



Exosome isolation by ultracentrifugation and precipitation methods from human adipose-derived mesenchymal stem cells and the effects on human colon cancer cell line

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ABSTRACT

Exosomes, one of the extracellular vesicles, mediate molecular information and intercellular communication between cells by containing various proteins, lipids, mRNA, metabolic enzymes and numerous essential molecules such as miRNAs. The therapeutic potential of paracrine factors of exosomes released from human adipose mesenchymal stem cells is still under investigation. In our study, we isolated exosomes released from human adipose mesenchymal stem cells by two different methods. To evaluate the cytotoxic effect of the obtained exosomes, we performed MTT assay in SW-480 cell line at 24, 48 and 72 hours. In the results obtained, no statistically significant difference was observed at 24 and 72 hours, while statistically significant differences were determined between the concentration groups at 48 hours.

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Introduction

Many cells release exosomes, tiny membrane vesicles with an endocytic origin. Additional groups reported that cultured reticulocytes secreted vesicles of endocytic origin, whereas exosomes were first identified as microparticles with 5'-nucleotidase activity produced from neoplastic cell lines [1]. Exosomes are endosomal-derived and the smallest extra vesicles (EV), measuring between 30 and 150 nm in diameter [2]. The unifying features of EVs and exosomes are that they operate as carriers of molecular information in cell-cell communication and convey cargo molecules important in both physiological and pathological processes to recipient cells [3]. In addition, the prevalence of exosomes in all bodily fluids indicates their stability in extracellular contexts and explains their potential for endocrine communication. Recent clinical interest in these vesicles has arisen due to their potential use in diagnostic applications and as part of innovative treatment techniques [4]. Secreted from almost all cell types, exosomes have become an area of extensive research, extending beyond immunology to neurobiology, stem cell and tumor biology, and their use as biomarkers or therapeutic tools in clinical applications [5]. Under the right circumstances, human mesenchymal stem cells (hMSCs) can develop into mesenchymal tissue lineages, including muscle, bone, cartilage, and adipose. They have also been extensively researched as a potential reserve cellular fraction for tissue maintenance and repair [6]. Recently, human adipose mesenchymal stem cells (hAMSCs) are become a potential source for stem cell banks and an ideal source of cell cultures for tissue engineering. Studies have shown that hAMSCs can be easily obtained from adipose tissue without ethical concerns or transplantation issues. These cells have high proliferation rates and multilineage differentiation capacity for cultivation in vitro [7]. In research about MSCs, bioactive compounds released by MSCs are thought to be the primary therapeutic approach with cell adhesion and differentiation; they have varying therapeutic benefits in conditions such as liver damage and arthritis. [8]. Abundant data have shown that human adipose mesenchymal stem cells derived exosomes (hAMSCs-exo) are involved in angiogenesis, immunomodulation, reduction of ischemia-reperfusion-induced injury, and other processes. It is then explained by the latest research studies such as myocardial repair, neuroprotection and neurotrophic effects, hepatic repair, renal repair, skin repair, and regeneration [9]. Exosomes are small extracellular vesicles that contain functional molecules from host cells and participate in intercellular communication. Among all the bioactive substances contained in these vesicles, microRNAs and nucleic acids are widely investigated. However, exosomal proteins play an equally important role in signal transduction and regulation [10]. The

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target cells of their microenvironment can absorb exosomes released into the extracellular environment, and biological fluids like blood, urine, breast milk, and cerebrospinal fluid can carry them to distant locations. When disease or damage disrupts the microenvironment, MSC-derived exosomes are essential for maintaining tissue homeostasis and allowing the tissue to respond to external stimuli. Therefore, it might provide insight into the paracrine activity regulation mechanism of MSCs that underlies their tissue-specific regeneration capabilities [11]. Considering all these properties of MSCs, their role in tumor development has also been intensively studied. Studies have reported that the effects of MSCs on the initiation and progression of tumor development are both pro-tumorigenic and anti-tumorigenic. Currently, this paradox is poorly understood due to the paucity of mechanisms governing the interactions between MSCs and tumor cells. These mechanisms suggest that MSCs may act on tumor cells through direct cell-cell interactions or by secreting various diffusible factors such as growth factors and cytokines [12]. MSC-derived exosomes (MSC-exo) have been proven to be promising therapeutic tools because they include anti-tumor mediators of MSCs on cancer cells, which can decrease tumor growth. Nevertheless, MSC-exo, which are divided into three categories—using MSC-exo as therapeutic carriers, using tumor suppressor MSC-exo as therapeutic vehicles, and suppressing tumorigenic MSC-exo as therapeutic targets offer a practical delivery vector that can prevent the degradation of therapeutic molecules due to their lipid bilayer membranes [13]. Exosome isolation is one of the most important steps for accurately detecting exosomal contents. Many methodologies are used to isolate exosomes, including ultracentrifugation, size exclusion chromatography, ultrafiltration, immunoaffinity isolation, microfluidic techniques, and polymeric precipitation method [14]. The research has led to the lack of a standardized technique for isolating exosomes. To assess the quantity and effectiveness of exosomes and to provide insight into previous researches, we employed ultracentrifugation and the Total Exosome Isolation Kit (TEI) in our investigation. Determining the exosomes lethal impact on colon cancer cells was another goal.

Material and Methods

Growing cells

Human adipose-derived mesenchymal stem cells (Merck, USA, catalog number: SCC038)) were purchased commercially. Human Mesenchymal-LS Growth Medium (SCM023, Merck) and Dulbecco's Modified Eagles Medium (DMEM) low glucose culture medium (Catalog number: 11885084, Gibco) were used for cell growth. For SW-480 cells, DMEM high glucose culture medium, 10% Fetal bovine serum (FBS) (ECS0182L, Euroclone) was used. Under a microscope, the growing cell morphology was examined (Figure 1. A, B).

Exosome isolation from cell culture medium

The hAMSCs grown in cell culture were incubated in a serum-free medium for 72 hours for exosome isolation. Cells kept in a serum-free medium were checked for density and viability under a microscope. Then, at the end of the period, the medium was collected and made ready for isolation using both ultracentrifugation and TEI kit methods.

Exosome isolation by ultracentrifugation

Exosome isolation using the ultracentrifugation method was performed at Kocaeli University Stem Cell and Gene Therapy Research and Application Center (KOGEM). Here, the amount of media collected for exosome isolation and the total number of cells were determined. Using 120 ml of cell culture medium, the steps of exosome isolation by ultracentrifugation method are briefly given (Figure 1. C). Accordingly, the collected medium was centrifuged at 300 xg for 10 min, 15.000 xg for 10 min and the supernatant was passed through a 0.22 µm sterile filter. Then 120.000 xg was centrifuged for 70 min and the supernatant was removed. After washing with PBS and centrifugation at 120.000 xg for 70 min, the exosome pellet was obtained and dissolved with PBS.

Exosome isolation by Total Exosome Isolation Kit method

The amount of medium required for exosome isolation and the total number of cells were determined. TEI reagent (Catalog number: 4478359, Thermo Fisher Scientific) exosome isolation kit was used. Isolation steps were then followed according to the kit instructions (Figure 1.D.). According to the amount of medium collected, 500 µl of TEI reagent was added to 1ml of cell culture media and left to incubate at 4 °C overnight. The next day, centrifugation was performed at 10.000 xg for one hour at 4 °C. The supernatant was then removed and the exosome pellet obtained was dissolved in phosphate-buffered saline (PBS). Isolated exosomes were kept at 2 to 8 °C for short-term storage and at -80 °C for long-term storage.

Protein quantification by Bradford method

A popular technique for animal proteins is the Bradford protein test [15], which is based on the Coomassie brilliant blue G-250 dye binding to a protein complex that produces absorbance at 595 nm. Here, the protein

content of exosomes isolated by ultra-centrifugation and kit method was evaluated by plotting the standard bovine serum albumin (BSA) graph to determine the stability of the analysis. For Bradford analysis, the soluble protein was extracted from the isolated exosome samples using RIPA buffer. Bradford analysis and protein extraction using RIPA buffer were carried out in accordance with Yıldırım et al. 2023 [16]. Since no separate method was used for exosome quantification and exosome treatment at different concentrations, the amount of protein determined by the Bradford assay was used.

MTT Assay

3-[4, 5- dimethylthiazol- 2- yl]- 2, 5 diphenyltetrazolium bromide (MTT), one of the cytotoxicity tests, is one of the tests that precisely measures cell viability and proliferation in vitro by measuring metabolic activity in cells [17,18]. Briefly, SW-480 cells were incubated overnight in 96-well tissue culture plates at 10,000 cells/well. Then, 24, 12, 6, 3, 1.5, 0.750, 0.375, 0.187 ng/μl hAMSC-derived exosomes were applied and incubated at 37 °C with 5% CO₂ for 24, 48, and 72 hours. At the end of the time, MTT was applied with a final concentration of 0.5 mg/ml and absorbance at 550 nm was taken at the end of 4 hours.

Statistical analysis

The software program Graph Pad Prism version 8.0.1 (GraphPad Software Inc., La Jolla, CA) was used to graph all of the results. The Graph Pad Prism One Way ANOVA test was used for all statistical analyses. The Shapiro-Wilk test, a numerical technique for assessing data normality, was used to verify that the results from the experimental investigations had a normal distribution. To look for significant differences between groups, Tukey's post hoc test was used in conjunction with a one-way analysis of variance for all normally distributed data. When $P < 0.05$, the results were deemed significant. The computer program Graph Pad Prism 9 was used to graph the results.

Results and Discussion

As mediators of chemical exchange and intercellular communication, exosomes are involved in both physiological and pathological processes. Exosomes can simultaneously transport a range of bioactive compounds and quickly deactivate or readily degradable components via several number of different pathways and locations. They can take part in immune system functions, tumor diagnostics and treatment, and tissue healing by securely delivering their cargo molecules to target cells [19]. In our study, it was aimed to grow hAMSCs healthily and perform exosome isolation studies using ultracentrifugation and kit protocol. In addition, protein quantification of the isolated hAMSCs-derived exosomes was performed. Then, the cytotoxic effects of the quantified exosomes on SW-480 cells were evaluated. Accordingly, the amount of exosomes isolated from 120 ml medium by ultracentrifugation method was determined as 3400 ng/μl by Bradford protein assay. The amount of exosomes isolated from 12 ml of medium with TEI kit was determined as 1193 ng/μl by Bradford protein assay.

Cells readily absorb MTT, which is used to measure cell viability and proliferation. It is then enzymatically converted to formazan, a black substance that builds up in cytoplasmic granules [20]. SW-480 cells, a colon cancer cell line, and hAMSC cells as exosome sources were used for MTT assay. SW-480 cells were seeded in 96-well plates and exosome treatment was performed. Exosomes were applied at 24, 12, 6, 3, 1.5, 0.75, 0.375, and 0.187 ng/μl and cell viability was determined by MTT assay. In the MTT study, cell viability in SW-480 cells after exosome treatment was determined in Figure 1. E, F, G. While no dose-dependent cytotoxic effect was observed in the results obtained, no statistically significant difference was observed when the control and PBS groups were compared at 24, 48 and 72 hours. However, the groups treated with varying quantities of exosomes at 24 and 72 hours did not differ statistically significantly from the control and PBS groups. Exosome treatment caused a statistically significant decrease in cells when the 0.75 ng/μl group, which was exosome treated for 48 hours only, was compared with the control group. When cell viability between exosome-treated groups was analyzed, statistically significant differences were found between 6 ng/μl and 0.75 ng/μl groups and between 0.75 ng/μl and 0.375 ng/μl groups.

Exosomes, which are tiny vesicles that range in size from 30 to 150 nm and are released by MSCs, can facilitate cell-to-cell communication by carrying a variety of biomolecules, including proteins, mRNAs, microRNAs, and long non-coding RNAs. In experimental models of tissue repair injury and aging, these nanoparticles exhibit regenerative benefits [23] Mesenchymal stem cells derived from bone marrow, adipose tissue, and the umbilical cord have been shown in numerous studies to be able to enhance wound healing by producing exosomes. Moreover, mesenchymal stem cell exosomes derived from adipose tissue are thought to promote vascularization, hastening the repair of diabetic skin wounds [24].

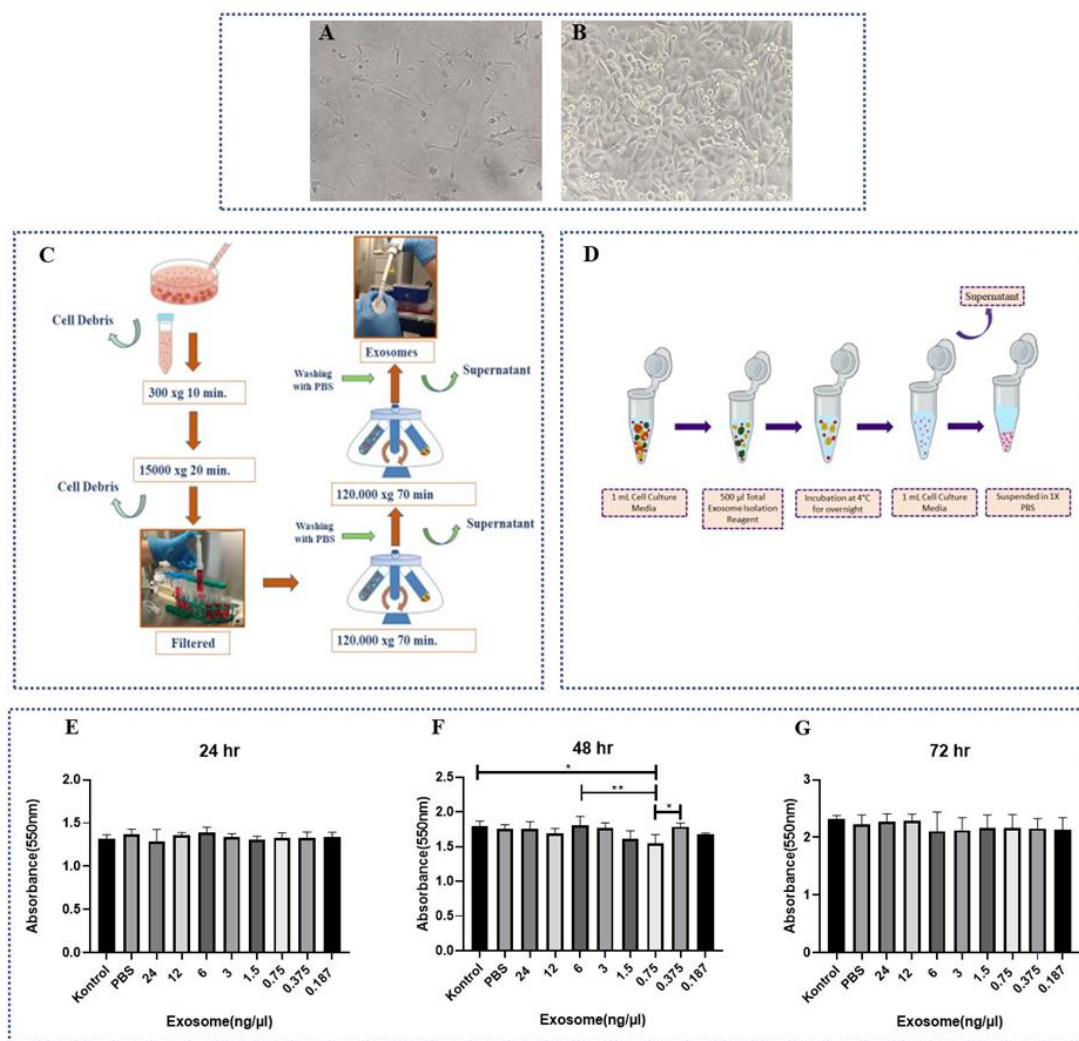


Fig 1 Application steps of two different methods used in exosome isolation with cell lines used in the study. MTT analysis results of 24h, 48h and 72 hours of exosome application. (A) microscopic image of hAMSCs (X10), (B) microscopic image of SW-480 cells, (C) exosome isolation by ultracentrifugation method, (D) exosome isolation by TEI kit method, (E) 24 h MTT analysis results, (F) 48 h MTT analysis results, (G) 72 h MTT analysis results. Some of the images in Figure 1. C are modified from Tang et al. 2017 and Willis et al. 2017 [21, 22].

Exosomes are believed to have antitumor capabilities and to be engaged in restricting the course of cancer, even though they have primarily been found to stimulate the growth of tumors. Exosomes and their contents have been shown in recent research to be a possible source of regulatory information on tumor progression and metastasis, particularly in the diagnosis of cancer. To ascertain the intricacy and functional diversity of exosomes, more research is required. Nevertheless, the typical physiological roles of exosomes in tissue homeostasis in the pathological remodeling of organs are unknown [25].

In our study, hAMSCs-exo were isolated using two different methods. The protein content of the exosomes obtained by ultracentrifugation and kit methods was calculated. There are many different methods in the literature on exosome isolation. In a study by Wang et al. 2015, they used three different methods to purify and analyze exosomes and to provide a better and more economical method for exosome research. These methods were the density gradient ultracentrifugation method, ultracentrifugation and ultrafiltration method and ExoQuick™ Extraction kit method. Exosomes obtained from THP-1 macrophage-like cells were isolated by 3 methods. As a result of Bradford analysis, the amount of protein obtained by the density gradient ultracentrifugation method was 1.09 µg/µl, the amount of protein obtained by the ultracentrifugation method was 1.38 µg/µl and the amount of protein obtained by the ExoQuick™ Extraction kit method was 1.82 µg/µl [26]. It was determined that the protein amounts obtained by ultracentrifugation and the kit method used in

our study were compatible with the studies in the literature and exosome isolation using the kit was more advantageous in terms of the amount of exosomes obtained.

Regarding exosome isolation, Helwa et al. 2017 conducted an exosome isolation study with commercial kits and ultracentrifugation method. Different kits and ultracentrifugation methods were evaluated in the results obtained. As a result of exosome isolation obtained from different amounts of serum samples, it was determined that the ultracentrifugation method had relatively larger particles than the kits. Centrifugation at such high speeds, however, may harm the physical characteristics of exosomes and the sensitivity of proteomic analysis by causing particles in the serum to fuse with impurities and other proteins. Using commercial exosome isolation kits has been linked to decreased exosome purity because more particles from reagents and components based on polyethylene glycol (PEG) may precipitate nanoparticles outside the exosome. Ultracentrifugation isolation is a feasible substitute for kit isolation of exosomes in serum samples isolated at varying volumes, as evidenced by the consistent diameter and concentration of exosome particles in serum samples. The conventional method for isolating exosomes, ultracentrifugation, has been used for a long time, but it requires expertise and an ultracentrifuge. Nonetheless, both kit and ultracentrifugation isolation produced consistent exosome particle diameters and concentrations in serum samples, suggesting that it is a good substitute for exosome isolation in serum samples isolated at various volumes. Despite being the conventional method for isolating exosomes for a long time, ultracentrifugation necessitates both experience and the availability of an ultracentrifuge. It is also technically delicate, time-consuming, and very tedious [27]. Based on this, we preferred the kit method in our study and continued our experimental studies. The size, concentration, morphology and purity of the exosomes obtained in exosome isolation are of utmost importance [28]. However, the characterization of exosomes is one of the most important steps. Our studies on the characterization of the exosomes isolated within the scope of our study are ongoing. However, within the scope of this article, it was aimed to isolate exosomes with only two different methods and to determine the cytotoxic effect of the isolated exosomes. The study requires an isolation procedure that meets these conditions and a standardized protein analysis. For this purpose, exosome isolation and quantification methods differ in the literature. Kechik et al. 2018 isolated exosomes from human saliva in their study. Then, they tried to characterize the obtained exosomes by SEM, NTA, Bradford and western blot analysis. They also evaluated the effect of protease inhibitors on the storage of exosomal protein. They concluded that the analyses largely agreed with one another. They specifically claimed that there was no discernible variation in exosomal protein concentration results across all settings in the protein assay. While clustering of exosomes was observed in SEM and NTA results, especially in NTA analysis, the concentration of isolated exosomes was calculated, but due to the clustering of exosomes, they stated that it is more reliable to use the exosomal protein concentration obtained from the protein assay result for in vitro studies of cells [29]. Based on this, we used Bradford analysis to determine the amount of exosomes isolated in our study and used the amount of protein for different concentrations of exosomes.

MTT assay of the isolated exosomes was performed in SW-480 colon cancer cells. Thus, cell viability and the cytotoxic effect of hAMSC-exo in colon cancer cells were evaluated. In Figure 1. E, F, it was concluded that there was no statistically significant difference between the control and PBS groups in the MTT analysis at 24 and 72 hours, and there was no statistically significant difference between the groups in the exosome-treated groups. In a study conducted by Hosna et al. 2018, antitumour effects were evaluated in different cancer cell lines by isolating exosomes from mesenchymal stromal cells. Accordingly, in the MTT analysis, no statistically significant difference was observed in the BT-474 cell line treated with exosome and the BT-474 cell line treated with exosome and doxorubicin [30]. In MTT analysis, no statistically significant difference was found between the control and PBS groups at 48 hours, but statistically significant differences were found between the control group and 0.75 ng/μl group, 6 ng/μl and 0.75 ng/μl group, 0.75 ng/μl and 0.375 ng/μl groups. Accordingly, there was a significant decrease between the control group and the 0.75 ng/μl group, a significant increase between the 6 ng/μl group and the 0.75 ng/μl group, and a significant decrease between the 0.75 ng/μl group and the 0.375 ng/μl group. In the literature, Kheradjoo et al. (2022) evaluated the cytotoxic effect of MSC-exo on retinoblastoma cells in a study they conducted. In the results obtained, it was stated that exosome application inhibited cell proliferation. At the same time, it was shown that exosome application induced apoptosis in retinoblastoma cells upregulated microRNA-143 expression and decreased Bcl-2 expression [31].

Conclusion

Overall, our study supports the feasibility of using these two isolation techniques to isolate sufficient exosomes. Thus, we conclude that the kit method is relatively more advantageous than the ultracentrifugation method. In summary, although the commercial kit in this study allows the isolation of a

limited amount of exosomes, it is an adequate alternative to the ultracentrifugation method and will encourage the understanding of the function of exosomes and support exosome studies. hAMSCs-exosomes have been shown to reduce proliferation in SW-480 cells. As a result of this study, characterization studies of exosomes are ongoing. In addition, the effect of hAMSCs-exosomes on SW-480 cells is being studied in more detail and transcriptome analysis studies are being carried out.

Abbreviations

EV:Extravesicles; hMSCs: Human mesenchymal stem cells; hAMSCs :Human adipose mesenchymal stem cells; hAMSCs-exo:human adipose mesenchymal stem cells derived exosomes; MSC-exo: MSC-derived exosomes; TEI:Total Exosome Isolation Kit; DMEM: Dulbecco's Modified Eagles Medium; FBS: Fetal bovine serum; PBS: Phosphate-buffered saline; BSA: Bovine serum albumin; MTT: 3-[4, 5- dimethylthiazol- 2- yl]- 2, 5 diphenyltetrazolium bromide.

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Availability of data and material

Please contact the corresponding author for any data request.

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