Journal of Research in Clinical Medicine
J Res Clin Med 2026;14:35479
doi:10.34172/jrcm.35479
https://jrcm.tbzmed.ac.ir
TUOMS PRESS

Original Article

# Storage Temperature Affected Cytoprotective Potential and Reciprocal Interaction of Exosomes with Endothelial Cells

**Narges Mardi[1,2], Reza Rahbarghazi[3,4*], Morteza Milani[1*], Amir Mehdizadeh[5], Mehdi Talebi[5,4], Mohammad Nouri[1]**

[1]Department of Medical Biotechnology, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[2]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[3]Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran
[4]Department of Applied Cell Sciences, Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran
[5]Hematology and Oncology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

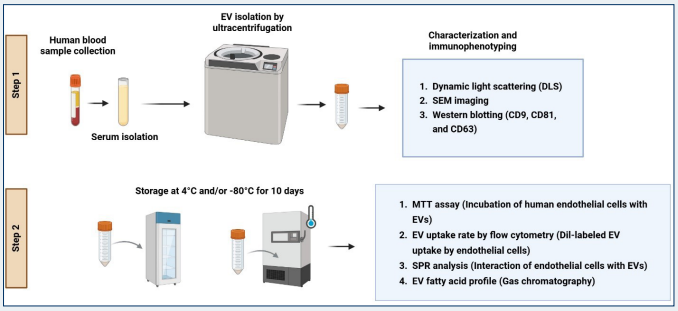
**Article info**

**Article History:**
**Received:** July 19, 2025
**Revised:** September 11, 2025
**Accepted:** September 22, 2025
**ePublished:** August 10, 2026

**Keywords:**
Endothelial cells, Extracellular vesicles, Physical interaction, Storage temperature, Viability, Uptake

**Abstract**

**Introduction:** Scalable approaches to prepare off-the-shelf extracellular vesicles (EVs) products are mandatory for clinical uses. Here, the possible effects of storage temperature were studied in human EVs.




**Methods:** Human serum Exos were isolated and characterized using western blotting, SEM, and DLS methods. The isolated Exos were pooled, randomly classified into two main groups [normal EVs (4°C) and frozen EVs (-20°C)], and stored for 10 days. After that, the cytoprotective effects of stored Exos were studied on HUVECs using an MTT assay. The uptake of Dil-labeled Exos was monitored by HUVECs using flow cytometry. Reciprocal interaction of EVs with HUVECs and EV fatty acid profile were assessed using surface plasmon resonance (SPR) and gas chromatography (GC).

**Results:** Western blotting confirmed the existence of specific tetraspanins (CD8, 63, and 81) on the samples. Based on the data, isolated EVs exhibited an average size of 40.1±25.6 nm with a zeta potential value of -11 mV. Compared to freshly isolated EVs and EVs stored at 4°C, frozen EVs increased the viability of HUVECs ($P<0.05$) with the lack of significant difference in the internalization rate of normal and frozen EVs ($P>0.05$). Of note, SPR analysis revealed the increase of EV-HUVEC interaction in EV stored at -20°C indicated higher relative response levels (RU) values (~1.245-fold). GC showed an increase in unsaturated fatty acid content in frozen EVs compared to EVs maintained at 4°C.

**Conclusion:** These data indicate that the storage temperature can influence the cytoprotective potential, cell interaction capacity, and fatty acid profile of human EVs.

## Introduction

The paracrine interaction among prokaryotic and eukaryotic cells can be orchestrated using extracellular vesicles (EVs).[1,2] Exosomes (Exos) are EV subsets with an average diameter between 50-150 nm and are produced by the intracellular endosomal system.[3] It has been elucidated that certain cargo and signaling molecules can be sequestrated onto the Exo lumen using intricate molecular complexes.[4] Of note, recent documents have pointed to the fact that the application of Exos has several advantages compared to whole-cell-based therapies.[5] These nanoparticles can be used for different therapeutic purposes such as gene therapy, on-target drug delivery, tissue regeneration, cancer therapy, and immunogenic vaccine fabrication.[6]

However, the administration of allogeneic Exos can circumvent limitations described for cell-based therapies.[7] For instance, Exo administration does not provoke allo-

***Corresponding Authors:** Reza Rahbarghazi, Emails: rahbarghazir@tbzmed.ac.ir, rezarahbardvm@gmail.com and Narges Mardi, Email: mardinarges@gmail.com

reactive T lymphocytes and splenic reticuloendothelial cells.[8-10] Meanwhile, the trap of Exos is less compared to transplant cells in tissues such as pulmonary tissue, splenic, and hepatic niches.[11,12] These features make the Exos a valid therapeutic biological element for the alleviation of pathological conditions.[13]

Despite the introduction of Exos as a promising cell-free therapy tool, their application in medicine faces critical limitations that need further investigation. For example, Exo isolation is time-consuming, expensive, and laborious, and the possibility of infection transfer is also possible.[14] For therapeutic purposes, repeated doses of Exos with certain intervals are mandatory, which necessitates the development of technologies for large-scale production and storage.[15,16] Exo stability, membrane integrity, content retainability, and biological function should be at the center of attention in terms of freezing and storage for short and long periods.[17]

Maroto et al examined the effects of storage temperature on bronchoalveolar lavage Exos in terms of size and content, evaluated by DLS, LC-MS/MS.[11] They indicated that the storage of Exos at different temperatures, 4 °C and -80°C, led to an increase in Exo diameter to 10 and 25%, respectively, compared to the freshly isolated Exos. Along with these changes, the zeta potential values and exosomal protein content were also reduced.[11] Other studies confirmed the modulatory effects of storage temperature on the Exo surface and luminal marker contents evaluated by proteomic analyses.[18] In contrast to these data, Park and co-workers indicated that the detrimental effects of 4°C on physicochemical properties were more prominent and related to freezing temperatures.[19] In support of this notion, Lee et al found that the storage of HEK293 cell Exos at temperatures below -70°C can preserve the integrity and functionality of Exos compared to higher temperature values.[20] These data have shown the lack of enough data related to the supportive/detrimental effects of storage temperatures on Exo function and physicochemical properties.

Here, we aimed to examine the possible effects of stored EVs at different temperatures (4°C and -20°C) on the survival rate and uptake by human umbilical vein endothelial cells (HUVECs) *in vitro*. It seems that data from the current study can help us design and develop freezing conditions for prolonged EV storage for therapeutic purposes.

## Material and Methods

### *Endothelial cell culture*

In this study, human umbilical vein cells (HUVECs; NCBI code: C554) were purchased from Pasteur Institute (Tehran, Iran). Cells were cultured in high-glucose content DMEM (DMEM/HG; Cat no: DC-99200-500; Dacell) culture medium containing 10% fetal bovine serum (FBS; Ref no: 10270-106; Gibco) and 1% Penicillin-Streptomycin (Cat no: BI-1203; Bioidea) inside the standard incubator (37˚C, 5% $CO_2$ and 95% relative humidity). Cells were subcultured using 0.25% EDTA-Trypsin solution (Cat no: XC-T1717; Biosera). In this experiment, cells of passages 3-6 were used for several analyses.

### *EV isolation*

To this end, human sera were collected in the Clinical Biochemistry Lab of Shahid Madani Hospital, affiliated with Tabriz University of Medical Sciences. All processes of this study were approved by the Local Ethics Committee of Tabriz University of Medical Sciences under award number 66627 and the ethical code of IR.TBZMED.VCR.REC.1400.158. All volunteers were asked to complete the informed consent. In this study, we used the remnant of individual sera without any interference in the therapeutic protocol. The collected samples were transferred to the Cell Culture Laboratory of the Faculty of Advanced Medical Sciences for EV isolation. In short, samples were centrifuged at 400 *g* for 5 minutes to exclude cells. The procedure was continued by the centrifugation of samples at 2000 *g* to pellet the cell debris. After that, using centrifugation at 12000 *g*, apoptotic bodies, organelles, and cell debris were eliminated. Finally, samples were centrifuged at 100,000 g (USA Optima) to obtain the Exo pellet. Before the cell culture procedure, samples were sterilized using 0.22 µm microfilters (Cat no: SEMCE0300228; BIOZOL GmbH; Germany). In this study, serum samples were pooled before EV isolation.

### *EV characterization*

*Scanning electron microscopy (SEM)*

Isolated EVs were collected and fixed using 2.5% glutaraldehyde solution (Sigma-Aldrich) for 20 minutes at 4˚C. After that, EVs were dehydrated using ascending EtOH solutions (60-100%) each for 20 minutes. The samples were then placed on aluminum foil and gold-sputtered. Samples were imaged using the SEM system [Model: MIRA 3; TESCAN, Czech Republic].

### *Transmission electron microscopy (TEM)*

The morphological properties of the isolated EVs were also examined by TEM assay. In this regard, 10 µg from extracted EVs was fixed with 2% paraformaldehyde (PFA) on the surface of a copper grid and air-dried; Then, the samples were negatively stained with 3% uranyl acetate. After washing, the samples were examined by TEM (LEO 906, Zeiss, Germany).

### *Western blotting*

For this purpose, collected EVs were lysed using RIPA lysis buffer, electrophoresed in 12% SDS-PAGE, and transferred onto PVDF membranes. The membranes were blocked with 1% bovine serum albumin (BSA) for 1 hour at room temperature (RT), and incubated with anti-human CD63 (Cat no: sc-5275; Santa Cruz Biotechnology), anti-human CD81 (Cat no: sc-166029; Santa Cruz Biotechnology), and anti-human CD9 (Cat no: sc-13118; Santa Cruz Biotechnology) antibodies for overnight at 4˚C. After three times PBST washes (each in 10 minutes), the membranes were incubated with HRP-conjugated secondary antibodies

(Cat no: sc-516102; Santa Cruz Biotechnology) for 1 hour at RT. After PBST washes (each in 10 minutes), immunoreactive bands were exposed using ECL (Bio-Rad) solution and X-ray films.

***EV storage at different temperatures***

To assess whether the storage temperature can affect the dynamic activity of isolated EVs, samples were maintained at two different temperatures (4 and -20˚C) for 10 days. After the completion of storage time, samples were subjected to different analyses.

***Cell survival rate***

The modulatory effects of isolated EVs stored at different temperatures on HUVECs viability, an MTT assay was performed. In brief, about 200 µL culture medium containing HUVECs ($2\times10^4$) was plated on each well of 96-well plates (SPL; Korea) and allowed to reach 70-80% confluence. After 24 hours, the supernatants were discarded and replaced with 200 µL (EV solution plus DMEM/HG) for 48 hours. To assess the survival rate, the supernatants were removed and replaced with 200 µL MTT solution (5 mg/mL; Cat No) and incubated at 37˚C for 3-4 hours. Then, 50 µL DMSO (Cat no: D4540; Sigma-Aldrich) was overlaid, and plates were agitated gently for 20 minutes. The optical density of each sample was read using an ELISA microplate reader at a wavelength of 630 nm. This assay was performed three times (each in octuplicate).

***Uptake of EVs by HUVECs***

The possible effect of storage temperature on EV uptake was measured using flow cytometry analysis. In brief, the Exos (~100 µg/ml exosomal protein) from different groups were stained with 20 µM Dil-CM (Cat no: C-7000; Thermo Fisher Scientific) at 37˚C for 20 minutes. To exclude non-bounded Dil-CM compounds, samples were washed with PBS using ultracentrifugation at 100,000 *g* for 1 hour. HUVECs were plated at an initial density of $1\times10^5$ per well of 24-well plates and allowed to reach 70-80% confluence. Thereafter, Dil-labeled Exos were added to the culture medium for 24 hours, and the percent of red-colored Dil-labeled cells was analyzed using flow cytometry (BD FACSCalibur) and FlowJo software (version 7.6.1).

***Monitoring EV-ECs using SPR analysis***

To assess whether storage temperature can affect the mutual interaction of EVs with cells, the physical interaction between EVs and HUVECs was examined. For this purpose, $4\times10^5$ were suspended in 500 µL DMEM/HG culture medium, plated on an Au SPR chip, and allowed cells attach to the surface. After 20-30 minutes, the plates were carefully transferred onto each well of 6-well plates with 3 ml culture medium (10% FBS and 1% Pen-Strep) and cultured under standard conditions. After reaching a single-cell monolayer, the EVs from different groups were introduced to Au SPR chips. Before analysis, the chips were washed carefully with fresh culture medium and placed into the SPR instrument holder (Model: MP-SPR Navi 210A, BioNavis Ltd., Tampere, Finland) at the Immunology Research Center, Tabriz University of Medical Sciences. After that, EVs (EV protein ~ 1.6 mg/ml) were perfused at a flow rate of 15 µl/min for 5 min into the SPR channel. The relative response levels (RU) values were monitored related to the interaction of Exos with the plated cells using a multi-parameter SPR device. Any changes in the SPR angle of both groups were recorded during the real-time injection.

***Fatty acid profile assessment using GC analysis***

To study whether storage temperature can affect the EV membrane fatty acids composition, fatty acid content was measured using CG according to a previously published experiment.[21] In brief, EV solutions were mixed with a methanol-hexane mixture and vortexed. The procedure was continued with the addition of acetyl chloride in a drop-wise manner and heating the samples at 100˚C for 1 hour. After cooling, the reaction can contribute to the formation of methyl esters, which were detected by GC. To neutralize the reaction, $K_2CO_3$ solution was added, and samples were subjected to centrifugation at 3000 rpm for 5 minutes to eliminate the protein fraction. The hexane phase was used for the analysis of methyl esters using a Teknokroma TR CN100 column ($60\times0.25$ mm) using a Buck Scientific gas chromatograph (Model: 610, SRI Instruments, Torrance, USA). Intensities were normalized to Tridecanoic acid (13:0) as an internal standard, and raw data were analyzed using PeakSimple, version 3.59 (SRI Inc). The percentage of saturated fatty acids (SFAs; Myristate, Palmitate, Stearate, and Pentadecanoate), monounsaturated fatty acids (MUFAs; Oleate), and polyunsaturated fatty acids (PUFAs; Linoleate) were analyzed, and expressed as a % of the total fatty acids.

***Statistical analysis***

Data (mean ± SD) are compared in each group using One-Way ANOVA with post hoc analysis. $P<0.05$ was considered statistically significant.

## Results

***EV characterization and immunophenotyping***

In this study, the isolated EVs were characterized in terms of size, morphology, and surface proteins (Figure 1A-D). SEM images exhibited typical spherical morphologies and an average size of 40.1 ± 25.6 nm (Figure 1A-B; yellow arrowheads). TEM imaging results confirmed the spherical and elliptical shapes of isolated EVs (Figure 1C). According to the DLS data, EVs had a zeta potential value of -11 mV ± 2.1 (Figure 1D). The proteomic analysis confirmed the existence of exosomal tetraspanins such as CD9, CD63, and CD81 in isolated samples (Figure 1E). These features indicate the success of the current protocol in the isolation and characterization of EVs, especially Exos. Further analyses in stored EVs indicated zeta potential values of −14.2 ± 1.3 mV and -10.6 ± 0.98 mV compared to the control group.

***EVs stored at -20°C affect endothelial cell survival rate***

To see whether storage temperature can affect the modulatory effect of EVs on human endothelial cell (EC) survival, an MTT assay was performed after 24 hours. Data indicated that the incubation of HUVECs with normal EVs (stored at 4°C) did not change the survival rate compared to the non-treated control group ($P > 0.05$). Statistically significant differences in terms of survival rate were achieved in groups that received frozen EVs (stored at -20°C) compared to control and normal EVs groups ($P < 0.05$). These features show that stored EVs at lower temperatures can increase the viability of HUVECs.

***Temperature storage did not affect EV uptake by HUVECs***

To see whether the storage temperature can affect the uptake ratio by human ECs, EVs were labeled with red fluorescence CM-Dil dye, and the entry rate was evaluated using flow cytometry analysis (Figure 2B-C). Data indicated a significant increase in fluorescent HUVECs in EV-treated groups compared to the non-treated control cells ($P < 0.0001$). These values were 90.8 ± 0.52% and 85.7 ± 2.52% in the Normal and Frozen EVs groups, respectively. The increase in fluorescence intensity is associated with the labeled EV uptake by HUVECs after 24 hours. Despite a slight change in fluorescence intensity in the Normal and Frozen EVs groups, these values did not yield statistically significant differences. These features indicate that storage temperature (4°C and -20°C) did not affect EV uptake by human ECs.

***SPR analysis revealed higher RU intensity in frozen Exos***

To assess the possible effect of storage temperature on EV-HUVEC interaction, an SPR analysis was done (Figure 3A-D). HUVECs were plated on SPR Au chips and allowed to adhere and acquire the normal morphologies, and analyzed using an SPR device (Figure 2A-C). Sedimentation and capture of EVs on the HUVEC-coated surface were monitored for 400 seconds. Data revealed the difference in EV density on the HUVEC-coated Au chips in terms of RU values. It was noted that the RU value increased in the Frozen EVs group than that of the Normal EVs group, indicating the difference in EV-HUVEC interaction. The injection of EVs stored at -20°C on HUVEC-coated chips yielded higher RU values (~1.245 ± 0.184-fold) compared to the Frozen EVs group. These features indicate that the interaction between the EVs stored at different storage temperatures differed when analyzed via a label-free and real-time SPR analysis.

***Fatty acid component of EVs was altered after being stored at different storage temperatures***

GC data indicated that the storage temperatures can affect the fatty acid profile of EVs after 10 days (Figure 4 and Table 1). Based on the data, our GC system detected six fatty acids, including myristate (14:0), palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2), and arachidonate (20:4) in EV samples. Of note, palmitate is the most dominant fatty acid component in Exo samples of 4°C (44.27%) and −20°C (44.28%) groups. Based on data, the percent of stearate was 0.24% in frozen EVs compared to 1.38% in normal EVs stored at 4°C. The levels of oleate and linoleate were increased in frozen EVs compared to the normal EVs (Figure 4 and Table 1). In contrast, arachidonic acid was diminished in EVs stored at −20°C (5.54 vs. 4.54%)

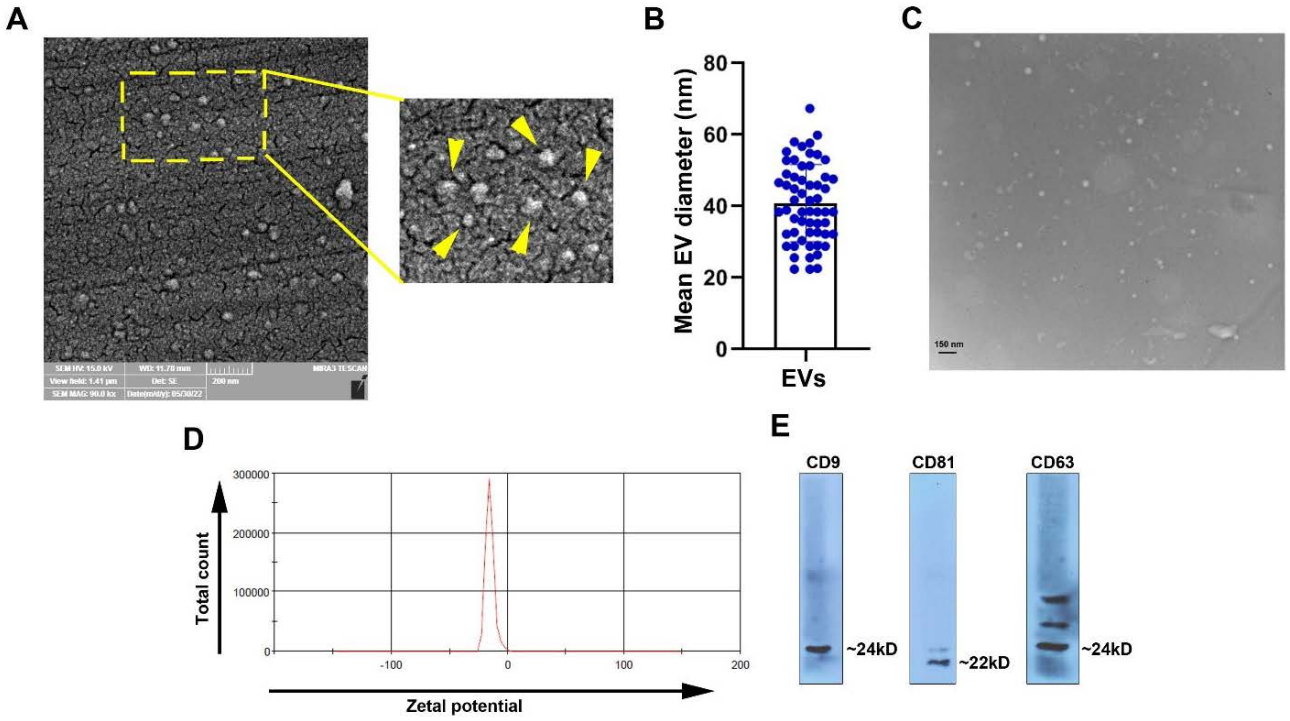



**Figure 1**. Physicochemical properties and immunophenotyping of isolated EVs (A-D). SEM images (A), and the mean size of values calculated based on 100 EVs (B). TEM images (C). DLS analysis revealed the existence of a negative charge on the EV surface (D). Western blotting of pooled EV samples (n=3) indicated the existence of typical exosomal tetraspanins CD9, 81, and 63 (E; Adapted with permission.[22] BioImpacts. 2026).

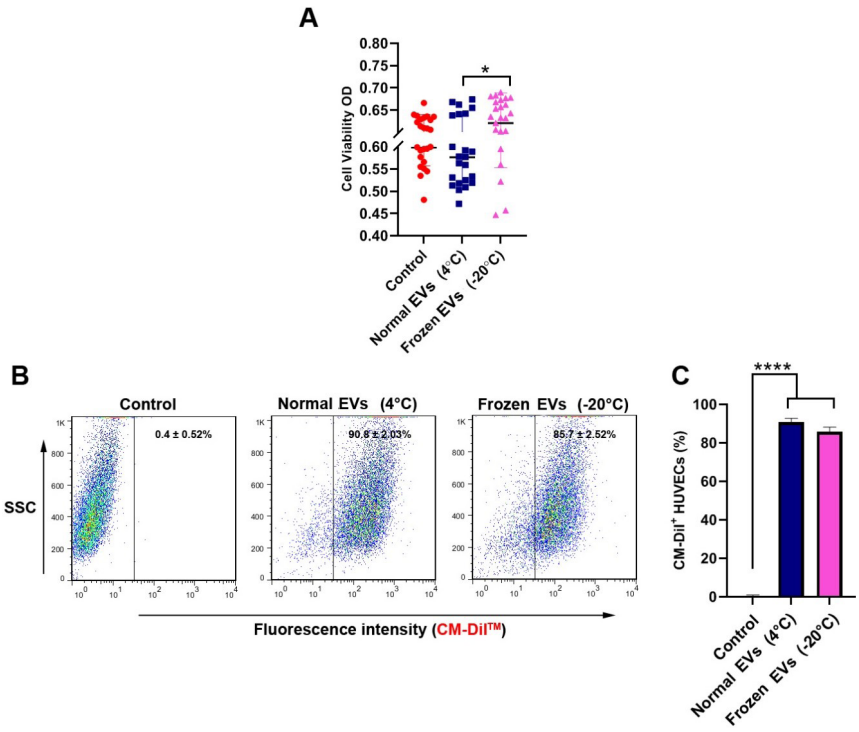



**Figure 2**. Monitoring the modulatory effects of EVs stored at 4 and -20°C on HUVEC survival rate (**A**) and uptake ratio after 24 hours (**B-C;** n = 3). Data indicated the increase in cell survival rate in frozen EVs compared to the control and normal EVs groups ($P < 0.05$). Flow cytometry analysis indicated the entry of DiI-labeled EVs in normal and frozen EVs groups compared to the non-treated control group ($P < 0.0001$; **B-C**). No significant differences were found in normal and frozen EVs in terms of uptake rate

compared to normal EVs. Interestingly, a slight increase in MUFA + PUFA/SFA ratio was found in frozen EVs (1.92 vs. 1.14) when compared to normal EVs stored at 4°C. These data indicate that storage temperature can influence the percentage of EV fatty acids.

## Discussion

In recent decades, the application of EVs, especially Exos, has been extended in human regenerative medicine for cell-free-based therapies.[23] However, these nano-sized particles cannot be stored for a long time at room temperature conditions.[20,24] Recent progress has revealed the efficiency of lower storage temperatures on the preservation of EVs for clinical demands.[11,20] Although the lack of standard EV and Exo isolation protocols, batch-to-batch heterogeneity, and metabolic status of parent cells affect the therapeutic outcomes, it has not been well-addressed whether storage temperature can influence the exosomal integrity and function compared to freshly isolated Exos.[24,25] Regarding the wide-scale application, accessibility, and feasibility of freezing at -20°C compared to low-temperature environments (-80, and -196°C). Here, we aimed to assess the possible modulatory effects of two different storage temperatures (4 and -20°C) on EV function and bioactivity. Here, stored EVs at 4°C and -20°C can affect the survival of HUVECs *in vitro*. These features were more effective in the group that received Frozen EVs (-20°C) compared to the control and Normal EVs (4°C) groups. One reason would be that the maintenance of EVs at -20°C or lower temperature environments can efficiently preserve the luminal cargo such as miRNAs, growth factors, and other peptides. While higher temperatures such as refrigerator conditions can lead to the loss of luminal cargo.[26] Lower temperature conditions diminish the catabolic activity of degrading enzymes and the stability of luminal cargoes.[27] It should not be forgotten that the type of storage conditions can differentially affect the EV cargoes and intensity. By the reduction of storage temperature, the leakage of specific cargo will be increased in EVs isolated from mouse bronchoalveolar lavage fluid.[11] This effect is closely associated with the formation of ice crystals, destruction of the lipid membrane, and osmotic imbalance during the freezing procedure.[28,17] Based on the current data, the stimulatory effect of EVs stored at -20°C on HUVEC survival could not be related to the uptake rate. In support of this claim, it was suggested that the storage of EVs at lower temperatures, *i.e.*, -80°C, can reduce the protein levels of certain factors related to cell growth inhibition, such as Annexins and other factors. Annexins are integral Exo proteins with diverse effects on target cell dynamic growth.[29] Whether Annexins can influence of HUVEC survival rate needs further investigation. The lack of significant differences in EV uptake by HUVECs after being stored at 4 °C and -20°C would be related to the involvement of several uptake mechanisms.[4] For example, the process of Exo uptake is orchestrated via various mechanisms such as direct fusion, receptor-mediated endocytosis, macropinocytosis, or phagocytosis.[3] In this case, we hypothesize that freezing and storage conditions can affect distinct Exo uptake mechanisms, the activity of another entry system in the target cells can compensate for the lack of functionality for specific pathways.[18,24] Commensurate

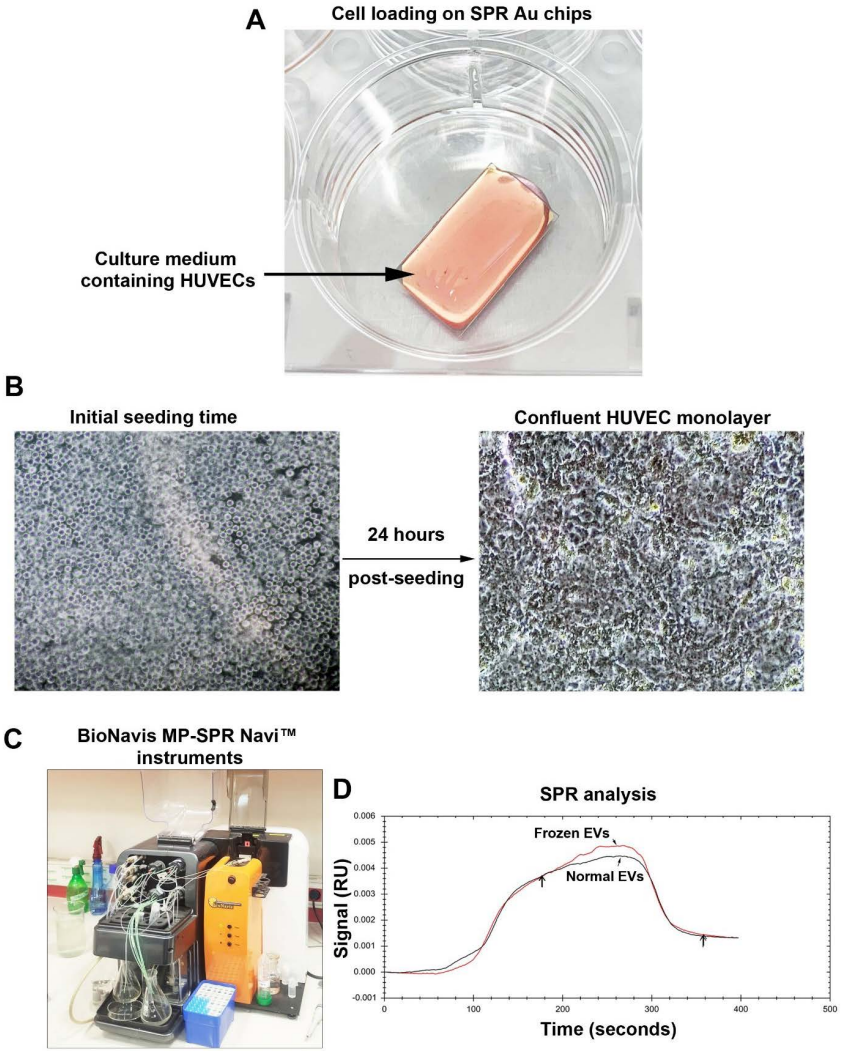



**Figure 3**. Analysis of the interaction between the normal and frozen EVs and HUVECs using an SPR system (**A-C**). 500 µL culture medium containing $4 \times 10^5$ HUVECs was loaded on SPR chips and allowed the cells to adhere (**A**). At the seeding time, cells exhibited round shape morphologies (**B**), indicated by bright-field images under an inverted microscope. After 24 hours, cells efficiently attached to the Au chip surface and appeared with normal morphologies similar to the culture system at the plastic surface (**B**). The injection of normal and frozen EVs led to changes in RU signals between both groups

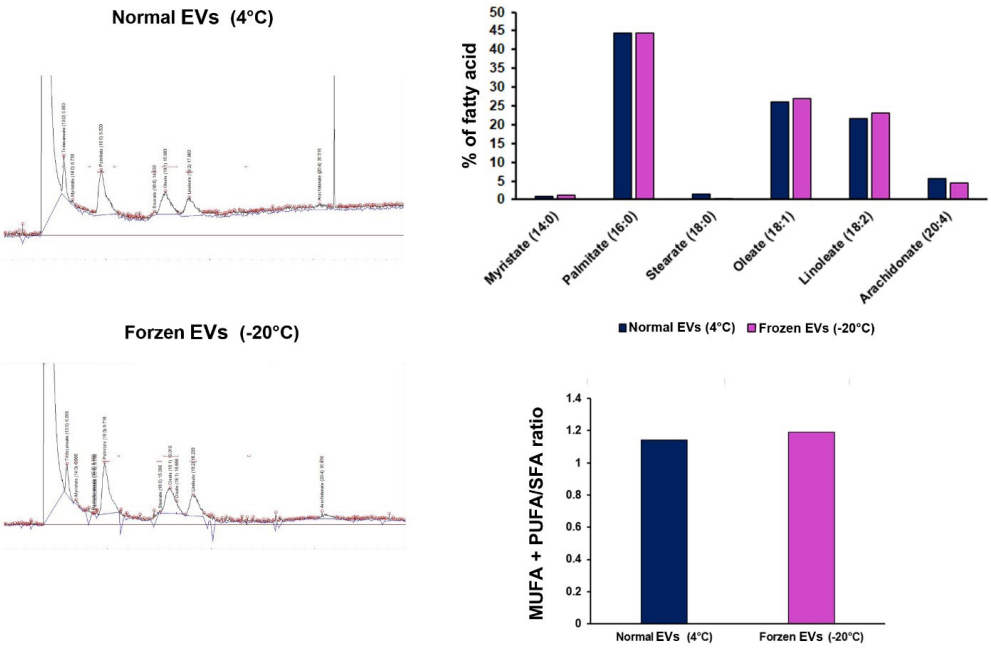



**Figure 4.** Effects of storage temperatures (4 and -20°C) on EV fatty acid profile after 10 days measured by gas chromatography. Data were obtained from three pooled samples

**Table 1.** Percent of fatty acids (SFA, MUFA, and PUFA) in Exos stored at 4°C and –20°C for 10 days.

| Component | Normal Exos (4°C) | | Frozen Exos (–20°C) | |
|---|---|---|---|---|
| | Area | Percent | Area | Percent |
| Myristate (14:0) | 0.837 | 1.00968672 | 1.894 | 1.084989 |
| Palmitate (16:0) | 36.698 | 44.26939455 | 77.306 | 44.28519 |
| Stearate (18:0) | 1.145 | 1.381232131 | 0.431 | 0.246901 |
| Oleate (18:1) | 21.706 | 26.184301 | 46.823 | 26.82283 |
| Linoleate (18:2) | 17.919 | 21.61598128 | 40.186 | 23.02078 |
| Arachidonate (20:4) | 4.592 | 5.539404321 | 7.924 | 4.539309365 |
| Sum | 82.897 | 100 | 174.564 | 100 |

* SFA: saturated fatty acid; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acid

with these descriptions, flow cytometry analysis revealed the lack of statistically significant difference in the entry of normal and frozen EVs into the HUVECs in the statistic culture system. To see whether EV storage at 4 °C and -20°C can influence interaction with vascular ECs, an SPR analysis was performed. Data confirmed the increase in RU signals in the group that received frozen EVs compared to normal EVs. The increase in EV-HUVEC interaction can be associated with several mechanisms. As mentioned in previously conducted experiments, the maintenance of Exos at refrigerator temperature can lead to a significant reduction of specific tetraspanins such as CD63 and other factors like HSP70.[20] It has been indicated that membrane HSP70 can stabilize the lipid structure and control the endocytosis process.[30] Thus, the reduced affinity of normal EVs stored at 4°C can be related in part, but not completely, to the loss of factor (s) involved in ligand-receptor-based uptake mechanisms. Another reason for enhanced RU values in frozen EVs is that lower temperatures are integral to the risk of EV aggregation.[31,32] This effect can increase EV sedimentation at the luminal surface of vascular cells and thus the possibility of thrombus formation.[33] However, a non-significant difference in uptake rate between the Normal and Frozen EVs groups rejects this possibility. Of note, the storage of EVs at lower temperature conditions can increase the average size; this effect can intensify the uptake rate and interaction with target cells.[32] It should not be forgotten that this effect will be more critical when on-target delivery of EVs to the injured site is important. GC data indicated the increase of MUF + PUFA/SFA ratio in EVs stored at below temperature (-20°C). Recent documents have confirmed the crucial role of lipids in the maintenance of Exo integrity, and biological properties.[34] It seems that the increase of MUF + PUFA/SFA ratio can increase the flexibility of stored Exos at below temperatures. Besides, lipidomic profile can influence the membrane dynamics such as lipid raft organization. The preservation of right lipid profile in the subcellular particles i.e., Exos can help to promotion of sorting, trafficking, induction of several signaling pathways, and other biological phenomena.[35] Along with these comments, several studies have confirmed that unsaturation degree, fatty acid chain length, existence of double bonds, and hydroxylation of lipids in the structure of membrane phospholipids and sphingolipids are integral to the biophysical properties of membrane. As mentioned above, higher PUFA contents can yield superior membrane flexibility, elasticity, permeability, and bending strain.[36,37] Several studies confirmed freezing can lead to the acquisition of round morphologies in EVs from different biological sources.[17,38,39] One reason would be the increase of PUFA and MUFA in the structure of Exos after being exposed to different low storage temperatures. Of course, saturated short-chain fatty acids, or unsaturated fatty acids increase the flexibility of Exo membrane via the reduction of hydrocarbon chain and Vander vales interactions, respectively.[40] Although, these features may increase the morphological adaption of Exos, it is also possible the reduction of SFA in Exo membrane leads to cargo leakage via the thinning of membrane thickness.[37] In another experiment conducted by Sokolova et al, they declared the reduction of mean Exo diameter (~50%) after incubation at 4°C for 8 days.[41]

## Conclusion

Taken together, EV storage at -20°C did increase the cytoprotective effects after being incubated with human ECs *in vitro*. No significant differences were obtained in terms of EV uptake between the HUVECs exposed to EVs stored at 4 and/or -20°C. It seems that the higher cytoprotective effects of EVs stored at -20°C are possibly related to the stability of growth factors and other signaling biomolecules. Meanwhile, the changes in EV membrane fatty acid profile are also determinant. Future studies should focus on the elucidation of underlying mechanisms associated with low storage temperatures.

**Acknowledgments**

Authors wish to thank the personnel of the Faculty of Advanced Medical Sciences for their help and guidance.

**Authors' Contribution**

Conceptualization: Reza Rahbarghazi, Morteza Milani
Methodology: Narges Mardi
Software: Reza Rahbarghazi
Validation: Reza Rahbarghazi
Formal analysis: Narges Mardi
Investigation: Narges Mardi, Amir Mehdizadeh, Mehdi Talebi, Reza Rahbarghazi
Resources: Mohammad Nouri
Data curation: Reza Rahbarghazi
Project administration: Mohammad Nouri
Funding acquisition: Mohammad Nouri
Visualization: Reza Rahbarghazi

Supervision: Reza Rahbarghazi, Mohammad Nouri
Writing–original draft: Narges Mardi
Writing–review & editing: Reza Rahbarghazi

**Competing Interests**
Authors declared that there is no conflict of interest related to this study.

**Ethical Approval**
All processes of this study were approved by the local ethics committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1399.055).

**Funding**
This study was supported by a grant from Tabriz University of Medical Sciences (Grant No: 66627).

**References**

1. Lin Z, Xiong Y, Sun Y, Zeng R, Xue H, Hu Y, et al. Circulating miRNA-21-enriched extracellular vesicles promote bone remodeling in traumatic brain injury patients. Exp Mol Med 2023;55(3):587-96. doi:10.1038/s12276-023-00956-8
2. Szwedowicz U, Łapińska Z, Gajewska-Naryniecka A, Choromańska A. Exosomes and other extracellular vesicles with high therapeutic potential: their applications in oncology, neurology, and dermatology. Molecules 2022;27(4):1303. doi:10.3390/molecules27041303
3. Mardi N, Salahpour-Anarjan F, Nemati M, Shahsavari Baher N, Rahbarghazi R, Zarebkohan A. Exosomes; multifaceted nanoplatform for targeting brain cancers. Cancer Lett 2023;557:216077. doi:10.1016/j.canlet.2023.216077
4. Mardi N, Haiaty S, Rahbarghazi R, Mobarak H, Milani M, Zarebkohan A, et al. Exosomal transmission of viruses, a two-edged biological sword. Cell Commun Signal 2023;21(1):19. doi:10.1186/s12964-022-01037-5
5. Choi H, Choi K, Kim DH, Oh BK, Yim H, Jo S, et al. Strategies for targeted delivery of exosomes to the brain: advantages and challenges. Pharmaceutics 2022;14(3):672. doi:10.3390/pharmaceutics14030672
6. Jafari D, Shajari S, Jafari R, Mardi N, Gomari H, Ganji F, et al. Designer exosomes: a new platform for biotechnology therapeutics. BioDrugs 2020;34(5):567-86. doi:10.1007/s40259-020-00434-x
7. Watanabe Y, Tsuchiya A, Terai S. The development of mesenchymal stem cell therapy in the present, and the perspective of cell-free therapy in the future. Clin Mol Hepatol 2021;27(1):70-80. doi:10.3350/cmh.2020.0194
8. Song J, Huang J, Chen X, Teng X, Song Z, Xing Y, et al. Donor-derived exosomes induce specific regulatory T cells to suppress immune inflammation in the allograft heart. Sci Rep 2016;7:20077. doi:10.1038/srep20077
9. Liu Q, Rojas-Canales DM, Divito SJ, Shufesky WJ, Stolz DB, Erdos G, et al. Donor dendritic cell-derived exosomes promote allograft-targeting immune response. J Clin Invest 2016;126(8):2805-20. doi:10.1172/jci84577
10. Heidari N, Abbasi-Kenarsari H, Namaki S, Baghaei K, Zali MR, Ghaffari Khaligh S, et al. Adipose-derived mesenchymal stem cell-secreted exosome alleviates dextran sulfate sodium-induced acute colitis by Treg cell induction and inflammatory cytokine reduction. J Cell Physiol 2021;236(8):5906-20. doi:10.1002/jcp.30275
11. Maroto R, Zhao Y, Jamaluddin M, Popov VL, Wang H, Kalubowilage M, et al. Effects of storage temperature on airway exosome integrity for diagnostic and functional analyses. J Extracell Vesicles 2017;6(1):1359478. doi:10.1080/20013078.2017.1359478
12. Morishita M, Takahashi Y, Nishikawa M, Takakura Y. Pharmacokinetics of exosomes-an important factor for elucidating the biological roles of exosomes and for the development of exosome-based therapeutics. J Pharm Sci 2017;106(9):2265-9. doi:10.1016/j.xphs.2017.02.030
13. Nong K, Wang W, Niu X, Hu B, Ma C, Bai Y, et al. Hepatoprotective effect of exosomes from human-induced pluripotent stem cell-derived mesenchymal stromal cells against hepatic ischemia-reperfusion injury in rats. Cytotherapy 2016;18(12):1548-59. doi:10.1016/j.jcyt.2016.08.002
14. Boriachek K, Masud MK, Palma C, Phan HP, Yamauchi Y, Hossain MS, et al. Avoiding pre-isolation step in exosome analysis: direct isolation and sensitive detection of exosomes using gold-loaded nanoporous ferric oxide nanozymes. Anal Chem 2019;91(6):3827-34. doi:10.1021/acs.analchem.8b03619
15. Harrell CR, Jovicic N, Djonov V, Volarevic V. Therapeutic use of mesenchymal stem cell-derived exosomes: from basic science to clinics. Pharmaceutics 2020;12(5):474. doi:10.3390/pharmaceutics12050474
16. Massa M, Croce S, Campanelli R, Abbà C, Lenta E, Valsecchi C, et al. Clinical applications of mesenchymal stem/stromal cell derived extracellular vesicles: therapeutic potential of an acellular product. Diagnostics (Basel) 2020;10(12):999. doi:10.3390/diagnostics10120999
17. Qin B, Zhang Q, Hu XM, Mi TY, Yu HY, Liu SS, et al. How does temperature play a role in the storage of extracellular vesicles? J Cell Physiol 2020;235(11):7663-80. doi:10.1002/jcp.29700
18. Cheng Y, Zeng Q, Han Q, Xia W. Effect of pH, temperature and freezing-thawing on quantity changes and cellular uptake of exosomes. Protein Cell 2019;10(4):295-9. doi:10.1007/s13238-018-0529-4
19. Park SJ, Jeon H, Yoo SM, Lee MS. The effect of storage temperature on the biological activity of extracellular vesicles for the complement system. In Vitro Cell Dev Biol Anim 2018;54(6):423-9. doi:10.1007/s11626-018-0261-7
20. Lee M, Ban JJ, Im W, Kim M. Influence of storage condition on exosome recovery. Biotechnol Bioprocess Eng 2016;21(2):299-304. doi:10.1007/s12257-015-0781-x
21. Rezabakhsh A, Nabat E, Yousefi M, Montazersaheb S, Cheraghi O, Mehdizadeh A, et al. Endothelial cells' biophysical, biochemical, and chromosomal aberrancies in high-glucose condition within the diabetic range. Cell Biochem Funct 2017;35(2):83-97. doi:10.1002/cbf.3251
22. Mardi N, Zarebkohan A, Biray-Avci C, Rahbarghazi R, Talebi M, Lotfimehr H, et al. Targeted delivery of doxorubicin using RSV F-protein modified breast cancer-derived exosomes in breast cancer-bearing mice. Bioimpacts 2026;16:33180. doi:10.34172/bi.33180.
23. Ma ZJ, Yang JJ, Lu YB, Liu ZY, Wang XX. Mesenchymal stem cell-derived exosomes: toward cell-free therapeutic strategies in regenerative medicine. World J Stem Cells 2020;12(8):814-40. doi:10.4252/wjsc.v12.i8.814
24. Wu JY, Li YJ, Hu XB, Huang S, Xiang DX. Preservation of small extracellular vesicles for functional analysis and therapeutic applications: a comparative evaluation of storage conditions. Drug Deliv 2021;28(1):162-70. doi:10.1080/10717544.2020.1869866
25. Tano K, Oulé MK, Doyon G, Lencki RW, Arul J. Comparative evaluation of the effect of storage temperature fluctuation on modified atmosphere packages of selected fruit and vegetables. Postharvest Biol Technol 2007;46(3):212-21. doi:10.1016/j.postharvbio.2007.05.008
26. Yuan F, Li YM, Wang Z. Preserving extracellular vesicles for biomedical applications: consideration of storage stability before and after isolation. Drug Deliv 2021;28(1):1501-9. doi:10.1080/10717544.2021.1951896
27. Fowler A, Toner M. Cryo-injury and biopreservation. Ann N Y Acad Sci 2005;1066:119-35. doi:10.1196/annals.1363.010
28. Ruzycka-Ayoush M, Nowicka AM, Kowalczyk A, Gluchowska A, Targonska A, Mosieniak G, et al. Exosomes derived from lung cancer cells: Isolation, characterization, and stability

studies. Eur J Pharm Sci 2023;181:106369. doi:10.1016/j.ejps.2022.106369
29. Rosenberger L, Ezquer M, Lillo-Vera F, Pedraza PL, Ortúzar MI, González PL, et al. Stem cell exosomes inhibit angiogenesis and tumor growth of oral squamous cell carcinoma. Sci Rep 2019;9(1):663. doi:10.1038/s41598-018-36855-6
30. Balogi Z, Multhoff G, Jensen TK, Lloyd-Evans E, Yamashima T, Jäättelä M, et al. Hsp70 interactions with membrane lipids regulate cellular functions in health and disease. Prog Lipid Res 2019;74:18-30. doi:10.1016/j.plipres.2019.01.004
31. Bosch S, de Beaurepaire L, Allard M, Mosser M, Heichette C, Chrétien D, et al. Trehalose prevents aggregation of exosomes and cryodamage. Sci Rep 2016;6:36162. doi:10.1038/srep36162
32. Lőrincz ÁM, Timár CI, Marosvári KA, Veres DS, Otrokocsi L, Kittel Á, et al. Effect of storage on physical and functional properties of extracellular vesicles derived from neutrophilic granulocytes. J Extracell Vesicles 2014;3:25465. doi:10.3402/jev.v3.25465
33. Wu X, Liu Y, Wei W, Liu ML. Extracellular vesicles in autoimmune vasculitis - Little dirts light the fire in blood vessels. Autoimmun Rev 2019;18(6):593-606. doi:10.1016/j.autrev.2018.12.007
34. Zhang Y, Liu Y, Liu H, Tang WH. Exosomes: biogenesis, biologic function and clinical potential. Cell Biosci 2019;9:19. doi:10.1186/s13578-019-0282-2
35. Schumann J, Leichtle A, Thiery J, Fuhrmann H. Fatty acid and peptide profiles in plasma membrane and membrane rafts of PUFA supplemented RAW264.7 macrophages. PLoS One 2011;6(8): e24066. doi:10.1371/journal.pone.0024066
36. de Carvalho C, Caramujo MJ. The various roles of fatty acids. Molecules 2018;23(10):2583. doi:10.3390/molecules23102583
37. Bodur M, Yilmaz B, Ağagündüz D, Ozogul Y. Immunomodulatory effects of omega-3 fatty acids: mechanistic insights and health implications. Mol Nutr Food Res 2025;69(10): e202400752. doi:10.1002/mnfr.202400752
38. Höög JL, Lötvall J. Diversity of extracellular vesicles in human ejaculates revealed by cryo-electron microscopy. J Extracell Vesicles 2015;4:28680. doi:10.3402/jev.v4.28680
39. Zabeo D, Cvjetkovic A, Lässer C, Schorb M, Lötvall J, Höög JL. Exosomes purified from a single cell type have diverse morphology. J Extracell Vesicles 2017;6(1):1329476. doi:10.1080/20013078.2017.1329476
40. Bajerski F, Wagner D, Mangelsdorf K. Cell membrane fatty acid composition of *Chryseobacterium frigidisoli* PB4T, isolated from Antarctic glacier forefield soils, in response to changing temperature and pH conditions. Front Microbiol 2017;8:677. doi:10.3389/fmicb.2017.00677
41. Sokolova V, Ludwig AK, Hornung S, Rotan O, Horn PA, Epple M, et al. Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. Colloids Surf B Biointerfaces 2011;87(1):146-50. doi:10.1016/j.colsurfb.2011.05.013