free culture conditions (**Figure 3).**



	P 3 Fibroblast (Exosome+ 2nd Ab)	P 12 Fibroblast (Exosome + 2nd Ab)
Control group	Non-starved (97.9%)	**Non-starved (95.6%)**
Unstained Exosome (Exosome – 2nd Ab)	16hr Starvation (74.1%)	**16hr Starvation (39.8%)**

Figure 3. Exosome-containing vesicles in young and aged fibroblasts cultured in serum-containing and serum-free DMEM medium by flow cytometry. The findings indicate a decrease in exosome production in young and aged fibroblasts cultured in serum-deprived conditions, but this decrease is not significant (A= negative control, B= young passage 3 fibroblasts cultured in serum-containing and serum-free medium, C= aged fibroblasts cultured in serum-containing and serum-free medium)

Exosomes count (AU)

To count exosomes and determine their relative amounts, a measurement known as the optional unit was employed. The optional unit represents the relative amount of the desired substance compared to a reference amount established by each laboratory. In this method, a standard solution of exosomes, verified by microscopy, was prepared in various dilutions (1/1, 1/2, 1/4, 1/8, and 1/16), along with a zero dilution that contained PBS buffer. For creating the standard curve, the first dilution (1/1) was treated as equivalent to 10,000 exosomes. The exosome amounts were then quantified using this curve through Mean Fluorescence Intensity (MFI) measurements **(Figure 4).**

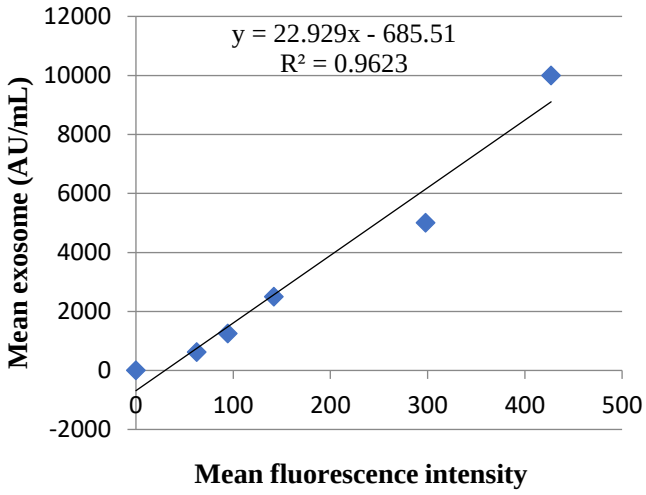
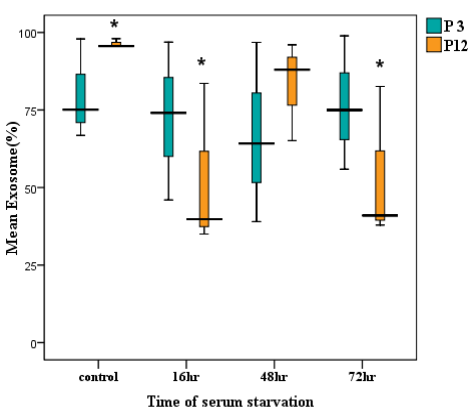


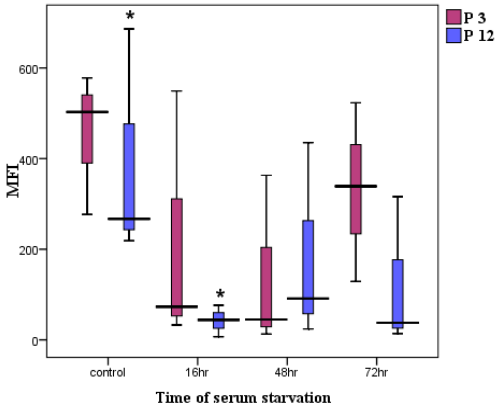
Figure 4. MFI standard chart.

Examination of the mean fluorescence intensity (MFI) of exosomes

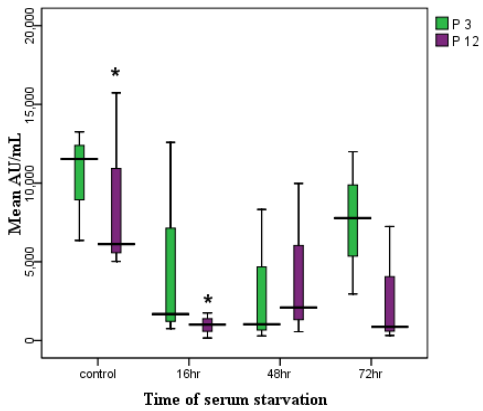
Flow cytometry results demonstrated that exosomes were secreted in the supernatant of fibroblasts cultured in both serum-containing and serum-free media, regardless of whether the cells were derived from young or old passages. This finding was further validated by measuring the average fluorescence intensity of CD81-positive exosomes. Analysis of the flow cytometry data revealed a significant reduction in the average percentage of exosomes produced by aged fibroblasts when cultured under serum-starved conditions for both 16 and 72 hours, compared to the control group (Figure 3-5-A). Additionally, the results indicated that exosome production in aged cells (passage 12) cultured in serum-containing medium, as well as in cells cultured in serum-free medium for 16 hours, was significantly lower (Figure 3-5-B). When calculating the relative number of exosomes using an optional unit, it was found that the number of exosomes in aged fibroblasts cultured for 16 hours under serum starvation conditions was also significantly reduced compared to the control group **(Figure 5-C)**



(A)



(B)



(C)

Figure 5. Examination of exosome abundance: A) Fibroblasts, after culture for 16 and 72 hours in serum-free DMEM, produced and secreted significantly fewer exosomes than the control group. B) The average fluorescence of exosomes also indicated a decrease in exosomes at passage 12 and after 16 hours of culture in a serum-free medium. C) Similar results were observed with relative counting of exosomes. (*: Significant difference, P3 = young fibroblasts, passage 3, P12 = old fibroblasts, passage 12, MFI = mean fluorescent intensity, AU = arbitrary unit, $P<0.05$, n=3)

Results from polyacrylamide gel electrophoresis

To investigate the protein profile of exosomes and the changes in protein expression under different culture conditions, we collected equal volumes of exosomal supernatants from both old and young fibroblasts cultured in serum-free and serum-poor mediums. All preparation steps were performed consistently across samples. As illustrated in Figures 3 to 5, our initial studies using SDS-PAGE analysis of the supernatants from fibroblasts cultured under serum-free and serum-poor conditions revealed a distinct change in the migration patterns of the protein contents of the exosomes over various durations of serum deprivation when compared to the control group. Notably, there was one exception: the supernatant from young fibroblasts cultured in serum-free conditions exhibited an electrophoretic pattern similar to that observed in serum-containing culture conditions (Figure 6).

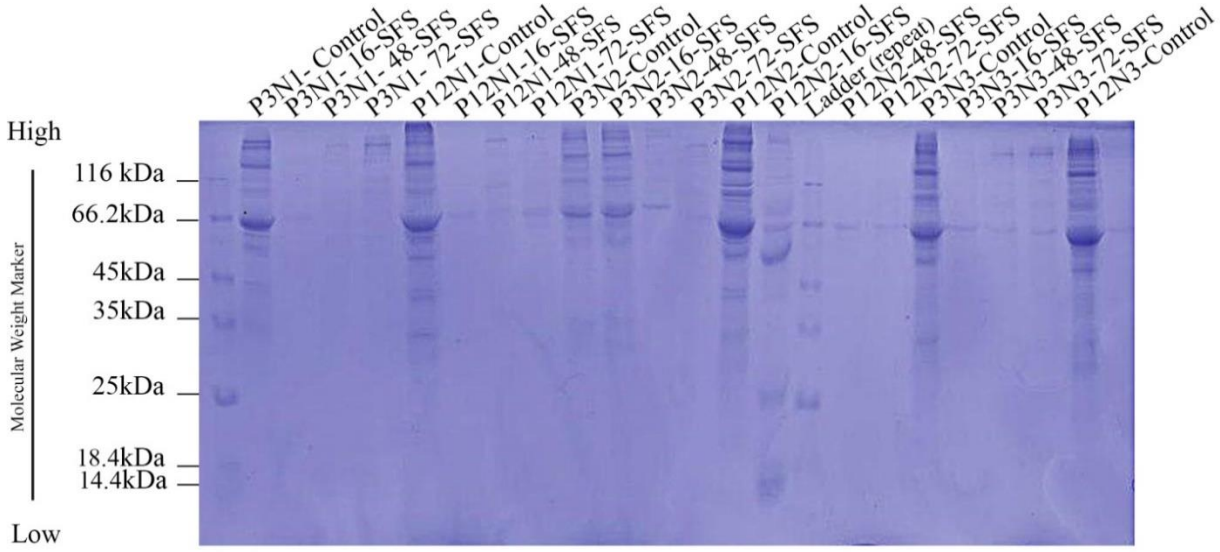


Figure 6. Electrophoretic pattern of proteins in exosomes in the supernatant of fibroblasts cultured in DMEM with and without serum (SFS). After concentrating the exosomes in the supernatant and examining their SDS-PAGE pattern, it is observed that the proteins in the exosomes have a molecular weight between 14-116kDa. Also, exosomes obtained from fibroblasts cultured in a serum-free medium have less protein than fibroblasts cultured in a serum-containing medium. Except one sample (P3N2-16-SFS) which has a protein pattern similar to its control group (P=passage, N=sample number, SFS=supernatant of fibroblasts cultured in serum-free DMEM).

Discussion

Exosomes are small membrane-bound vesicles secreted by various cells into the extracellular environment via endosomes. These vesicles play a significant role in intercellular communication and have garnered considerable attention from researchers due to their involvement in biological processes, including cancer (12). Despite advancements in biology and the identification of exosomes, the mechanisms of their action and the intricacies of cellular communication through exosomes in various processes and diseases remain a topic of debate (13). Previous research indicated that fibroblasts secrete proteins in serum-free conditions that can induce fibroblast migration and accelerate wound healing in animal models, such as rats. The results of the present study demonstrate that fibroblasts secrete exosomes in cell culture, and this secretion does not significantly decrease under serum-starved stress (14). However, it might be notable when considering larger sample sizes. In one sample, serum-starved conditions appeared to increase exosome production, although this increase was not statistically significant (15). This could be

attributed to the origin of the fibroblast cells used, as they were derived from neonatal skin samples, and the youthful age of the cells may influence the outcomes. The study found a significant decrease in exosome production from aged fibroblasts cultured under both serum-containing and serum-starved conditions for 16 hours. This was confirmed through measurements of mean fluorescent intensity (MFI) and arbitrary units (AU/ml) (16). Additionally, a significant reduction was recorded between fibroblasts cultured for 72 hours in serum-starved conditions compared to the control group. This decrease was determined by assessing the average percentage of beads containing exosomes. However, no significant difference was found between the control group and fibroblasts cultured for 48 hours under serum starvation. It can be speculated that the decline in exosome production during the first 16 hours occurs because the cells experience stress due to serum deprivation and lack the necessary time and capacity to adapt to the new conditions (17). After 48 hours, the cells likely adapt to their environment, leading to an increase in exosome secretion for intercellular communication. However, after 72 hours, exosome production decreased due to the lack of vital growth factors resulting from serum deprivation. Previous studies have shown that serum deprivation diminishes the production of fibroblast growth factor-2 (FGF-2) in hepatoma cells. FGF-2 is a polypeptide that regulates cell growth and is a powerful stimulator of endothelial cell growth, playing a crucial role in wound healing (18). Another study indicated that exosomes containing TGF-β1 secreted from damaged epithelial cells can activate fibroblasts and initiate wound-healing responses. This suggests that wound healing may be hindered by a decrease in the expression of such factors (19). Other research has found that serum deprivation for 48 hours in cardiomyocyte cells increased exosome secretion, which in turn induced angiogenesis in neighboring endothelial cells, indicating that cells tend to secrete higher levels of exosomes under stress conditions. This finding contradicts the results of the present study (20). Additionally, the current study reveals that human skin fibroblasts secrete a diverse array of proteins through exosomes, and this protein secretion capacity varies with the duration of serum deprivation, generally decreasing compared to serum-containing conditions (21). Notably, an increase in exosome production and a consequent rise in protein content were observed in the supernatant from passage 3 fibroblasts that were exposed to serum-free conditions for 16 hours. Consistent with this observation, studies have shown that human fibroblast cells from the WI-38 line, derived from lung tissue, enter the G0/G1 phase of the cell cycle under serum deprivation, with a significant increase in the expression of the P53 gene (22). Moreover, another investigation revealed that, in lung cancer cells, exosomal secretion increases under cellular stress conditions, such as DNA damage, which correlates with enhanced expression of the P53 gene and subsequent production of TSAP6 (Tumor Suppressor Activated Pathway-6) protein. The protein composition of these vesicles also changes in response to these stressors. The P53 gene is a tumor suppressor gene that typically regulates cell division in the G1/S phase (23).

Exosomes originate from the inward budding of the endosomal membrane, leading to the formation of multivesicular bodies (MVBs) that may either merge with lysosomes for degradation or discharge their contents into the extracellular environment. The makeup of exosomes is extremely diverse and mirrors the physiological condition of the originating cell. They include proteins like tetraspanins (CD9, CD63, CD81), heat shock proteins, and different cytokines that can affect the behavior of recipient cells (24). The molecular content of exosomes is essential for their role in facilitating intercellular communication and aiding tissue regeneration. Exosomes from different cell types have been demonstrated to improve wound healing via multiple mechanisms. Fibroblasts, essential to the wound healing process, release exosomes that aid in cellular migration, proliferation, and remodeling of the extracellular matrix (ECM). For example, research conducted by Zhang et al (25). Showed that tiny particles from fat stem cells (ASCs) helped heal skin wounds faster by improving skin cells and boosting collagen production in mice(25). Histological studies showed elevated production of collagen I and III in the initial phases of wound healing after exosome therapy. In a similar vein, a study by Ahmadpour et al. demonstrated that exosomes from fibroblasts greatly enhanced skin wound healing in a rat model of full-thickness skin ulcers (26). The research indicated that treatments with both low and high doses of exosomes led to enhanced re-epithelialization and granulation

tissue development when compared to the control groups. These results highlight the promise of exosomes derived from fibroblasts as a new treatment approach for improving wound healing. The ways in which exosomes enhance wound healing are complex. A crucial element is their function in regulating inflammation. Exosomes have the ability to transport anti-inflammatory cytokines that assist in modulating the inflammatory reaction during tissue healing. For instance, exosomes generated from dermal fibroblasts have demonstrated the ability to reduce pro-inflammatory cytokine secretion in endothelial cells subjected to elevated glucose levels, indicating a protective function against inflammation-related harm (27). Additionally, exosomes promote angiogenesis—the creation of new blood vessels—by transporting pro-angiogenic factors like vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). Research conducted by Chen et al. (2021) showed that subcutaneous administration of exosomes from diabetic fibroblasts enhanced angiogenesis and sped up wound healing in diabetic rats by stimulating the Akt/β-catenin signaling pathway (28). This underscores the possibility of utilizing exosome-based treatments to enhance healing results in wounds related to diabetes. Although exosomes derived from fibroblasts have demonstrated encouraging outcomes, research comparing various exosome sources shows differing levels of effectiveness. For example, exosomes derived from antler stem cells (AnSC-expos) have been shown to enhance regenerative skin wound healing more efficiently than those from bone marrow mesenchymal stem cells (bMSC-expos). AnSC-exosomes not only accelerated the healing process but also enhanced the quality of the healed skin by preventing fibroblast-to-myofibroblast transition (FMT), a process related to scarring. On the other hand, studies suggest that ASCs-Exos might impede collagen production in the later phases of wound healing to minimize scar development (25, 29). This twofold function—facilitating initial recovery while possibly reducing scarring—demonstrates the intricacy of exosome roles and their effects on tissue repair that depend on the context.

Clinical Implications and Future Directions

The potential of exosome-based treatments for wound healing is significant. Their capacity to improve fibroblast activity and adjust inflammatory reactions makes them strong candidates for medical uses. Ongoing research is concentrated on enhancing techniques for isolating and characterizing exosomes to guarantee reliable quality and effectiveness for therapeutic applications. Future research should additionally examine the long-term impacts of exosome therapies on wound healing results and assess their safety profiles in clinical environments. Moreover, comprehending how various environmental elements affect exosome release and structure will be vital for creating targeted treatments designed for particular wound types or patient groups. Exosomes thus play a vital role in intercellular communication, having important consequences for wound healing. Their varied molecular content enables them to influence multiple facets of tissue repair, such as inflammation, angiogenesis, and ECM remodeling. As studies progress in clarifying the mechanisms driving their effects, therapies utilizing exosomes are anticipated to assume a more significant position in regenerative medicine and medical treatments focused on improving wound healing.

Conclusion

In summary, it has been concluded that human skin fibroblast cells can produce and secrete exosomes even in response to serum deprivation and during the aging process. These exosomes transport proteins that help sustain cell life under the severe metabolic conditions resulting from serum deprivation and maintain cellular communication by effectively utilizing growth factors in their environment. This altered exosome production may influence wound healing in both young and elderly individuals, potentially reducing the wound healing capacity associated with aging, which may be linked to a decrease in exosome production. A pertinent question that arises is: What specific proteins do exosomes secrete in serum-free cell culture conditions that can impact cellular communication between fibroblasts? By identifying these proteins more precisely, it is possible to understand the relationship between exosomes and fibroblasts, as well as their connection to various diseases. Additionally, this knowledge could help in regulating intercellular communication by developing specific antibodies against these proteins. Various studies have indicated that exosomes and their contents can have diverse effects on cells and even on

distant tissues. Currently, it is believed that exosomes can be utilized in the treatment of diseases. Recent research has demonstrated that exosomes play a crucial role as mediators in cell therapy for various conditions. These vesicles have several advantages over cells in the treatment of disease, for example, exosomes have a lipid bilayer membrane that makes them resistant to lyophilization and manipulation while maintaining their biological activity. Also, exosomes induce minimal toxicity and immune response due to their small size, reducing the likelihood of their phagocytosis by immune cells. Exosomes become effective after delivery and entry into the body, and they are easier to transport compared to living cells due to their non-living nature. Additionally, exosomes can cross the blood-brain barrier. However, concerns remain regarding the use of these vesicles. For instance, how specific and efficient are exosomes in delivering proteins and RNAs into target cells or tissues? How can the amounts of specific and desired contents within exosomes be regulated? Finally, how can large quantities of exosomes be harvested for therapeutic use? This research can serve as a model for addressing these questions. Its findings have opened a new perspective on understanding the mechanisms of intercellular communication under stress conditions, such as during wound healing or the onset of tumor growth, where fibroblasts play a crucial role in these homeostatic and pathological processes. Future studies should focus on determining the specific proteins that make up these exosomes. Additionally, the effects of these exosomes on the growth and function of target cells, such as vascular endothelial cells or epidermal cells, as well as on various cancer cell lines, should be investigated. Lastly, the development of antibodies against exosomal proteins could help explore potential changes in communication between these cells.

Suggestions

a) Utilize techniques such as Immunoblotting to analyze the protein profile of exosomes present in the supernatant of fibroblasts in greater detail.

b) Employ molecular methods to investigate the expression of genes that are involved in the secretion of exosomes by fibroblasts.

c) Examine the metabolism of these cells under serum-starved conditions by measuring the activity of various enzymes, such as acetylcholinesterase, in exosomes using more precise and efficient techniques.

Author contribution

NGH reviewed and edited the article, collaborating on multiple aspects of the project. **AM** contributed to data analysis, collected the data, and co-wrote the article. **BS** conceptualized the project, supervised its development, and thoroughly edited the final version. All authors reviewed and approved the completed article.

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Conflict of interest

There is no Conflicts of interest/competing interests.

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