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REVIEW

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Different storage and freezing protocols for extracellular vesicles: a systematic review

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Abstract

Background Extracellular vesicles (EVs) have been considered promising tools in regenerative medicine. However, the nanoscale properties of EVs make them sensitive to environmental conditions. Optimal storage protocols are crucial for maintaining EV structural, molecular, and functional integrity. This systematic review aimed to gather evidence on the effects of various storage protocols on EV characteristics and integrity.

Strategy A comprehensive search was conducted for original studies investigating the impacts of storage temperature, freezing techniques, freeze-thaw cycles, and stabilizing strategies on EV concentration, size distribution, morphology, cargo content, and bioactivity. Results from 50 included studies were analyzed.

Results Data indicated that rapid freezing procedures and constant subzero temperatures (optimally − 80 °C) resulted in appropriate EV quantity and cargo preservation. Subjecting EVs to multiple freeze-thaw cycles decreased particle concentrations, RNA content, impaired bioactivity, and increased EV size and aggregation. Electron microscopy revealed vesicle enlargement, and fusion, along with membrane deformation after being exposed to substandard storage protocols. The addition of stabilizers like trehalose helped EVs to maintain integrity. Of note, storage in native biofluids offered improved stability over purified EVs in buffers.

Conclusion Data emphasize the critical need for precise storage protocols for EVs to ensure reproducible research outcomes and clinical applications. Further studies using reliable methods are necessary to create specific guidelines for improving the stability of EVs in various applications.

Keywords Extracellular vesicles, Storage, Cryopreservation, Integrity, Stability

Introduction

Extracellular vesicles (EVs) have emerged as one of the most promising tools in regenerative medicine and drug delivery. These nano-sized and cell-derived particles play important roles in intercellular communication and also have potential applications in the field of diagnostics and therapeutics. However, the clinical translation of EV-based therapies faces significant challenges, especially in maintaining EV stability during storage, transfer, and administration.

EVs are heterogenous populations and consist of three main categories based on their biogenesis and

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size: exosomes (Exos, 30–150 nm), microvesicles (MVs, 50–1000 nm), and apoptotic bodies (several micrometers) [1, 2]. EVs are released during different physiological and pathological conditions and based on their distinct formation mechanisms, each class of EVs have a different cargo profile that resonates with their diverse biological functions [3–5]. These lipid bilayer-enclosed particles can transfer bioactive molecules, including proteins, nucleic acids, lipids, and metabolites, between cells and modulate recipient cell behavior as well as function [6, 7].

Exosomes are formed via the invagination of the endosomal membrane in endosomes and multivesicular bodies (MVBs), leading to the formation of numerous intraluminal vesicles (ILVs) with various cytoplasmic contents [8–10]. Exos generally can harbor certain tetraspanins (e.g., CD63, CD9, and CD81), heat shock proteins (HSP70), and endosome-associated proteins (i.e., TSG101, Alix) [11–14]. MVs are directly produced and released via the outward budding of the plasma membrane [9]. Apoptotic bodies originate from apoptotic cells containing various cellular components, including organelles and fragmented DNA. Unlike Exos and MVs, apoptotic bodies are a result of cell death and are typically engulfed and cleared through phagocytosis by local immune cells or native non-immune system cells [15]. It is thought that both Exos and MVs contain proteins, as well as nucleic acids (mRNAs, miRNAs, lncRNAs, and DNA), lipids, and metabolites [4, 11, 16]. Cargo sorting is regulated by complex molecular pathways and mechanisms inside the cells that sequester selectively of the contents [17]. Thus, the cargo of EVs is not a random sample of cellular content but it is a selective sorter that represents their parental cells' cytoplasmic condition under physiological and pathological status [4, 16]. Since EVs can mimic the cytoplasmic state of their parental cells and carry functional biomolecules, this capacity makes them putative therapeutic tools for various pathological conditions [9]. However, there is a need to overcome challenges in EV production and preservation up to years, especially optimizing storage conditions for maintaining the integrity and functionality of EVs, to realize their therapeutic potential.

To date, EVs have been isolated from diverse biofluids including blood, urine, saliva, breast milk, semen, follicular and amniotic fluid, and synovia [12, 18–23]. EVs can also be obtained from tissue lysates or cultured [16, 24]. Recently the application of EVs has expanded in biomedical fields due to their suitable biodistribution and delivery platform eligibility [9, 16, 25]. It has been observed that EVs are powerful and minimally invasive agents with remarkable therapeutic potential in various diseases [8, 26], however, the clinical application of EVs requires standardized protocols to isolate, characterize, and store EVs to maintain their structural integrity and dynamic activity [9, 27, 28].

Cryopreservation is a commonly used technique to preserve original characteristics such as concentration, size, morphology, and functional properties of EVs after storage [4, 29]. However, the freezing process often causes vesicle rupture, cargo lost, aggregation, and precipitation (Fig. 1). However, there is no universally optimal protocol for varied EV types, and therefore attempts

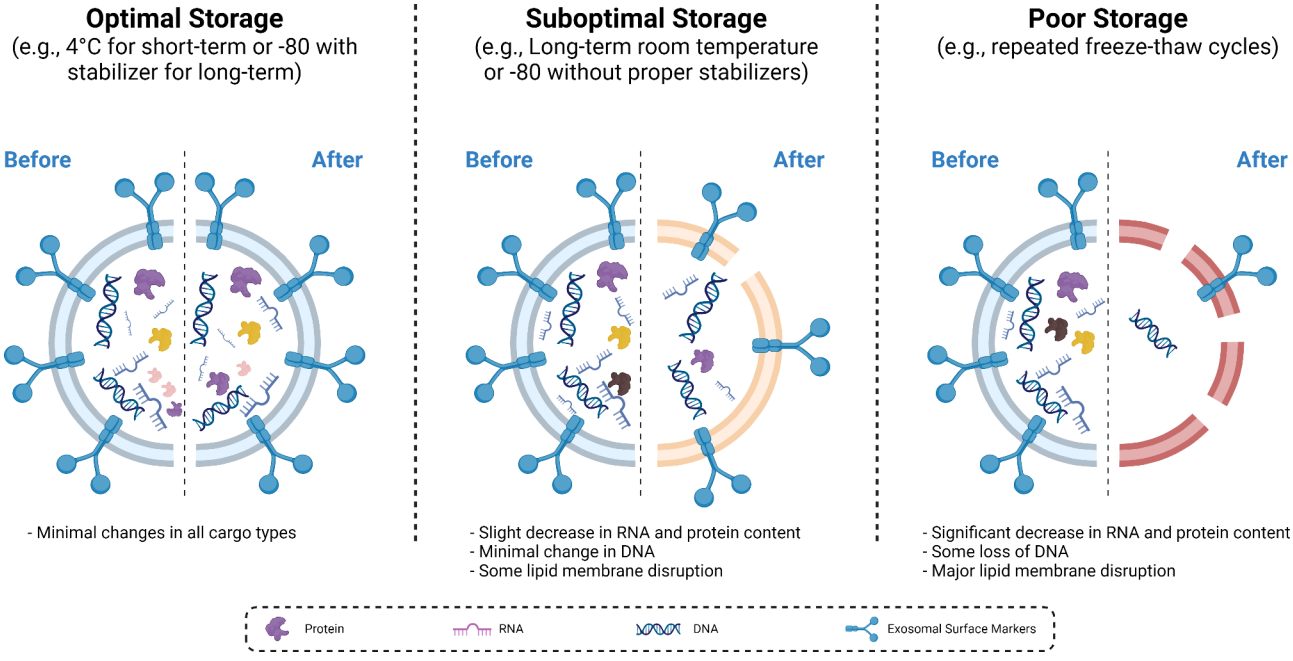

Fig. 1 EV cargo stability during optimal, suboptimal, and poor storage conditions. The figure was created with BioRender.com

should be focused on the establishment of standard storage conditions based on nanovesicle source and intended future application [4, 29, 30]. Cryoprotectants such as dimethyl sulfoxide (DMSO), and glycerol have been used to minimize cryodamage, however, numerous experiments have indicated the possible cytotoxicity and inhibition of specific downstream processes [22].

This systematic review aims to comprehensively evaluate various storage and cryopreservation methods for EVs, and assess their possible impact on physicochemical parameters, and functional qualities for potential clinical applications. This information will help identify optimal storage conditions and could advance EV-based therapies and establish standardized protocols for EV storage and cryopreservation. Moreover, the influencing factors such as temperature, freezing rate, cryoprotectant type and concentration, and post-thaw processing were highlighted. Eventually, this review highlights areas where further research is needed to support the translation of EV-based therapies from bench to bedside.

Methods

This systematic review aimed to find the appropriate protocol to store and freeze EVs without causing prominent damage in short- and long-term storage. The PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines [31] were implemented for data extraction and analysis.

Search strategy

A comprehensive search using PubMed and Scopus databases was conducted for the published literature up to August 2024. The search strategy was a combination of Boolean operators, keywords and Medical Terms (MeSH) related to "extracellular vesicles", "storage", "freezing", "cryopreservation", "stability", "functionality" and "integrity". The search was limited to articles published in English. In addition to a systematic search in PubMed and Scopus databases, a supplemented search was conducted to ensure comprehensive coverage of including papers. To this end, the reference lists of included studies were screened and a hand search on Google Scholar was conducted for additional relevant studies. These additional sources are represented as "other" in the flow chart of PRISMA (Fig. 2).

Selection criteria

Studies were included if they reported on "the impact of storage and freezing conditions on the integrity of EVs". All studies measuring the vascularization rate (EV release of blood cells during storage time) were excluded since those studies did not examine the stability capacity of EVs themselves. Reviews, editorials, commentaries, letters, and conference abstracts were also excluded. Two authors (SA and NJ) independently screened titles and abstracts and non-related articles were excluded. Full-text articles were obtained for eligible studies and were independently assessed by three authors (SA, NJ, and AG).

Data extraction

Data were extracted using a standardized data extraction table. The following information was extracted from each study: source of EVs and isolation methods, storage and freezing conditions, methods used to assess EVs integrity and stability (concentration, size, morphology, protein/DNA/RNA contents), and main findings. Any disagreements between authors during the data extraction process were resolved through discussion or consultation with a fourth author (MM).

Outcomes

Literature search

The initial systematic search resulted in 626 papers from PubMed, Scopus, and other databases out of which 170 duplicates were removed resulting in 456 total papers. Subsequently, title and abstract screening identified 58 potentially related papers, which were further assessed in full text. Studies assessing the influence of storage temperature, freezing method, free-thaw cycles, and stabilizing agents on EV parameters such as concentration, size, morphology, protein and DNA/RNA content, and functional bioactivity consisting of the final 50 papers were considered, while the excluded studies primarily focused on RBC vascularization (EV release during storage periods) or employed methodologies beyond the scope of our study. The study selection process, following PRISMA guidelines [31], is presented in the flow diagram (Fig. 2). The comprehensive summary of papers is presented in Table 1.

Storage temperature and duration effects

Studies revealed that storage temperature significantly influences EV recovery and integrity depending on the specific temperature range. The effect of various storage temperatures on EVs as reported in multiple included studies will be summarized in the following.

Impact of lower temperatures

Most of the studies indicated that storing EVs at -80 °C yielded better results in terms of particle concentration, RNA content, morphology, and biological functionality for long-term preservation compared to higher temperatures [4, 11, 12, 16, 20, 21, 25, 27, 29, 30, 32–40]. Additionally, it was also stated that ultra-low storage at -80 °C preserved EVs' integrity and function in short-term storage (more than 1 week) [41]. These findings were consistent across various EV sources, including

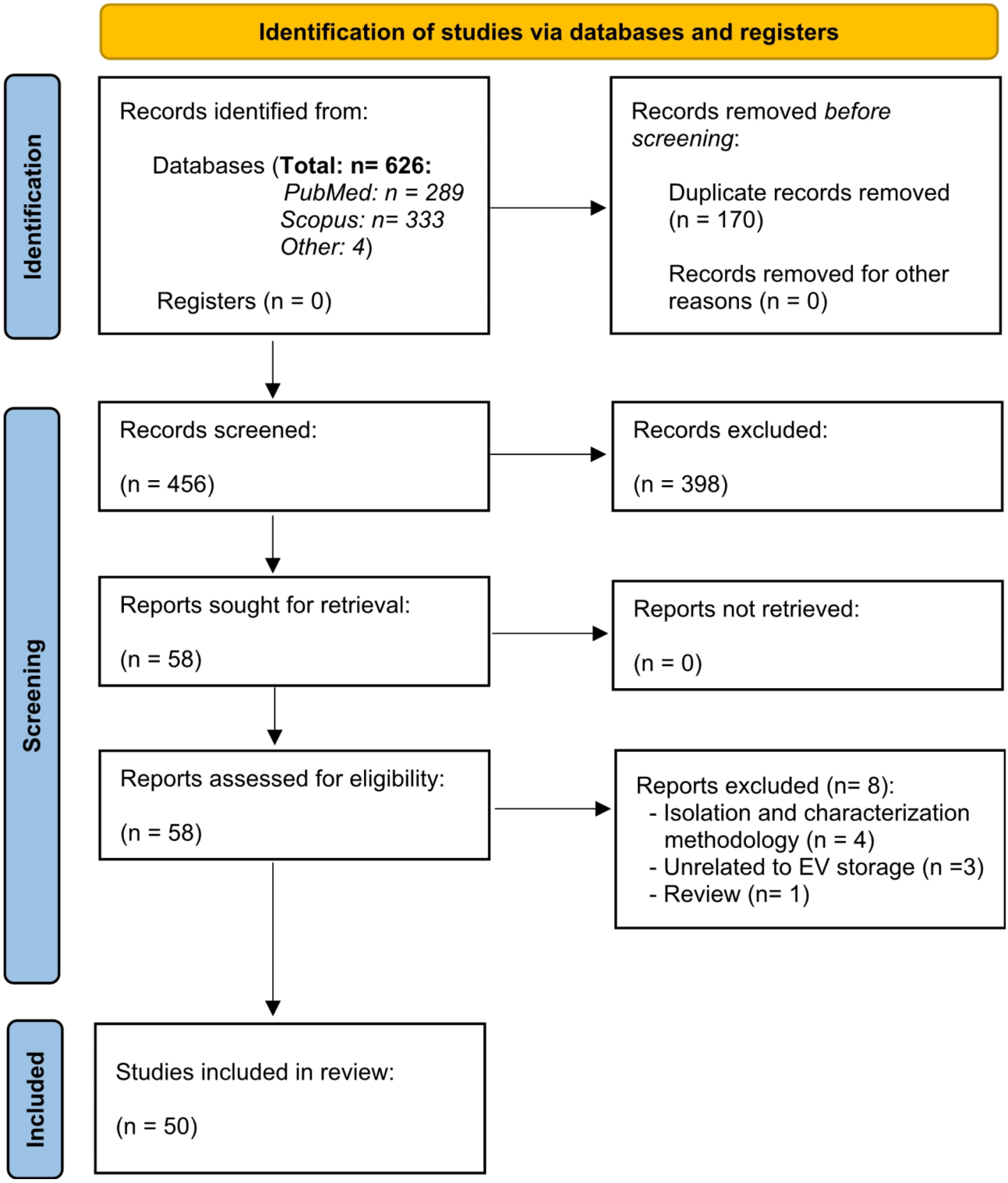

Fig. 2 PRISMA flow diagram. The identified documents were carefully reviewed, and studies meeting the eligibility criteria were included in the systematic review as illustrated in this flow diagram

Table 1 Summary of Original Research Studies. This table outlines key information, including the EV source and isolation methods, the evaluation of storage conditions, durations, freeze-thaw cycles on EVs parameters and overall conclusions

Ref.	Source/ Isolation/ Storage	Characterization	Morphology	Protein, RNA, and DNA evaluations	Biological function	Highlights
[3]	**human adipose stem cells (hASCs)** Isolation: tangential flow filtration (TFF) Storage: encapsulated in microneedles (EV@MN) made of hyaluronic acid (HA) at -20, +4, and + 25 °C for six months, and up to 10 freeze-thaw cycles.	• Significant decrease in EVs in PBS over time • No significant decrease up to three months, negligible decrease after six months for EVs in EV@MN (> 85% remained)	-	• Protein activity lost in PBS at any temperature in 2 weeks, while preserved at over 99% in EV@MN at 4 °C or -20 °C storage.	EV@MN preserves EV functions (cell proliferation and fibroblast migration) for up to six months.	• HA in EV@MN preserved EV bioactivity after 6 months at 4 °C • EVs in PBS lost activity quickly • EV@MN retains over 99% activity at 4 °C or -20 °C during short-term storage
[53]	**Mouse J774A.1 cells** Isolation: size exclusion chromatography (SEC) Storage: Engineered EVs (E-EVs) in PBS at 37 °C (1 week), RT, and − 80 °C (1 year); E-EVs loaded into microneedles (MN-EVs) at RT (12 months) supplemented with trehalose and cellulose	**E-EVs in PBS**: • 70 to 90% of E-EV's cargo remained intact for 3 h, up to 45% by day 7 • RT and − 80 °C stored E-EVs had a larger mean diameter **MN-EVs**: • Maintained count and size for 12 months at RT.	• RT and − 80 °C E-EVs showed aggregation and some membrane disruption. • MN-EVs maintained intact membranes, over 12 months at RT.	-	• Only a 3% loss in bioactivity of MN-EVs over 12 months at RT. • Almost no biological activity of E-EVs in PBS at RT for 12 months • Improved EVs stability with trehalose and cellulose in MN-EVs.	• EVs encapsulated in microneedles remained stable at RT for at least one year with no impact on their bioactivities.
[33]	**HEK293T cells conditioned media (CM)** Isolation: ultracentrifugation, TFF, SEC. Storage: +4 °C, -20 °C, or -80 °C; multiple freeze/thaw cycles; resuspended in various buffers e.g. human albumin and trehalose (PBS-HAT); different tube types tested.	• Concentrations decreased at any temperature in PBS. • Unaffected EVs at 4 °C for 8 days. • **Up to 26 weeks**: 90% loss at -20 °C, less at -80 °C; better size preservation at -80 °C versus − 20 °C. • **2 years Long-term storage**: Highest count in PBS-HAT at -80 °C, with less size increase; additives improved freezing recovery.	**TEM after 20 weeks in various conditions**: No consistent differences in shape, diameter, or intactness	• EV protein in PBS remained stable for 1 week at + 4 °C, declined at -20 °C after 8 weeks, and had better long-term stability at -80 °C. **RNA**: • 50% decline in 1 week at + 4 °C in PBS, significant loss in long-term at -80 °C, but stable for 2 years in PBS-HAT buffer.	• PBS with cryoprotectants kept EVs stability significantly. • Long-term storage in PBS (2 years) reduced cellular uptake.	• Storage in PBS hurt EV; PBS-HAT represented a promising solution • Human serum albumin reduces EV adsorption to tubes • Buffers maintained EV stability
[11]	**Human umbilical cord-MSCs (hUC-MSC) conditioned media** Isolation: Ultrafiltration and SEC Storage: Immediate, RT, 4 °C, -20 °C (7 days), -80 °C (1 month); one freeze-thaw cycle	• No particle count differences, unaffected by storage. • -80 °C storage had more uniform and smaller EVs. • Size increased at -20 °C. • Zeta potentials unaffected.	• Size differences between − 20 °C storage and RT • TEM shows a distinct bilayer at -80 °C (more homogeneous EVs)	• Better protein markers preservation at -80 °C • No significant miRNA differences, slight reduction at 4 °C	• No impact on the effect of EVs on sarcoma cell growth with storage	• Storing at -80 °C maintained EV size uniformity and integrity

Table 1 (continued)

Ref.	Source/ Isolation/ Storage	Characterization	Morphology	Protein, RNA, and DNA evaluations	Biological function	Highlights
[54]	**Leaf-Derived EVs (LEVs)** Isolation: centrifugation Storage: -20 °C, 4 °C, 25 °C, 45 °C (4 weeks); freeze-thaw (0, 1, or 3 cycles); storage media: 1,3-butylene glycol, Saliguard TMO	• LEVs stable at -20 °C • Larger sizes at higher temperatures • Freeze/thaw cycles made LEVs larger and aggregated • Similar zeta potentials, except LEVs-TMO at 45 °C turned negative after 4 weeks	**Freeze-thaw cycles caused LEV**: • Increase and vary in size. • Aggregation and disruption.	• Protein levels in LEVs and LEVs-TMO decreased over time, especially at higher temperatures. • LEVs-TMO at 4 °C had the highest protein levels. • No significant impact on protein content with freeze-thaw cycles.	• LEV uptake decreases with freeze/thaw cycles • LEVs-TMO uptake stable after freeze/thaw cycles • LEVs at 25 °C uptake was better than − 20 °C and 4 °C • Low LEV uptake at 45 °C (aggregated)	• LEVs in TMO: Stable at 4 °C for 4 weeks • Freeze-thaw cycles altered LEV size
[9]	**Cell culture supernatant** Isolation: ultracentrifugation, freeze-drying at -80ºC. Examined after Lyophilization Storage: PBS, 20 mM HEPES buffer, 8.5% sucrose for lyophilization	• EV size, surface charge, and PDI consistent after freeze-drying with cryoprotectant. • Lyophilization did not reduce EV counts.	• Morphology changed with more debris and aggregates without 8.5% sucrose in the lyophilization buffer.	• No significant differences in total protein content	• Cellular uptake confirmed EV functionality post-lyophilization	• Lyophilization preserved EVs' physicochemical properties and functionality
[62]	**U937 and CT26 cells.** Isolation: centrifugation Storage: Lyophilized and stored at 4 °C, 25 °C, 3 months, and 6 months. Storage media: Sucrose or trehalose, with/without polysorbate 80.	• No significant size changes • Trehalose or sucrose plus polysorbate 80 could maintain EV size after lyophilization	Well-preserved	• Protein concentration, structure, and activity declined during storage. • Trehalose, sucrose, and polysorbate 80 maintained protein levels before and after lyophilization.	• EVs in cryoprotectant had a similar bioactivity, antioxidant enzyme activity, and reduced heart infarct size like fresh EVs	• Trehalose provides higher storage stability than sucrose. • Trehalose with polysorbate 80 maintained EV bioactivity.
[30]	**Human BDMSCs** Isolation: ultracentrifugation, freeze-drying Storage: RT, 4 °C, -20 °C, -80 °C for 1 week, 4 weeks, 6 weeks; up to five freeze-thaw cycles	• EV size increased after 28 days at -20 °C • Mode size increased after 5 freeze-thaw cycles at -80 °C	• EV morphology and size retained after lyophilization	• Protein content stable at RT and 4 °C. • CD63 and TSG101 are stable across temperatures. • Stable upon five freeze-thaw cycles.	• Bioactivity decreased after 5 freeze-thaw cycles. • Frozen EVs led to lower IL-6 secretion. • Lyophilization preserved EV bioactivity.	• 4 °C and lyophilization were best for long-term bioactivity. • EV proteins were stable in storage conditions. • Freeze-thaw cycles and long-term storage harmfully affected bioactivity.
[32]	**Murine melanoma B16BL6 cells** Isolation: ultracentrifugation, lyophilization Storage: -80 °C and RT (1 week); cryoprotectant: Trehalose	• Lyophilization without trehalose led to aggregation • Wider PDI compared to -80 °C storage	• Not aggregated Exos at -80 °C and lyophilized with trehalose. • No significant changes compared to -80 °C storage.	• Trehalose preserved protein and RNA integrity in lyophilization Exos.	• Luciferase activity and cytokine release stimulation potential of EVs remained stable at RT	• Lyophilization with trehalose preserves Exos. • RT storage did not affect exosome content or function. • RT maintained protein, RNA, pharmacokinetic, and physicochemical properties.

Table 1 (continued)

Ref.	Source/ Isolation/ Storage	Characterization	Morphology	Protein, RNA, and DNA evaluations	Biological function	Highlights
[22]	**Human umbilical cord-MSCs conditioned medium** Isolation: centrifugation, lyophilization Storage: 4 °C, -20 °C, -70 °C, -196 °C (liquid nitrogen) for 2–3 weeks; storage media: DMSO (2.5 to 10%).	• Microvesicle concentration stable at 4 °C for 1 and 2 weeks (95.0% and 64.8% of initial levels), followed by over 50% after the third week. • Lyophilization extended shelf life	-	-	-	• Microvesicles sensitive to freezing • Lower DMSO (< 5%) preserved 90–95% of microvesicles and outperforms higher DMSO (7.5–10%)
[29]	**Purified plasma (pPFP) and BV2 microglia cell line** Isolation: qEV SEC columns, ultracentrifugation. Storage: -80 °C with/without preservatives (trehalose), lyophilization; various freeze-thaw cycles; storage media: Trehalose, DMSO, Glycerol, Protease Inhibitor, Sodium Azide	• Storage at -80 °C reduces EV yield and counts after 6 months. • Freeze-thaw cycles decreased EV yield and increased size. • Stored EVs became more positively charged after 6 months.	• Reduced EVs yield, and increased particle size after freeze-thaw cycles.	• Increased contaminant protein concentration during storage	-	• Storage at − 80 °C was best for EVs • −80 °C preservation of EVs in their biofluids is preferable over isolated EV • Freeze-thaw damaged EVs' membrane • Time affects protein and size
[55]	**Human umbilical cord-MSCs** Isolation: centrifugation for apoptotic vesicles, ultracentrifugation for exosomes Storage: lyophilized or encapsulated in hyaluronic acid hydrogel (EV- HA) and stored at -80 °C or RT for 2 months; storage media: Trehalose, polyvinylpyrrolidone	• No change in size and slight changes in total numbers and zeta potentials after lyophilization.	• Cryo-EM showed intact membrane structures of lyophilized apoptotic vesicles (apoVs).	• Minimal decrease in tissue factors expression in lyophilized apoVs.	• Lyophilized apoVs and EV- HA, maintained their procoagulant ability at both RT and − 80 °C.	• Lyophilization or encapsulation in hydrogels fulfilled storage challenges, kept bioactivity, and facilitated the transportation of EVs.
[64]	**RO cells (ACC452)** Isolation: ultracentrifugation, SEC Storage: Lyophilized and stored up to 6 months; stabilizers: sucrose, poloxamer 188, polysorbate 20, polyvinylpyrrolidone	• PBS-EVs lost intact vesicles. • Sucrose reduced freeze-thaw particle growth. • P188 and sucrose preserved the highest number of intact vesicles (size, zeta value, and concentration)	-	-	-	• EV stability is affected by freezing and drying • P188 and especially sucrose preserves EV stability for 6 months • Storage at 2–8 °C suitable for at least 1 month
[34]	**bEnd.3 endothelial cells** Isolation: ultracentrifugation. Storage: 4 °C, -20 °C, and − 80 °C (up to 28 days); freeze-thaw: 1–5 times (to 4 °C)	• Widened size range for all storage conditions • Most significant enlargement at -20 °C	• TEM observations: aggregations, membrane destruction, fusion.	• Protein and CD63 levels decreased post-storage. • Decreased protein levels after one week at 4 °C but stable at -80 °C. • No significant total RNA decreases at 4 °C within a week	• Storage reduced EV's autologous uptake in vivo and ex vivo	• Storage affects the size, quantity, content, cellular uptake, and biodistribution
[12]	**Human semen** Isolation: ExoQuick (EQ) precipitation Storage: -80 °C in biofluid (30 years)	• No impact from storage duration	• Morphology unchanged after freezing and storage • No aggregation, fusion, or membrane damage	• Stable RNA and protein in Exos when frozen. • Some cargo components, like AChE activity, decreased	-	• Freezing duration did not affect semen Exos and their protein content

Table 1 (continued)

Ref.	Source/ Isolation/ Storage	Characterization	Morphology	Protein, RNA, and DNA evaluations	Biological function	Highlights
[35]	**MIN6 supernatants** Isolation: ultracentrifugation, ultrafiltration. Storage: 4 °C (1 day) or -80 °C (up to 1 year); four freeze-thaw cycles; storage media: PBS or trehalose	**Trehalose**: • Increased EV count and yield, maintained zeta potential, uniform size distribution, and reduced mean size. • Protected from freeze-thaw cycles compared to PBS.	More particles in trehalose -EVs, with less aggregation and fusion	• No differences in total protein and RNA concentrations.	• Enhanced bioactivity in trehalose EVs more than PBS (stimulation of TNF-alpha). • Freeze-thaw: trehalose preserved EV bioactivity better than PBS.	• Trehalose preserved count, purity, size, charge, and cargo and inhibted aggregation. • Exos in trehalose were more bioactive than in PBS.
[48]	**Bronchoalveolar lavage fluid** Isolation: ultracentrifugation. Storage: +4 °C (4 days), -80 °C (4 days); one freeze-thaw cycle	• +4 °C increased exosome size slightly. • -80 °C significantly increased size and PDI. • Thawing from − 80 °C disrupted ζ values and structure.	• Fresh and + 4 °C: separated, membrane-encapsulated. • -80 °C: nanovesicle Aggregation, diminished ζ values, and multi-lamellar membranes.	• +4 °C storage: Lost 457 proteins. • -80 °C storage: Lost 315 proteins.	• Altered biological function.	• Freezing made Exos enlarged and formed multilamellar vesicles. • Storage led to leakage of non-membrane-integrated proteins.
[36]	**A549 cell line** Isolation: ultracentrifugation Storage: short-term at RT (PBS, PBS with trehalose or DMSO, 14 days); long-term at 4 °C, -20 °C, -80 °C (PBS, PBS with trehalose or DMSO, 8 weeks)	• Appropriate short-term stability in all preservatives • Best long-term stability at -80 °C with trehalose • No change in size distributions in short or long-term storage • Reduced concentration at -20 °C and 4 °C	• No significant aggregation in 2-month-storage	• Stable exosome protein for 2 weeks at RT and 2 months at low temperatures • 20% decrease in PBS at RT	-	• Exos stored in PBS with trehalose had the best stability in terms of concentration, zeta potential at RT and low temperatures
[45]	**Placental cell culture media** Isolation: ultracentrifugation Storage: RT, 4 °C, -20 °C (14 days); one freeze-thaw cycle	• No concentration differences at RT or 4 °C • Storage at -20 °C reduced concentration with no significant size changes on days 7 and 14	-	• Stable protein levels at RT or 4 °C. • Unaffected DNA by storage conditions.	• EVs at RT or 4 °C retained endothelial cell activation prevention ability • Reduction observed for EVs at different durations and temperatures	• Placental EVs stable at RT or 4 °C for 14 days • Storage at -20 °C reduces EVs concentration • Functional activity unaffected at -20 °C
[42]	**KSHV-infected HUVECs** Isolation: ultracentrifugation Storage: -70 °C, -20 °C, 4 °C, 37 °C (25 days)	• Higher temperatures caused higher decreases over time. • On day 16, -70 °C had more particles. • Counts decreased at -20 °C and − 70 °C by day 25 • Size decreased over time at all temperatures, especially at -70 °C.	-	• No notable alterations in Surface protein stability of EVs up to day 8. • A decline in protein levels at 37 °C by day 16 • Protein levels remained stable at other temperatures by day 25.	• Activity maintained only at 4 °C and − 70 °C by day 25. • -20 °C less effective by day 16. • 4 °C slightly better than − 70 °C, possibly due to freeze-thaw. • 37 °C lost activity in 4 days.	• EV number decreased during storage • 4 °C offers good stability • -20 °C storage reduced activity • Storage at -70 °C preserved EV activity • EVs at 4 °C more active than − 70 °C
[57]	**CSF from glioblastoma patients** Isolation: ultracentrifugation Storage: RT (1 and 7 days), -80 °C (7 days); freeze-thaw (1, 2, or 3 cycles)	• 37–43% reduction after 3 freeze-thaw cycles in EV count.	**TEM**: • No membrane damage. • No morphology change.	• No significant changes in miRNA levels	-	• EV miRNAs stable at RT for 7 days • Single freeze-thaw cycle did not affect EVs or miRNAs • Decreased parameters after two freeze-thaw cycles

Table 1 (continued)

Ref.	Source/ Isolation/ Storage	Characterization	Morphology	Protein, RNA, and DNA evaluations	Biological function	Highlights
[19]	**Human whole saliva primary culture** Isolation: gel filtration chromatography, ultracentrifugation Storage: 4 °C (up to 20 months); slow freeze-thaw cycles; storage media: Tris-buffered saline	• Stable size after 20 months at 4 °C • Intact in its biofluid (saliva) for 28 days at 4 °C • Resistant to NP-40 and Triton X-100 detergents	• Morphology unchanged during storage • Membrane integrity preserved	• Stored Exos showed some protein degradation. • Key exosomal marker proteins remained intact.	-	• Salivary Exos were stable at 4 °C • Storing in whole saliva-preserved Exos for at least 28 days at 4 °C
[20]	**Human plasma** Isolation: ultracentrifugation, Immunoaffinity pull-down, OptiPrep density gradient separation. Storage: 4 °C, -20 °C, -80 °C (3 months)	-	• Exos were detected in all conditions in their biofluid • Aggregation in plasma samples stored at 4 °C after both 30 and 90 days.	• TSG101 detected in Exos from plasma-stored samples	• Exosome uptake remained stable and active by cells after 30 days at -20 °C	• Exos were stable for 90 days in plasma
[4]	**Mice BM-conditioned media** Isolation: ultracentrifugation Storage: 1 month (4 °C, -20 °C, -80 °C, -196 °C); one freeze-thaw cycle; storage media: PBS, PBS + trehalose + DMSO (TRE)	• Cryopreserved Exos increased in size at -20 °C in PBS, not in TRE. • At 4 °C, exosome size decreased in PBS and increased in TRE.	• Morphology stable at -80 °C (PBS, TRE) • Membrane disruption and fusion at 4 °C • Less aggregation in TRE	-	• Trehalose avoided biological functionality lost during storage (EV uptake and migration potential)	• Storing at -80 °C with trehalose preserved structure, integrity, and bioactivity of EVs
[21]	**Urine samples (healthy, diabetic, normal/micro/macroalbuminuria)** Isolation: ultracentrifugation Storage: -20 °C vs. -80 °C, up to 4 years	• Particle concentration decreased after 14 days • Stable particle size and size distribution	• Stored at -80 °C up to 24 months, maintained particle size, concentration, structure, and EV protein markers	• TSG101, CD9, CD63 levels decrease after 4 months at -20 °C. • Healthy control EVs show detectable EV markers at -20 °C for 1.5 months, comparable to -80 °C. • Lower RNA yield at -20 °C compared to -80 °C storage.	-	• Temperature affects EV protein markers - Urine (biofluid) storage resulted in EV-enriched protein markers
[16]	**Epithelial ovarian cancer (EOC) tissue lysates** Isolation: ultracentrifugation, centrifugation Storage: lysates or tissues at -80 °C for 15 days	-	• Membrane structure and morphological diversity maintained after − 80 °C storage	• CD81 slightly reduced in frozen tissue lysate EVs • TSG101, ALIX, and Flotillin-1 levels stable	• No significant difference between fresh and frozen tissue-derived EVs • Freezing did not affect cellular uptake	• Freezing did not affect EV's uptake or structure • Cryopreservation is suitable for EV membrane structure and size maintenance
[28]	**HT-29 human colorectal adenocarcinoma cell line** Isolation: ultracentrifugation Storage: -80 °C (8 weeks), then thawed at + 4 °C and stored (up to 48 h) in various tubes; one freeze-thaw cycle	• Significant concentration loss in ordinary tubes at 48 h resulted from EV adsorption on tube walls. • Particle counts reduced in all samples in PBS at + 4 °C, particularly in ordinary tubes.	• No aggregation, fusion, or membrane disruption during storage or freeze/thaw cycles	-	-	• Adsorption of EVs onto tube walls causes concentration losses. • Surface block with excess protein or BSA or use of Eppendorf Protein LoBind tubes can alleviate EV adsorption.

Table 1 (continued)

Ref.	Source/ Isolation/ Storage	Characterization	Morphology	Protein, RNA, and DNA evaluations	Biological function	Highlights
[56]	**Plasma samples and BM1 cells** Isolation: ultracentrifugation, qEV columns Storage: -80 °C (10–12 days); slow freezing; one freeze-thaw cycle; cryoprotectant: 10% DMSO	• Lower concentrations in stored EVs. • Similar size distribution for fresh and stored EVs.	-	• Freezing reduced RNA yield in EVs • Slow freezing and rapid thawing did not fully recover RNA levels • Freeze-thaw cycles caused RNA loss • DMSO-protected RNA yield	-	• Cryopreservation resulted in EV and RNA loss. • 10% DMSO improved RNA yield in cryopreserved samples.
[49]	**Human erythrocytes** Isolation: ultracentrifugation Storage: 4 °C (up to 7 months); resuspended in PBS-citrate	• Light scattering intensity decreased in the first week and remained constant afterward (may be due to vesicle adhesion)	• After 6 weeks at 4 °C: empty and degraded vesicles seen. • EVs stability at 15–60 °C: morphology almost preserved.	-	-	• EVs were stable at various pH levels, osmolarities, and temperatures • Minimal changes observed when stored at 4 °C
[50]	**Human Milk & Infant Formulas** Isolation: ultracentrifugation Storage: 4 °C, -80 °C with/without glycerol and DMSO	• 4 °C caused loss of Exos in human milk after 4 weeks • No significant loss at 4 weeks in frozen samples or with preservatives • No significant size changes.	-	• Stored milk at -80 °C or > 24 h resulted in low RNA yield.	-	• Exosome-sized vesicles Lost in human milk at 4 °C after 4 weeks - No significant loss in frozen samples
[37]	**HEK 293 conditioned medium** Isolation: Exo-Quick kit. Storage: Short-term (4 °C to 90 °C, 30 min), long-term (-70 °C to RT, 10 days)	-	• Altered exosome morphology during storage • Increased dispersion in 10-day RT • maintained morphology at -70 °C storage	**Short-term storage**: • Stable exosome markers at 4 °C, 37 °C, and RT. • Slight loss at 60 °C. • All proteins degraded at 90 °C **Long-term storage**: • Stable markers below – 20 °C; lost in higher temperatures.	-	• High temperatures degraded exosomal proteins • Freezing conditions were best for long-term storage • Above – 20 °C was not ideal for exosome preservation • Cold storage is recommended for long-term preservation.
[13]	**Peripheral blood from metastatic colorectal cancer patients** Isolation: ExoQuick or PureExo® kit Storage: serum at 4 °C (24, 72, 168 h); RT (6, 12, 24, 48 h); up to 5 freeze-thaw cycles	-	-	• EV markers were consistent in different conditions. • High DNA stability, especially at 4 °C, while decreased over 48 h at RT. • Freeze-thaw cycles caused significant DNA decline.	-	• Serum EVs and their DNA contents were stable under different storage conditions. - Freeze-thaw cycles had the most significant impact on EV stability.
[25]	**LPS-stimulated THP-1 cells** Isolation: ultracentrifugation, ExoEasy purification. Storage: 4 °C, -80 °C (up to 1 month); thawed at RT	• No difference in EV number at 4 °C or -80 °C over time	-	-	-	• 4 °C or -80 °C were suitable for EV storage up to one month • Best preserved at -80 °C

Table 1 (continued)

Ref.	Source/ Isolation/ Storage	Characterization	Morphology	Protein, RNA, and DNA evaluations	Biological function	Highlights
[38]	**Human milk** Isolation: Ultracentrifugation. Storage: -80 °C (up to 6 months)	• No significant trends in count and size	-	-	-	• Storage did not affect Exos
[27]	**Endothelial progenitor cells (EPC)** Isolation: centrifugation with PEG Storage: -80 °C (2 months); storage media: shear-thinning gel (STG)	-	-	• Frozen EVs maintained RNA and miRNA purity. • Minimal degradation over 8 weeks at -80 °C.	• -80 °C maintained EV's bio-functionality during storage (antibacterial, angiogenesis)	• Fresh and frozen EVs stored at -80 °C maintained function for over 2 months
[39]	**Cardiac progenitor cells conditioned medium** Isolation: TFF, HiScreen Capto Core 700 column Storage: 4 °C–– 80 °C for 7 days in various tubes; buffers: PBS with Tween 20 or BSA	• EVs stored at 4 °C or -80 °C decreased particle counts over time. • Storage in glass tubes led to EV loss compared to other tube materials. • Better count and size preservation in Tween or BSA.	-	• No differences in protein content between storage temperatures.	• -80 °C had stronger cell migration effects in-vitro compared to 4 °C after 7 days • Tween 20 and BSA assisted EVs to preserve their function	• Polypropylene tubes enhanced EV recovery. • BSA and Tween 20 protected EVs. • 4 °C or -80 °C suitable for short-term EV storage. • -80 °C is better for longer preservation
[58]	**Blood samples** Isolation: centrifugation Storage: Various tubes; plasma: -80 °C; one freeze-thaw cycle	• EVs decreased after a single freeze-thaw cycle.	-	-	-	• EV concentration decreased after a freeze-thaw cycle
[65]	**First-morning urine sample** Isolation: ultracentrifugation, immunoaffinity, chemical precipitation Storage: -80 °C (6 months, long-term), RT (1 month, short-term); with or without preservative; one freeze-thaw cycle	• Slight decrease in EVs after 1 month at RT. • Size distribution of EVs unaffected by 1-month RT storage.	• EVs aggregate during long-term storage at RT.	• Stable protein and RNA contents at RT for 1 month • Higher protein and RNA content at -80 °C for 6 months • ALIX and TSG101 levels remained unchanged • Small non-coding RNAs unaffected by storage conditions	- EVs' biological function was not affected by storage conditions.	• Urine EV RNA was stable at RT for short- and long-term storage • RT is not suitable for protein analysis, but acceptable for short-term (1 month). • Cryoprotectants maintained EV stability. • Freeze/thaw harmed EVs and RNA integrity.
[59]	**Plasma** Isolation: ExoQuick kit Storage: Short-term: 4 °C (2 weeks), -20 °C, -80 °C (up to 2 months); long-term: -20 °C (3 or 5 years); up to 2 freeze-thaw cycles	• No significant size and concentration differences were observed after storage	-	• Exosomal miRNAs were stable in different storage conditions.	-	• Exosome miRNAs stable across storage conditions.
[51]	**HEK293T-palmGFP cell line conditioned medium**. Isolation: ultracentrifugation Storage: 4 °C or RT (up to 12 weeks); 3–4 freeze-thaw cycles; storage media: NaCl-HEPES, PVP nanofibers	• Particle count decreased after 2 weeks at 4 °C and RT	-	• EV marker (CD81) decreased after 4 °C and RT storage.	-	• Polymer base preservation improved EV stability. • Electrospinning offers practical vesicle storage stability.

Table 1 (continued)

Ref.	Source/ Isolation/ Storage	Characterization	Morphology	Protein, RNA, and DNA evaluations	Biological function	Highlights
[61]	**Mouse fibroblast transfected cell culture media** Isolation: ultracentrifugation Storage: 4 °C or -80 °C for 21 days; up to 3 freeze-thaw: cycles	• Count and size remained consistent after storage • Count increased with the first freeze-thaw, but decreased with subsequent cycle	-	• A slight decrease in protein content with 2 freeze-thaw cycles.	-	• EVs were stable in storage, freeze-thaw, and high salt.
[63]	**Genetically engineered HEK293T cells** Isolation: ultracentrifugation Storage: 4 °C, -80 °C (up to 7 days); storage media: PBS, culture media, trehalose, BSA-HEPES	• Significant particle reductions and size increase in PBS over time • Storage in culture media (before isolation) resulted in significant particle loss compared to isolated EV storage	• EVs in PBS may fuse or aggregate immediately after resuspension	• EVs in cryoprotectant buffer had higher DNA copies than EVs in cultured media or PBS at 4–– 80 °C storage over 7 days.	• EVs in buffer showed higher binding capacity to target cells • Storing EVs in PBS or buffer for 24 h at 4 °C did not influence EV targeting capacity.	• Storage in PBS negatively affected EV integrity and functions. • Cryoprotectants prevented EV loss and maintained EVs' capacity.
[60]	**Mouse brain tissue homogenate** Isolation: ultracentrifugation Storage: RT, 4 °C, -20 °C, -80 °C, -196 °C (0.5 to 7 days); storage media: PBS, DMSO	• Significant concentration difference across temperatures in the PBS group, but not in the DMSO group	• Damaged microparticles (MPs) during storage • DMSO had no protective effect on MPs • Freezing caused agglomeration, lysis, or fusion.	-	• Procoagulant ability decreased at all temperatures.	• No protective effect from DMSO on cryopreserved MPs. • Size, morphology, and biological function of MPs were downgraded by cryopreservation.
[41]	**Dairy cow foremilk samples** Isolation: ultracentrifugation Storage: milk samples at 4 °C and – 20 °C (7 days) before EV isolation; EVs at -80 °C up to 1 month	• No significant differences in particle size and concentration over 7 days of storage.	• No differences in morphology, aggregates, or contaminations over 7 days.	• No significant changes in protein, EV markers, RNA, and miRNA levels over storage time	-	• Storage of milk EVs at 4 °C for one week did not affect its protein concentration and markers
[47]	**Human serum from autopsy cases** Isolation: ultracentrifugation Storage: 4 °C, 20 °C, 30 °C (3 days)	• Size distributions not significantly changed by storage temperatures or periods • Samples stored at 20 °C and 30 °C showed increased smaller-sized (< 33 nm) particles	-	• Protein and miRNA levels unchanged at 4 °C and 20 °C, but reduced at 30 °C	-	• Exos were stable up to 3 days at 4 °C and 20 °C, and 1 day at 30 °C.
[52]	**Human corneal stromal stem cells (CSSC) conditioned medium** Isolation: Total Exosome Isolation Reagent Storage: 4℃ and – 80℃ up to 4 weeks for EVs; RT for EVs lyophilized with trehalose	• Similar concentration of EVs after storage at 4 °C and – 80 °C. • Better preservation of particle concentration and size with the addition of trehalose during lyophilization.	• Trehalose protected lyophilized EVs from aggregation.	• Trehalose avoided EV markers depletion after lyophilization • Total RNA and miRNA levels of EVs stable up to 7 days after lyophilization.	• Trehalose-lyophilized EVs had the best anti-inflammatory and anti-fibrotic effects. • EV integrity and function were better preserved at -80 °C than at 4 °C.	• Short-term storage (4 weeks) did not significantly alter EV integrity and function. • Storage at -80 °C is optimal for EVs preserving • Lyophilization with trehalose is effective in preserving EVs.
[44]	**Human serum exosome** Isolation: ultracentrifugation Storage: Pooled plasma stored at -80℃ before exosome isolation for up to 6 months; Isolated Exos stored at 4 °C (7 days), – 20 °C (1 month), or – 80 °C (up to 6 months)	• The highest concentration in one-week storage was at 4 °C and for longer storage was at – 80 °C • Freeze-thaw cycle in short-term damaged Exos	• Storage at 4 °C–– 20 °C caused amorphous, deformation and shrink Exos	• No difference in exosome protein markers at 4 °C for 1 week • In long-term storage higher temperatures had lower protein markers	-	• 4 °C is better than – 80 °C for 1 week storage • –80 °C for long-term storage is better • Plasma storage was better than isolated Exos in PBS

Table 1 (continued)

Ref.	Source/ Isolation/ Storage	Characterization	Morphology	Protein, RNA, and DNA evaluations	Biological function	Highlights
[46]	**Urine samples derived Exos** Isolation: ExoLution kit Storage: +4 °C (2, 7 or 14 days); +20 °C (2 days); +40 °C (2 days), -80 °C (2, 4 or 30 days) Freeze-thaw: whole urine up to 2 cycles	-	-	• +20 °C and +40 °C caused gradual mRNA degradation. • No significant changes up to 7 days at +4 °C	-	• High-temperature storage of urine samples causes mRNA content to lose • +4 °C is better for less than 7 days of storage, longer storage should be at -80 °C
[43]	**Serum samples derived EVs** Isolation: centrifugation Storage: 25 °C, 37 °C, 4 °C, and −20 °C up to 3 months	-	-	• EV miRNAs Stable at −20 °C up to 3 months • Higher temperature caused EV miRNA degradation faster • A single freeze-thaw cycle caused EV miRNA degradation of up to 70%	-	• Short-term storage at −20 °C is suitable for up to 3 months • Adding protectant significantly slowed down the degradation of EV miRNAs
[40]	**Lymphocyte-derived EVs** Isolation: ultracentrifugation Storage: -80 °C, −20 °C, 4 °C, RT, and 37 °C up to 1 month and freeze-drying Various cryoprotectants were used. Fast and slow freezing were tested.	• Stable EVs concentration at -80 °C for 30 days, and reduced EVs number at RT • EVs strongly damaged at 37 °C	-	• Total protein increased, and EV marker decreased at RT, 4 °C, and −80 °C after freezing in liquid nitrogen due to EV membrane damage	-	• Lyoprotectant maintained EV integrity upon lyophilization

conditioned media, biofluids, and tissue extracts. For instance, one-month storage of EVs from human umbilical cord mesenchymal stem cells (hUC-MSCs) at -80 °C did not significantly affect their uniform size, integrity, and bioactivity, while EVs stored at -20 °C showed a significant particle aggregation and size increase [11]. Similarly, HEK293T and MSC-derived EVs preserved their size, concentration, morphology, and RNA/protein content better when stored at -80 °C versus −20 °C up to 26 weeks [33].

Although storage at -80 °C has been shown to provide promising preservation potential, -196 °C (liquid nitrogen) is less commonly used in the studies and there is very limited data on the comparison of these two ultra-low temperatures. The available data seems to report a better outcome from storing EVs at -80 °C compared to liquid nitrogen. For example, unlike −80 °C, both microvesicles and exosomes from mice bone marrow MSCs stored in liquid nitrogen for one month exhibited a size reduction [4]. Another study showed less EV concentration loss when stored at -80 °C compared to liquid nitrogen [34]. One study demonstrated membrane disruption in EVs freezing in liquid nitrogen followed by storing at -80 [40]. Therefore, based on the evidence −80 °C remains the most practical and commonly recommended option for long-term EV preservation since it effectively maintains particle integrity without the need for ultra-low temperatures (Fig. 3).

Freezing at -20 °C exhibited moderate preservation, better than 4 °C but not as reliable as -80 °C. For instance, one study recommended the superiority of -70 °C over −20 °C for EV maintenance and storage [42]. Another study demonstrated good preservation of EV protein, RNA content, and cellular uptake at -20 °C compared to 4 °C after 28 days [34]. In another study, it is mentioned that short-term storage at −20 °C is suitable for up to 3 months to preserve EV miRNAs [43]. Oppositely, a study reported amorphous, deformation and shrink Exos after 1 month storage at −20 °C [44]. Therefore, −20 °C could serve as an alternative for mid-term storage when −80 °C facilities are unavailable. However, further studies could clarify the exact effectiveness of −20 °C for EV preservation.

Impact of moderate temperatures (4 °C)

Moderate temperatures like 4 °C are often used for short-term storage, especially when EVs are needed within a few days. In some studies, storing at 4 °C was thought to yield appropriate conditions for preserving EV size, quantity, cargo, and bioactivity over short-term durations

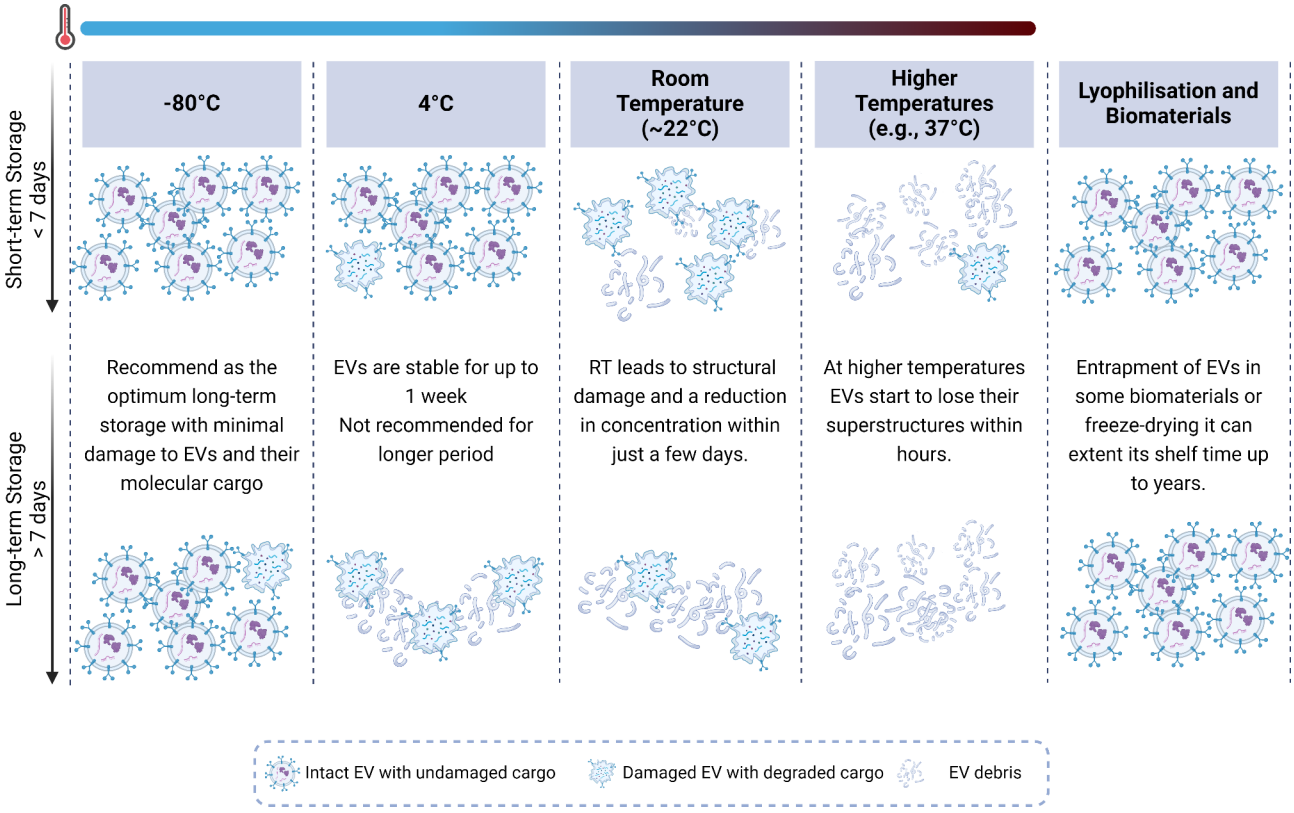

Fig. 3 Temperature and time effects on EV stability. The figure was created with BioRender.com

of up to 1 week [22, 25, 39, 42, 44–46]. For example, eight days of storage of HEK293T-EVs at 4 °C did not adversely affect their count, structure, and RNA and protein content [33]. Similarly, no significant changes in morphology, RNA content, or biological function were observed in hUM-MSCs-derived EVs after two weeks of storage at 4 °C [45]. In a study associated with postmortem serum and exosomal miRNA expression profiling, it was found that Exos may be stable at 4 °C for 3 days [47]. Another study claimed that a week of refrigeration of bovine milk samples at 4 °C before EV isolation did not adversely affect their quality [41]. Additionally, bronchoalveolar lavage fluid EVs retained their biological functionality and membrane integrity when stored at 4 °C for 4 days of storage, indicating that moderate temperatures without freezing are a safer choice for short storage durations to lessen the damage to macromolecules by ice crystal formation [48]. These findings suggest that 4 °C is a viable short-term storage option (Fig. 3).

However, as the storage period exceeds one week, the stability of the EV decreases. For instance, some studies have reported size increase, membrane disruption, aggregation, and protein degradation in EVs stored at 4 °C for longer periods [22, 34, 36, 44, 46, 49–52]. This indicates that 4 °C may be suitable for short-term storage, however, its effectiveness diminishes significantly with time and it is generally not recommended for long-term preservation.

Nevertheless, in a study, long-time EV storage at 4 °C for 20 months preserved their membrane integrity [19]. One study reported no significant differences in EV number in different storage temperatures at 4 °C or -80 °C for up to one month [25]. Another study reported better preservative results for erythrocyte EVs when stored at 4 °C compared to -80 °C up to 6 months [49].

Impact of moderate temperatures (room temperature and above)

Storage at room temperature (RT, ~ 25 °C) or higher leads to rapid and substantial degradation of EVs. Most studies show that EVs stored at room temperature exhibit significant decreases in particle concentration and integrity within just a few days. Storing EVs above 4 °C reduces stability even at room temperature (RT) and decreases EV quality [13, 30, 36, 37, 40, 51, 53]. For instance, mouse J774A.1 cells-derived EVs stored at RT for 12 months showed almost no biological activity [53]. Additionally, RT storage of HEK293T-EVs displayed significant size enlargement and membrane disruption [51]. Even over shorter periods, higher temperatures of >+20–30 °C in other studies caused a reduction in EV markers, proteins, and RNA cargo within days [40, 43, 46]. The EV

degradation process accelerates at higher temperatures, such as 37 °C within hours to days [37, 40, 42–44, 54]. Therefore, EVs should kept away from higher temperatures, otherwise, they might lose all their bioactivity after just four days of storage.

Storage duration effects

The period of storage influenced EV integrity independent of temperature, although the results are not consistent. For instance, in urine and plasma EVs, particle concentration and RNA content were decreased after being stored for more than 6 months at -80 °C [21, 29]. However, another study reported no significant change in the count and size of Exos after freezing milk for 6 months at -80 °C [38]. There are very limited studies that investigate the long-term sustainability of EVs (more than one year), which is a critical limitation in the field of studying the sustainability of EVs. Most of the studies do not extend beyond 6–12 months with sufficient focus on the impact of ultra-low temperature, storage medium, and cryoprotectants on EV stability during extended periods. Although relevant papers suggest that storage at -80 °C generally preserves EV up to several months, whether this remains effective over the years has yet to be determined in multiple comprehensive studies.

Zeta potential and polydispersity index

Zeta potential and polydispersity index are critical physical properties that play a significant role in understanding the stability and uniformity of EVs during storage. Zeta potential measures the surface charge of EVs, which influences their colloidal stability and aggregation tendencies under different storage conditions. A high zeta potential indicates better stability, reducing the risk of aggregation over time. Changes in zeta potential during storage may reflect alterations in EV surface properties or aggregation. The polydispersity index reflects the size distribution of EVs, with lower values indicating a more uniform population. Monitoring these properties during storage can provide valuable insights into EV stability and potential functional changes. However, few studies evaluated the impact of parameters like temperature, freeze-thaw cycles, and storage period on the zeta potential of EVs. One investigation found that the zeta potential remained consistent during short-term storage at room temperature or refrigeration [36]. In contrast, significant declines in zeta potential values were observed after freezing at -80 °C and thawing, indicating possible structural disruption [48]. Another study noted that the zeta potential of EVs was unchanged after 3 months of -80 °C storage, but became more positive after 6 months, suggesting alterations to surface charge over time [29]. Another trial reported slight changes in zeta potential values with EV lyophilization [55]. Changes to zeta potential could reflect cargo leakage, membrane instability, and vesicle aggregation during suboptimal storage.

Freezing methodology

Rapid freezing protocols implementing direct liquid nitrogen vitrification or snap freezing exhibited comparatively better maintained EV particle numbers compared to slow freezing, without cryoprotectants in both methods [11, 16]. It should be noted that freezing in both −80 °C and liquid nitrogen can lead to membrane disruption of EVs to some extent, however, lesser damage was reported in one study at -80 °C than in liquid nitrogen [16]. Controlling the rate of reducing temperature (-1 °C/minute) partially recovered RNA yield versus unfrozen controls [56]. One investigation found EV diameter increased after storage at -20 °C but not at 4 °C [34], whereas another study found similar results at -80 °C versus 4 °C [48]. Hypothetically, there is the possibility to implement cryoprotectants in rapid freezing protocols to enhance the efficiency and protective effects. Since cryoprotectants like DMSO, glycerol, or trehalose can prevent ice crystal formation, there might be less membrane disruption in EVs. Further research is needed to develop optimum EV-specific freezing protocols to enhance the stability and functionality of EVs.

Freeze-thaw effects

Multiple studies revealed that repeated freeze-thaw cycles reduced EV number and RNA content coincided with increased mean size, aggregation rate, and weakened bioactivity [13, 29, 30, 34, 35, 43, 54, 56–60]. Additionally, studies consistently report that multiple freeze-thaw cycles can lead to membrane disruption that negatively affects the structural integrity of EVs. For instance, one study reported a 23–36% loss of EVs derived from various blood cells after a single freeze-thaw cycle [58]. Another found a 37–43% reduction in EVs quantity after 3 freeze-thaw cycles [57]. An approximately 70% EV miRNA degradation was seen followed by a single freeze-thaw cycle in a report [43]. In plasma-derived EVs, RNA yield declined with each additional freeze-thaw procedure [56]. Another study reported that three cycles of freezing to -80 °C and thawing at RT increased vesicle size and polydispersity index (PDI) [35]. Two investigations reported minimal effects on EV number, RNA, or miRNA content after one freeze-thaw cycle, while significant declines occurred after two cycles [59, 61]. Most of the published reports focused on the effect of freeze-thaw cycles at -80 °C, and there is a gap in the comparison of the effect of freeze-thaw cycles between −20 and −80 °C. However, since −80 °C storage generally shows better EV preservation post-storage thawing, it is possible that freeze-thaw cycles at -20 °C might result in greater losses. In only one study, it was reported that freeze-thaw cycles resulted in

decreased biological activity in the endothelial gap closure assessment in -20 °C EVs compared to the −80 °C [30]. These findings emphasize the critical importance of minimizing freeze-thaw cycles in EV transport and storage. Designing experiments or protocols that implement only a single-time freeze-thaw cycle or alternative storage methods such as lyophilization may help to reduce the harmful effects of repeated freezing and thawing.

Morphological effects

By using transmission electron microscopy (TEM), scanning electron microscopy (SEM), cryogenic electron microscopy (cryo-EM), and flow cytometry, it became clear that suboptimal storage temperatures and repeated freeze-thaw cycles cause EV size enlargement, aggregation, membrane disruption, and structural deformation [4, 20, 29, 34, 37, 48, 49, 54]. In one study, storing at 4 °C for 6 weeks yielded empty and degraded EVs [49]. In another study, -80 °C led to multi-lamellar membrane formation [48]. Only one study found minimal morphological differences after storage for up to one month at -80 °C versus 4 °C [25]. The observed morphological changes highlight the sensitivity of EVs to suboptimum storage conditions and also emphasize the need for careful optimization of storage protocols to maintain EV structural integrity for their biological function preservation and therapeutic potential maintain.

Protein and RNA content

Western blotting analysis revealed that storing samples at -20 and −80 °C can result in favorable outcomes for the preservation of EV tetraspanins (CD63, CD9, CD81, TSG101, and HSP70) compared to higher temperatures such as 4 °C and RT. The most favorable results were detected at -80 °C however longer storage periods led to greater protein degradation [11, 12, 33–37, 48]. In one study, these markers became hard to detect with the Dot Blotting technique within 7 days in EVs stored at RT [11]. In another study, significant decreases in protein content were noticed within 8 weeks for EVs stored at both −20 and −80 °C [33]. Similarly, multiple studies reported that storing at lower temperatures, closer to -80 °C led to appropriate EV-associated RNA compared to storing at higher temperatures [21, 27, 33, 46]. In contrast to RNA content, EV-related DNA seems to be fairly stable at different temperatures, since it was not affected by storage temperature and period [13, 45]. For instance, the DNA content of serum EVs remained relatively unaffected under different storing conditions, including 4 °C and RT [13]. Freeze-thaw cycles, however, can cause a significant loss of DNA content [13]. In biofluids like milk and urine, storage at -80 °C caused better EV protein and RNA preservation compared to -20 °C or maintenance at RT [21, 50, 57]. In this case, one study noticed no significant differences in RNA yield between EVs stored in their physiological biofluids at RT for 7 days compared to -80 °C [57].

Functional effects

In addition to physical and molecular changes, few studies evaluated the functional potential of stored EVs on recipient cells. One study found that EVs stored at 4 °C exhibited reduced endothelial cell gap closure compared to fresh EVs [30]. Another study reported reduced bioactivity after 5 freeze-thaw cycles despite preserved cytokine secretion from DC2.4 cells [32]. Two studies observed impaired EV uptake and activation of target cells following inappropriate storage [34, 54]. Remarkably, in two studies, freeze-dried EVs preserved their function when lyophilized with trehalose [52, 62]. These studies emphasize the importance of validating storage protocols both for structural and molecular preservation and the desired biological activity maintaining of EVs for their following intended applications.

Stabilizing agents

Phosphate-buffered saline (PBS) as the most commonly used buffer to resuspend isolated EVs, may result from EV aggregation, decline in count, and loss of cargo and bioactivity [3, 4, 33, 35, 36, 43, 53, 60, 63, 64]. Several studies disclosed that using chemical stabilizers and cryoprotectants helped maintain EV integrity during handling and storage. Accordingly, the application of protease inhibitors, trehalose, and human serum albumin inhibited the EV number loss enriched from various sources [4, 29, 32, 33, 35, 36, 52, 62, 63]. For instance, one study reported no variations were found in EV concentration following storage for up to one year at -80 °C supplemented with trehalose [35]. Two years of storage at -80 °C of EVs in PBS supplemented with human serum albumin and trehalose minimally degraded their RNA and protein content compared with EVs in PBS with no supplements [33]. Other cryoprotectants like DMSO, glycerol, and sucrose helped maintain particle quantity, RNA content, and bioactivity during freezing and thawing procedures [4, 9, 22, 29, 33, 48, 50, 56, 62]. One study observed aggregation occurred when trehalose was not used as a cryoprotectant for lyophilized EVs [32]. Another study observed that freeze-thaw cycles and lyophilization with stabilizers like sucrose and poloxamer 188 (P188) can protect EVs from swelling. This strategy also maintained EVs' morphology, protein content, and bioactivity for 6 months at both RT and −80 °C [64]. Utilizing supplementing buffers and stabilizers such as trehalose, Tween-20, or bovine serum albumin (BSA), it is possible to preserve EV particle count, cargo, and function for at least one week of storage [39, 63]. In contrast, only in one study it was claimed that DMSO did not exert

any protective effects after a week of storage at 25 °C, 4 °C, -20 °C, -80 °C, and −196 °C [60]. By preserving EVs inside hydrogel-forming microneedles, EV parameters were stable for up to 12 months at 4 °C or RT [3, 53]. Based on evidence, the use of appropriate stabilizing agents can lead to the preservation of EV integrity during storage and transportation. However, the choice of stabilizer should be based on various factors. For example, the source and type of EV, storage conditions, and intended downstream applications must be carefully considered to ensure optimal preservation of EV stability while avoiding toxicity.

Storage in biofluids

Several studies revealed that storage of EVs in native biofluids like urine, milk, but also cell culture media can lead to better-preserved particle concentration, RNA content, and surface protein expression compared to purified EVs stored in buffer solutions [11, 16, 19–21, 29, 44, 57]. The observation that EVs are better preserved in their native biofluids compared to purified EVs in buffer solutions may be attributed to several factors. Firstly, native biofluids provide a complex, physiological environment that may help maintain EV integrity. This environment includes proteins, lipids, and other molecules that could act as natural stabilizers to reduce the likelihood of EV aggregation or degradation. Secondly, native biofluids likely provide a more suitable osmotic environment for EVs compared to artificial buffers, thereby preserving their cargo, such as RNA, and surface proteins more effectively than in artificial buffer solutions. This hypothesis suggests that storing EVs in their natural context may offer a more supportive environment with enhanced long-term stability. Further research is needed to uncover the mechanisms behind biofluid preservation and develop synthetic media that mimic these protective effects.

Lyophilization, hydrogels, and biomaterials for conserving EV stability

Recently, emerging methods such as lyophilization (freeze-drying) and the use of hydrogels have been considered as potential solutions to overcome the limitations of traditional liquid phase storage. In lyophilization, EVs are stored in a dry and solid state that maintains their structural integrity and biological functionality even at room temperature. Similarly, hydrogels provide an encapsulation environment that captures and stabilizes EVs. This approach not only can enhance EV stability but also facilitate controlled release in therapeutic applications. Several studies investigated the potential of lyophilization techniques and biomaterials like hydrogels for preserving EV stability during the storage period (Fig. 4). Lyophilization was found to maintain EV size, morphology, protein content, and bioactivity when appropriate cryoprotectants were simultaneously used [3, 9, 22, 30, 32, 55, 62]. According to several reports trehalose and sucrose with polysorbate 80 provided effective lyophilization and prevented aggregation [9, 32, 52, 62]. In one study, authors reported well-preserved, spherical vesicles after lyophilization with trehalose or sucrose and polysorbate 80 [62]. Lyophilized EVs showed similar cellular uptake and functional effects related to freshly isolated EVs [9, 62].

A study demonstrated that RT storage of lyophilized EVs with trehalose did not affect their protein, RNA contents, or functional properties [32]. Similar results were obtained without using cryoprotectant [30]. Hydrogels have also shown promising platforms for stabilizing and storing EVs. The encapsulation of EVs in hydrogel microneedles composed of hyaluronic acid preserved EV integrity [3]. This approach protected the EVs even after 6 months of storage, maintaining cargo integrity and biological activity. Overall, lyophilization and incorporation into stabilizing hydrogel matrices emerge as an effective approach for long-term EV preservation. Nevertheless, a study reported that lyophilization had limited ability to mitigate the effects of storage on samples and both lyophilization with and without cryoprotectant failed to outperform the effectiveness of storing samples at -80 °C [29]. Therefore, additional studies are urgently needed to address the critical issues related to the application of hydrogel for EV protection and storage.

Storage tube material effects on EVs

The adsorption of EVs onto storage tube walls during refrigeration can result in significant vesicle loss [28]. It is reported that about a 32% reduction occurred for total EV count after 48 h of storage at 4 °C. This effect would be related to EVs binding on ordinary polypropylene tubes. However, the use of specialized low protein-binding tubes prevented over 50% of these adsorption-mediated losses. These features indicate that the tube material significantly impacts the recovery of EVs after low-temperature storage [28]. Of note, use of glass tubes resulted in lower particle recovery versus plastic tubes after 1 week at 4 °C (Fig. 4) [39].

Discrepancies

There are several controversies regarding the effects of -20 °C versus 4 °C storage on the physicochemical properties of EVs. In one study, it was suggested that the preservation capacity of -20 °C was better in terms of EV size distribution, concentration, and RNA content compared to 4 °C [34], while another reported reduced EV concentration at -20 °C, but not at 4 °C [42]. Also as mentioned before, in a study abnormal and shrink Exos were observed after 1 month storage at −20 °C [44]. Similarly, there were various results for the impacts of short storage

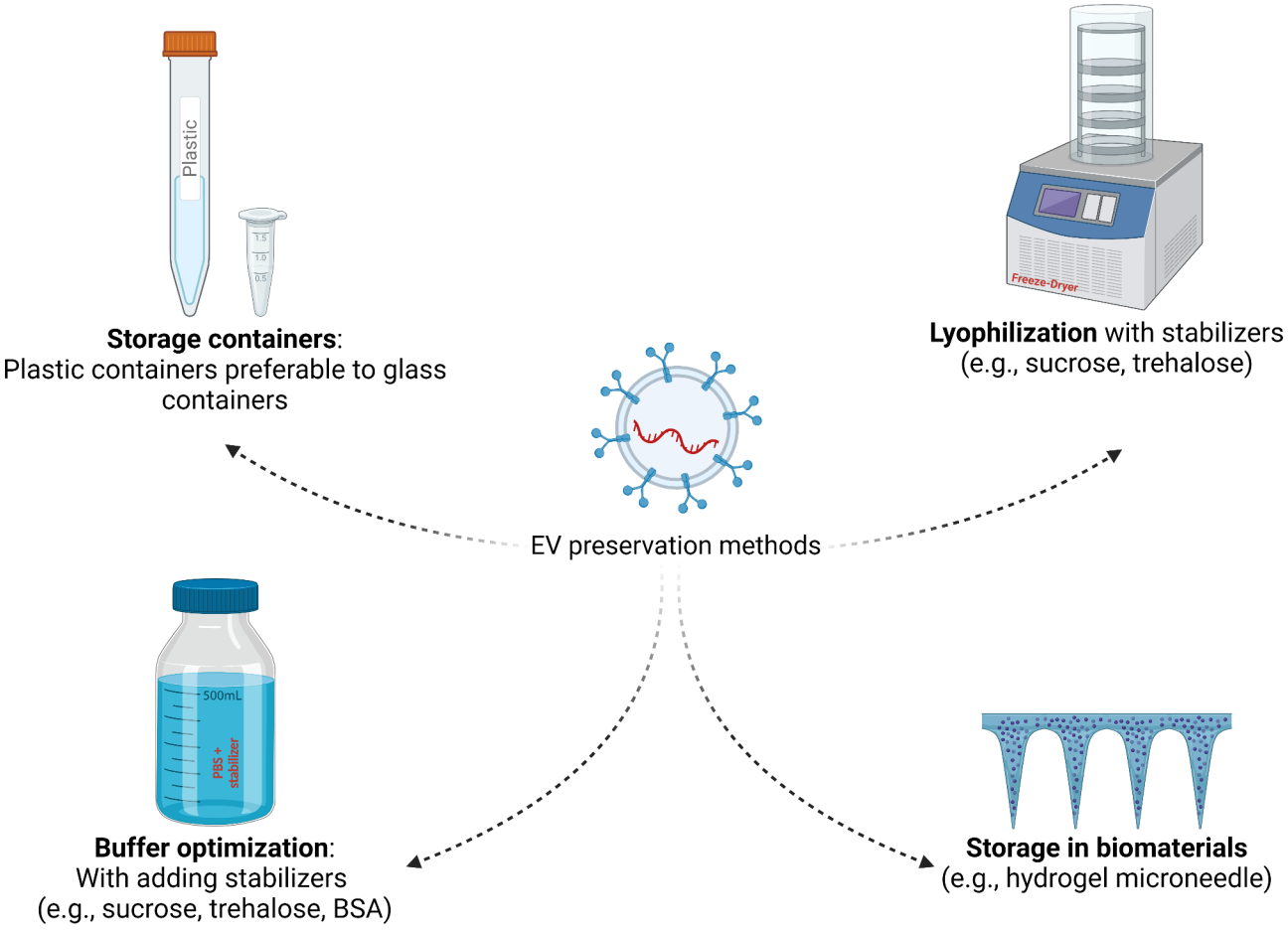

Fig. 4 Storage methods and additives for EV preservation. The figure was created with BioRender.com

at RT. One study observed no significant differences in EV number after 14 days at RT [45], while several other studies noted decreased EV concentrations at RT starting from 48 h [13, 30, 36, 37, 51]. Another study concluded that RT is suitable for short-term storage (less than a month), however, longer storing durations at RT will affect EVs protein and RNA contents [65]. These inconsistencies in the tolerance of EVs to RT might be due to different isolation and assay methods.

Discussion

In this systematic review, 50 original studies were included. These studies examined various storage conditions and how they can affect the physicochemical features of EVs, such as their initial number, size distribution, shape, content, and functional activity. EVs have a nano-scale physical structure, which makes them very sensitive to surrounding microenvironmental conditions. Optimized protocols are required to maintain EV structural, molecular, and functional integrity during handling, freezing, long-term storage, and thawing.

EVs help cells communicate with each other by exchanging important substances such as nucleic acids, proteins, lipids, and metabolites [9, 16, 25]. The potential clinical application of EVs can be directly affected by alterations in their cargo content resulting from inappropriate storage durations, temperatures, and freeze-thaw cycles. Multiple studies in the current systematic review mentioned that storing EVs resulted in a loss of their ability to affect recipient cells irrespective of storage temperature. All biological properties like cell uptake, migration, and cytokine secretion stimulation were affected by different degrees [30, 32, 33, 39]. Since EVs are gaining continuous attraction as drug delivery platforms, cell-free therapeutics, and clinical biomarkers, maintaining their functional properties during processing and storage using methods is vital. However, the wide range of methods of isolation, optimal temperature, freeze-thaw limits, effective stabilizing substances, various sources of EVs, and functional assessments make it extremely complex to establish consistent and broadly applicable procedures.

Despite the existence of conflicting data and lack of complete convergence, some consistent findings became

apparent. In general, storage at -80 °C outperformed higher temperatures for preserving EV quantity, cargo, and morphology, especially for longer than 1 week (Fig. 3). Rapid freezing helped better maintain EV parameters, emphasizing the importance of quickly transitioning samples to ultra-low temperatures to face minimum freezing damage [11, 16]. Subjecting EVs to repeated freeze-thaw cycles caused consistent damage which emphasizes the need for protocols with the least need for or without thawing and refreezing [13, 29, 30, 34, 35, 54, 57, 58]. Storing EVs in their natural biofluids, rather than resuspending them in standard buffers, makes them more stable which indicates the benefit of keeping their physiological conditions in the storage process [11, 16, 19–21, 29, 44, 57].

In addition to conventional methods, emerging storage techniques such as Lyophilization and biomaterial encapsulation helped to reduce the detrimental effects of storage on EVs. Lyophilization, when coupled with cryoprotectants, has been shown to preserve EV morphology and cargo content over long periods at room temperature. Likewise, hydrogels provide a protective matrix for EVs which enhance stability and functionality while offering the potential for controlled therapeutic release. These methods prevent vesicle clumping and leakage, addressing challenges in preserving EVs in liquid form. Optimizing lyophilization conditions can maintain EV structure and function, even at RT. However, scalability for clinical-grade EVs needs more research. Using hydrogel matrices to stabilize EVs is another option, extending their lifespan and enabling controlled drug release. Exploring the synergy between EV encapsulation and their release could uncover ideal materials and methods. Developing integrated processes for EV isolation, lyophilization, and encapsulation to meet GMP standards will advance clinical use (Fig. 4). The development of standardized protocols for the use of these innovative approaches will be key to utilizing their maximum potential in EV-based therapies. This technique offers advantages for ease of storage and transportation, particularly for clinical use where refrigeration might not always be feasible.

Storage tubes could also alter EV parameters [28, 39]. Standard tubes can cause EVs to adhere to the wall, leading to inaccurate measurements and potential research and therapy problems [28, 39]. Using anti-adhesion coated tubes can be a simple solution to improve the reliability of EV studies and production. Exploring alternative approaches like lyophilization and bio scaffolds can eliminate plastic tube-related issues (Fig. 3).

This review revealed several key controversies on the best storage temperatures and durations to preserve EV integrity across different studies. These discrepancies likely come from differences in EV isolation method, storage, and analyze, emphasizing once again, the importance of standardized protocols. Additionally, since various isolation methods can unintentionally contain contaminants that affect EV stability, further research using reliable techniques is needed to establish the ideal isolation methods, temperature ranges, and appropriate buffers.

Another issue is that many studies only assessed EV stability for short durations, usually just days to weeks. Long-term monitoring over months or even years could provide a more accurate understanding of their stability. Based on the increasing interest in EV-based therapies, especially in clinical settings, it is crucial to develop practical and reliable protocols that can maintain EV function for long periods. Multiple studies suggest that storage at very low temperatures, such as -80 °C, can effectively preserve EV integrity over months, but further research is needed to determine whether these conditions are sufficient for long-term storage over one or two years. In addition, innovative preservation techniques such as lyophilization and encapsulation in hydrogels may demonstrate a remarkable potential for long-term EV stability, however, these approaches also require further validation for periods longer than one year. Only a few studies explored how storage affects surface charge (zeta potential) and aggregation mechanisms, which should be a focus of future research. Probably storage conditions like freezing, thawing, and long-term storage can alter zeta potential, leading to aggregation [29, 48]. A more comprehensive investigation of EV physical properties is necessary to better grasp the factors causing their instability. Several inconsistencies were observed among studies regarding the effects of storage temperature and the use of cryoprotectants on EV integrity. This might be due to variations in experimental conditions, including differences in EV isolation methods, the source of EVs (e.g., biofluids vs. tissue extracts), and the specific experimental design used in the studies. For example, different results under similar storage conditions could be explained that studies using centrifuge-based separation techniques may yield EVs that are more prone to aggregation due to the applied high gravity force compared to those obtained through size-exclusion chromatography. Additionally, variations in EV cargo (e.g., RNA, protein) may also contribute to inconsistent findings, since EVs from different sources may represent different cargo profiles that may cause different outcomes from the same storage protocols in various studies. Future studies should focus on standardizing isolation and experimental conditions to reduce variability and improve reproducibility in EV storage research.

Conclusions

This systematic review highlighted the significant impact of storage conditions and stabilizing strategies on key EV parameters such as concentration, size, structure, cargo content, and functionality. The findings emphasized the importance of maintaining physiological conditions, implementing rapid or immediate freezing, minimizing freeze-thaw cycles, and using stabilizing additives to preserve EV quality. These insights are crucial for developing standardized procedures to ensure consistent EV stability for both research and clinical applications. However, further high-quality studies are needed to optimize storage conditions, freeze-thaw limits, and cryoprotectants for different types of EVs. In addition, lyophilization and biomaterial-based strategies offer promising alternatives for preserving EVs, enabling solid-phase storage and sustained release. Developing specific lyophilization and encapsulation protocols for EVs can address key challenges in storage and delivery, making EVs more accessible for therapeutic applications. Finally, the review highlighted the importance of storage tube surface properties, emphasizing the need for optimizing tube coatings, understanding EV-surface interactions, and exploring tube-free storage methods like lyophilization to enhance EV storage consistency and analysis.

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Author contributions
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Data availability
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
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Consent for publication
Not applicable.

Competing interests
Amin Tamadon was employed by PerciaVista R&D Co. The other authors declare that their research was conducted without any commercial or financial relationships that could be perceived as potential conflicts of interest.

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