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Preservation and Storage Stability of Extracellular Vesicles for Therapeutic Applications

Anjana Jeyaram[1] and **Steven M. Jay**[1,2,3,4]

[1]Fischell Department of Bioengineering, University of Maryland, 3234 Jeong Kim Engineering Building, College Park, Maryland 20742, USA.

[2]Program in Molecular and Cellular Biology, University of Maryland, College Park, Maryland, USA.

[3]Greenebaum Comprehensive Cancer Center, University of Maryland, College Park, Maryland, USA.

Abstract

Recently, extracellular vesicles (EVs)—including exosomes, microvesicles, and others—have attracted interest as cell-derived biotherapeutics and drug delivery vehicles for a variety of applications. This interest stems from favorable properties of EVs, including their status as mediators of cell-cell communication via transfer of biological cargo and their reported ability to cross biological barriers that impede many delivery systems. However, there are many challenges to translation and widespread application of EV-based therapeutics. One such challenge that has yet to be extensively studied involves EV preservation and storage, which must be addressed to enable use of therapeutic EVs beyond resource-intensive settings. Studies to date suggest that the most promising mode of storage is −80°C; however, understanding of storage-mediated effects is still limited. Additionally, the effects of storage appear to vary with sample source. The lack of knowledge about and standardization of EV storage may ultimately hinder widespread clinical translation. This mini-review reports current knowledge in the field of EV preservation and storage stability and highlights future directions in the area that could be critical to eventual development of EV therapies.

Keywords

exosomes; extracellular vesicles; preservation; stability; storage

INTRODUCTION

Extracellular vesicles (EVs) are cell-derived products that are useful for a variety of applications as both primary therapeutics and drug delivery vehicles (1). As with cells and other cell-derived products (proteins, etc.), maintenance of EV biological activity during storage is both critical and challenging. Yet, little has been reported about EV preservation. Though storage of EV-based therapeutics is not expected to be as difficult as cell-based

[4]To whom correspondence should be addressed. (smjay@umd.edu).

therapy preservation, some limitations will undoubtedly apply. Ultimately, effective preservation and storage of EVs will be critical in determining the scope of their therapeutic application. Here, we summarize current knowledge of EV preservation and storage techniques in the context of their uses as primary biotherapeutics as well as drug carriers. We further highlight critical areas for future study towards overcoming current limitations to enable effective preservation and storage of therapeutic EVs.

EVS AS PRIMARY BIOTHERAPEUTICS—EVS ISOLATED FROM BIOFLUIDS

As EVs are released in the body as a form of cell-cell communication, they can be isolated from biofluid samples for therapeutic applications. In many cases, it is not practical or most useful to isolate EVs from a patient sample immediately after biofluid collection. Studies have shown that the biofluid storage method can impact EV yield, composition, and function (summarized in Table I). It is important to understand what these changes are and how to avoid them for reproducible therapeutic outcomes, which are essential for clinical translation.

Semen

Seminal EVs have been demonstrated as potentially useful in the development of new prostate cancer diagnostics (2) and have recently been shown to possess anti-HIV-1 activity (3). However, it is often not possible to isolate EVs immediately upon collection of clinical samples; thus, studies investigating sample storage methods are vital to enabling therapeutic use of semen-derived EVs. To this end, Madison *et al.* showed that human seminal plasma or blood serum samples could be stored at − 80°C for short periods of time without significantly impacting EV yield or bioactivity (3,4). Welch *et al.* showed preservation of semen EV physical properties, including morphology, concentration, and size after short (2 years) and prolonged freezing (30 years) at − 80°C (5). The prolonged freezing of the semen also did not significantly alter EV total RNA content. The protein concentrations and proteome profiles of these EVs as determined by Bradford analyses and protein footprinting (silver stain), respectively, were also independent of length of freezing.

However, while semen EV morphology remained preserved, there was a significant decrease of EV-associated acetylcholine-esterase (AChE) activity after prolonged freezing. AChE is a plasma membrane protein incorporated into EVs during biogenesis, making AChE enzymatic activity a commonly used marker for EVs. This decrease in AChE activity may have important functional and biological effects. Welch *et al.* demonstrated that while freezing for two years did not significantly impact semen EV bioactivity, prolonged freezing of the semen before EV isolation impaired the ability of EVs to prevent HIV-1 infection of cells (5). The significant correlation between decreased AChE activity and absence of HIV-1 inhibition suggests that prolonged freezing of biofluids can impair critical therapeutic bioactivity of isolated EVs, depending on the mechanism of action. Although the small sample size limits conclusions, this report suggests the need for additional studies on the effects of biofluid storage prior to EV isolation.

Urine

EV collection from urine is of great interest for biomarker development, and this biofluid may also serve as a convenient medium for collection of EVs to be re-tasked for therapeutic purposes via drug loading methods (6–8). Zhou and colleagues showed that storage temperature is critical; EVs were effectively preserved at − 80°C, whereas storage at −20°C resulted in a significant loss of EVs compared to fresh urine. EV recovery after freezing could be maximized by intensive vortexing of the samples after thawing, resulting in 87.4 and 100% recovery from − 20 and − 80°C storage, respectively (9). Further studies suggest EV degradation can occur within two hours of urine collection and confirmed that the optimal storage of urine samples for EV preservation is − 80°C with the addition of protease inhibitors (10).

Milk

Milk-derived EVs have already been utilized as drug carriers and are also reported to have intrinsic therapeutic properties (11–13). Bovine milk-derived EVs have been stored at − 80°C for four weeks without any changes in physical properties, and percent loading of paclitaxel in these EVs also remained stable over this time (14). Milk EVs stored at − 80°C also remained mostly free of coagulation for several months and showed minimal loss of activity (11). Others have found that storing unprocessed breast milk at – 80 or 4°C causes the death of cells present in breast milk and results in contamination of the breast milk EV population, with the majority EVs formed after storage-induced stress on the cells (15). These findings are particularly critical given the surge of interest in the potential therapeutic applications of milk-derived EVs.

Blood Components

EVs isolated from various components of the blood, including plasma, platelets, and serum, have been employed for a variety of diagnostic and therapeutic purposes (16,17). In contrast to other biofluids, one study showed that long-term storage and freeze-thaw have been reported to not have a critical impact on EV yield (18), while another demonstrated that platelet samples stored as a liquid at room temperature yielded less EVs with decreased procoagulant activity than those frozen with 6% DMSO as a cryoprotectant (19). This effect may be due to effects on EV-associated proteins, which are reported to be sensitive to acute sample storage temperature as well as the period of time before initial sample processing (18). Interestingly, separate studies showed that storage of plasma at 4, − 20, or − 80°C as well as two freeze-thaw cycles, did not result in significant degradation of EV-associated RNA (20,21). An additional report detailed that even after storage of plasma samples at room temperature for over 42 h or − 80°C for 12 years, EVs could still be isolated without degraded RNA levels (22). DNA contained in serum EVs also remained stable in different environments, including one week at 4°C, one day at room temperature, and after repeated freeze-thaw cycles (23). Despite this apparent stability of blood component EV-associated nucleic acids after freezing, fresh plasma yielded a more pure sample of EVs than those isolated from frozen and thawed plasma samples (24) as well as a higher yield of vesicles (25). Additionally, protein and nucleic acid aggregation was observed after seven years of storage at − 80°C (24).

Broncheoalveolar Lavage Fluid

There is growing interest in the use of airway EVs for treating pulmonary diseases, including asthma and chronic obstructive pulmonary disease (COPD). Airway EVs can be isolated from broncheoalveolar lavage fluid (BALF); however, as with other biofluids, there is limited knowledge on the effect of storage conditions on the integrity of these EVs. Maroto *et al.* concluded that storage conditions can significantly destabilize surface characteristics, morphological features, and protein content of BALF-derived EVs (26). Importantly, this study demonstrates that storage conditions impact the proteomic content of the EVs, with distinct populations being lost during storage at 4°C as well as − 80°C. Maroto *et al.* hypothesized that these differences are most likely due to the dissociation of membrane-integrated proteins, rather than the loss of internal EV proteins. This change in protein composition and morphology after − 80°C is contradictory to the results of studies in frozen whole plasma (21), likely due to concentrated plasma proteins minimizing freezing effects. Overall, this study highlights the importance of understanding the impact of storage of different biofluids on EV populations.

EVS AS PRIMARY BIOTHERAPEUTICS—EVS ISOLATED FROM CONDITIONED MEDIUM

In addition to biofluids, EVs can also be isolated from conditioned medium of cell cultures. To our knowledge, there are no studies reporting the impact of storage of conditioned medium before EV isolation, although it is notable that the International Society for Extracellular Vesicles (ISEV) recommends storage of EVs in phosphate buffered saline at − 80°C in siliconized vessels to prevent adherence of EVs to surfaces (27). There have, however, been some studies on the impacts of storage conditions on EVs after isolation from conditioned medium. In a patent owned by Capricor Therapeutics detailing the process for producing EV formulations from cardiosphere-derived cells (28), EV concentration was determined to remain stable after one week of storage at 4, − 20, and − 80°C, as quantified by Nanoparticle Tracking Analysis. Despite this stability in size over one week, miRNA levels decreased during this period when EVs were stored at 4 or − 20°C and continued to decrease over 90 days. Within 30 days, the EV miRNA content dropped to below 50% of the initial amount at both 4 and − 20°C. miRNA levels when stored at − 80°C showed little change over 90 days. The reduction of miRNA when stored at lower temperatures was ameliorated by using Plasmalyte A, Ringers, and Plasmalyte A + Dextrose, although initial buffer content was not reported. The Plasmalyte A-containing buffer yielded EVs with the highest bioactivity, as assessed by changes in macrophage phenotype after treatment with EVs. While lyophilization was not directly compared to storage in the various buffers, the process did not cause any significant reduction in *in vitro* or *in vivo* bioactivity in comparison to EVs that had not been lyophilized. Further, lyophilization and rehydration of EVs did not result in substantial losses of miRNAs. While this patent includes some of the most extensive studies on EV stability reported to date, it remains unknown whether this information can be translated to EVs from other source cells, which will vary in surface lipid and protein composition as well as cargo content. This potential variability is reinforced by studies of liposome stability, which show that the specific surface composition has a critical

role (29). Further, it would be useful to determine if the EV concentrations and overall bioactivity remain stable beyond seven days.

Lőrincz *et al.* provide a convincing argument that storage significantly alters both the physical and functional properties of antibacterial EVs derived from human neutrophilic granulocytes (30). Storage at 4°C for just a single day was shown by flow cytometry to result in significantly decreased EV number and antibacterial effect. Upon storage at − 20°C, EV number was preserved, but a shift in size as well as almost complete loss of antibacterial function by 28 days was observed. In contrast, storage at − 80°C prevented significant changes in physical and functional properties at 28 days (Fig. 1).

In total, these studies confirm that storage conditions are critical to EV function. Specifically, they agree that − 80°C storage is optimal for preserving isolated EV samples, which is unfavorable due to increased costs and limitations in transportation. Also, the mechanism of storage-induced changes in EV bioactivity remains unclear. Further understanding of this mechanism may allow for rational design of improved strategies to prevent loss of function of EVs in storage, which would significantly broaden the potential therapeutic applications for EVs.

EVS AS DRUG DELIVERY VEHICLES

Most methods of exogenous EV loading involve manipulation of the EV structures to introduce cargo into the vesicles, potentially altering the stability of these vesicles in comparison to those that are endogenously bioactive. Currently, to our knowledge, there are no published studies on the storage stability of exogenously loaded EVs and their cargo. However, there is evidence that the mechanism of loading can alter EV properties. A commonly used method of cargo loading for EV-based gene and drug delivery is electroporation (31), a process in which the temporary formation of pores in the EV membranes allows for cargo diffusion into the vesicles. Although this method is widely used, it has been shown to induce precipitation of nucleic acids, such as siRNA, and yield low siRNA incorporation into EVs (32). While endogenous *in vitro* functionality of the EVs remains unchanged, Johnsen *et al.* reported that electroporation can induce EV aggregation (33). This same study also demonstrated that RNA may be released from the EVs or degraded after electroporation depending on buffer choice, but further studies are necessary to confirm this and to clarify the dynamic nature of any cargo loss or degradation. These effects also remain to be characterized for additional methods of EV loading. Overall, potency and morphology of cargo-loaded EVs after storage must be evaluated prior to their translation as drug delivery vehicles.

FUTURE DIRECTIONS

EVs have tremendous potential to alter the biotherapeutic landscape and impact treatment of myriad diseases and injuries. However, before this potential can be realized, a systematic understanding of how to best preserve the function of EVs during storage must be developed. Adverse effects of storage on EV function reported in the studies cited herein suggest the need for controlling and improving storage conditions to preserve EV

therapeutic utility. One potential approach to accomplish this is the use of disaccharide stabilizers in EV storage buffers (34,35). For example, trehalose is a natural, non-toxic sugar widely used as a protein stabilizer and cryoprotectant by both the food and drug industries and has been validated as a cryopreservative for labile proteins, vaccines, and liposomes as well as cells and organs for transplant. It works by decreasing intracellular ice formation during freezing and preventing protein aggregation (36). Bosch *et al.* have demonstrated that 25 mM of trehalose (TRE) in PBS may be an attractive way to protect EVs during storage (37). TRE itself was shown to not alter any physical characteristics of the EVs, the RNA or protein profiles, or typical EV markers. However, there was a significantly higher particle count in TRE compared to PBS, along with reduced mean size and size distribution. This translated into higher purity (ratio of particle to protein concentration) of reconstituted EV formulations with TRE, possibly via reduced EV aggregation as the result of trehalose interference with interparticular attractive forces. This report indicates that further study of trehalose and related stabilizers, such as sucrose, is warranted.

Beyond modifying the method of EV storage, it remains to be determined how EV purification methods impact the stability of EVs. The potential for the preparation method to impact overall EV function is supported by studies showing the importance of sample processing in the recovery and purification of EVs (9,10,15) and by data illustrating the impact of the cell culture environment on EVs (38). However, additional mechanistic studies are needed given that different EV isolation methods can produce products that vary in yield, purity, and integrity (39–41).

To date, a primary limitation to studying EV storage effects and mechanisms has been inconsistency in EV characterization and analysis. While one study showed that NTA and electron microscopy analysis illustrated no change in vesicle size from 1 to 10 freeze-thaw cycles (− 20°C) (42), another reported that once isolated, a single freeze-thaw cycle lowered the EV count by 10–15%, as detected via flow cytometry (43). Elsewhere, Almizraq *et al.* demonstrated that red blood cell-derived EV characteristics varied after storage based on the method of detection (44); a comparison of tunable resistive pulse sensing, flow cytometry, and dynamic light scattering showed varying changes in EV concentration after storage. Thus, utilization of standardized EV characterization methods, such as those recommended by ISEV (27), would aid progress in this area.

CONCLUSION

Evidence to date suggests that the most promising mode of storage for EVs remains − 80°C; however, application of this mode may be limited by cost (20,45) and poses challenges in transportation. Thus, alternatives such as lyophilization and the incorporation of additives may be necessary to improve EV storage stability. Increased understanding of how to best store therapeutic EVs would allow EV-based approaches to meet their full versatile potential as a new class of promising therapeutics and drug carriers.

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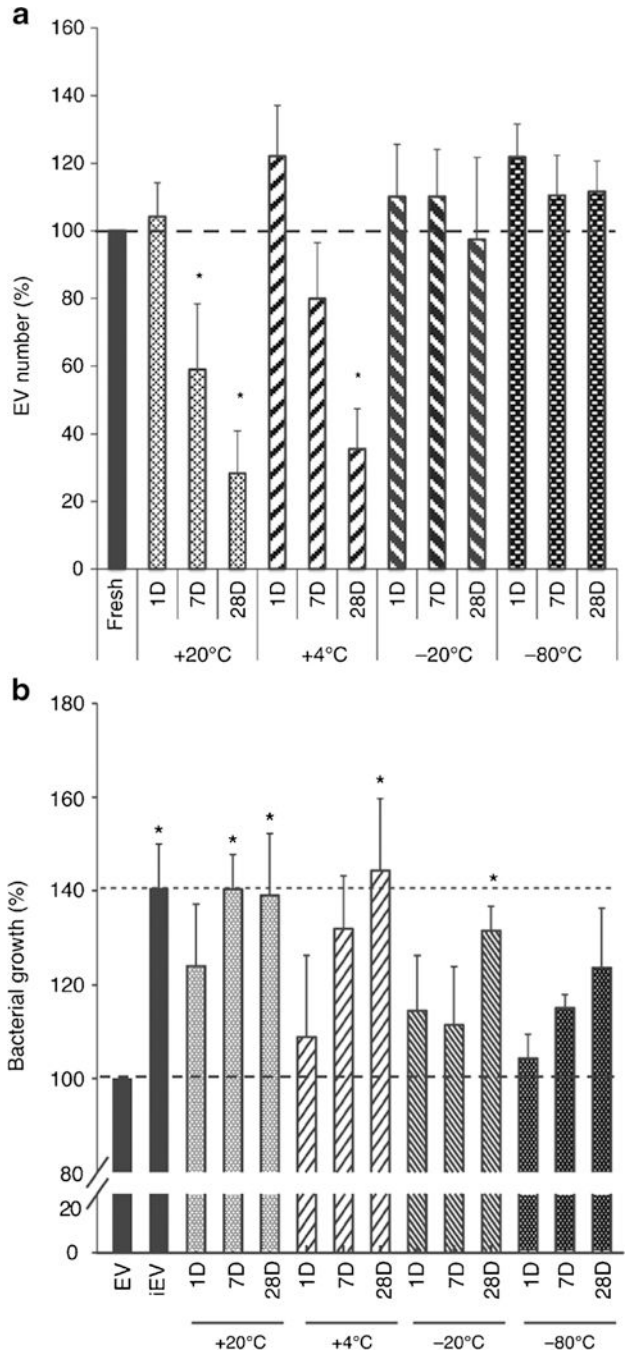

Fig. 1.

Effect of storage conditions on number and antibacterial capacity of neutrophilic granulocyte-derived EVs. **a** Changes in EV number after storage in comparison to a fresh sample were evaluated by flow cytometry. EVs were stored at + 20, +4, − 20, or at − 80°C for up to 28 days ($n = 4–8$, $*p < 0.05$ by t test). EV number decreased significantly at + 20 and + 4°C, but not at − 20 or at − 80°C. **b** Bioactivity after storage was evaluated by incubating bacteria with either fresh or stored samples of EVs. Heat-denatured EVs (iEV) were used as a negative control. Storage conditions of + 20, +4, − 20, and − 80°C were

assessed up to 28 days, with a decrease in bioactivity observed with increased length of storage. ($n = 4–6$, $*p < 0.05$ by t test). Adapted via open access from © 2014 Ákos M. Lőrincz *et al.* (32)

Table I.

Summary of Tested Storage Conditions for Various Biofluid Samples and the Impact on EVs

Biofluid	Storage condition	Duration	Physical characteristics	Contents	Activity	Other
Semen	− 80°C	2 years	No change[5]	No change[5]	No change[5]	–
		30 years	No change[5]	↓ AChE activity[5]	↓ HIV-1 inhibition[5]	–
Urine	RT	2 h, 1 day, 1 week	↓ EV yield over time[10]	–	–	–
	− 20°C	1 week	–	↓ EV-associated protein recovery (27.4%)[9]	–	–
	− 80°C	1 week	–	87.4% EV-associated protein	–	–
			↓ EV yield over time[10]	recovery[9]		
	4°C + vortexing	2 h, 1 day, 1 week		–	–	–
	− 20°C + vortexing	2 h[9], 1 day[9], 1 week[9, 10]	↓ EV yield over time[10]	86% EV associate protein	–	–
		2 h[9], 1 day[9], 1 week[9, 10]	↓ EV yield over time[10]	recovery[9]		
	− 80°C + vortexing			100% EV-associated protein recovery[9]	—	–
	− 80°C + protease	2 h, 1 day, 1 week	↓ EV yield over time	–	–	–
	inhibitors		(higher yield than without inhibitors)[10]			
Milk	4°C	2 h	–	–	–	Contamination by stress-induced EVs[15]
	− 80°C	2 h[15], 4 weeks[14], 6 months[11]	No change[11]	–	No change[11]	Contamination by stress-induced EVs[15]
	− 80°C (no cells or cream)	2–8 weeks	–	No change in CD63 and CD9 expression[15]	–	Less contamination by stress-induced EVs[15]
Blood (general)	RT – 160°C	Short term (days), long term (months)	–	–	–	Varying EV signal intensity (most stable in heparin blood collection tubes)[18]
Serum	RT	6, 12, 24, 28 h	–	No change in CD63, TSG101[23]; stable DNA; decrease in DNA concentration after 24 h[23]	–	–
	4°C	24, 72, 168 h	–	No change in CD63, TSG101[23]; stable DNA; relatively stable DNA	–	–

Biofluid	Storage condition	Duration	Physical characteristics	Contents	Activity	Other
				concentration with slow decrease after 72 h[23]		
	Freeze thaws	1, 3, 5 cycles	–	No change in CD63, TSG101[23]; stable DNA; dramatic decrease in DNA concentration[23]	–	–
Platelets	− 80°C + DMSO	1 h	↑ EVs[19]	–	↑ procoagulant activity[19]	–
Plasma	RT	0–42 h	–	No change in RNA levels[22]	–	–
	4°C	2 weeks	–	No change in RNA levels[20]	–	–
	− 20°C	2 weeks	–	No change in RNA levels[20]	–	–
	− 80°C	2 weeks	–	No change in RNA levels[20]	–	–
		1 month	No change in yield[21]; increase in EV yield[25]	No change in CD63, miRNA expression, or protein content[21]		
		20 months	Decrease in EV yield[25]	–	–	–
		7 years[24]; 12 years[22]	No change in morphology[24]	No RNA degradation[22]; ↑ total protein[24]; ↑ protein/nucleic acid aggregation[24]	–	–
Broncheoalveolar lavage fluid (BALF)	4°C	4 days	↑ Size; ↓ zeta potential[26]	Change in protein composition[26]	–	–
	− 80°C	4 days	Multilamellar vesicles; ↑ size; ↓ zeta potential[26]	Change in protein composition[26]	–	–

Superscript numbers refer to corresponding references

RT room temperature