HOW TO STORE EXTRACELLULAR VESICLES:

A COMPREHENSIVE GUIDE ON EV STORAGE ACROSS A VARIETY OF SAMPLES

APPLICATION NOTE

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1 / INTRODUCTION

Extracellular vesicles (EVs) can be released by every living cell, from complex multicellular eukaryotic organisms, such as our human body, to unicellular protist parasites, from bacteria to plants and fungi. Understandably, the unique molecular composition and roles EVs play in each of these organisms is unimaginably diverse and lays the base to specialised advanced EV research.

Due to their nanoscale dimensions, concentrations and the complexity of the fluids in which they are found in vivo or in vitro, the most critical step of any EV analysis is their isolation. Ideally, EVs must be separated from non-vesicular components and concentrated to sufficient quantities suitable for the desired analysis tool. Unless immediate EV isolation and analysis is possible, short- or long-term sample storage (either pre or post EV isolation) will be required. In order for results to provide a meaningful reflection of the original physiological state of EVs, researchers need to be confident that pre-analytical processes such as sample collection, handling, processing or isolation have not altered the recovery and physical properties or biological functions of the EVs. Even if, by overcoming pitfalls in the aforementioned processes, there is still quite an array of available EV analysis methods, with pros and cons matched to the needs of the research, which can still provide significant variable data from the same EV sample. Comprehensive review on EV analysis approaches can be found in other published work[1].

There are many reports that different pre-analytical treatments have varying effects on the results of downstream analyses; indeed, a major obstacle in EV research is the enormous degree of variation introduced by different methods of processing, storage and isolation. Not only is isolation of completely pure EV populations (e.g. exosomes, microvesicles or apoptotic bodies, mostly, but not fully distinctive by size) incredibly difficult, if not impossible by current techniques, but also different EV subtypes may be influenced differently by pre-analytical factors. Thus, standardised protocols for the isolation and storage of EVs that maintain their function and integrity combined with robust and reliable methods of characterisation are essential if consistent and accurate results are to be obtained. The treatment of any EV containing matrix (biological fluid or culture media) prior to or during isolation is known to affect the EVs in the sample and further analysis, most likely due to the effect of non-EV cohabiting components, such as cells and soluble molecules. Furthermore, in order to use the immense potential of samples stored in biobank repositories, influence of storage on EVs must be unravelled and fully understood.

Herein, we present a brush of considerations for the pre- and post- EV isolation handling and storage of EVs from most commonly used biofluids to help researchers achieve optimal results which are reproducible and reliable.

2 / PRE-EV ISOLATION CONSIDERATIONS

Due to the lack of comparable large-scale studies on the matter applying most up to date and rigorous EV isolation methods, we present only brief considerations for the treatment of biological fluids or culture media prior or after EV isolation to minimise EV loss (or artificial EV production) and isolation of artefacts that interfere with EV analysis[2,3]. In all cases, we advise that (where possible) for each researcher to evaluate the effects of their specific processing and storage procedures on their EV population of interest.

2.1 Blood

Blood derived EVs are valuable rich sources for biomarker discovery research, since many conditions/diseases can have significant impact on EV composition. Thus, in general, researchers should aim to use consistent protocols and ensure that all details are meticulously recorded, if using human individuals as testing subjects. The effects of collection and handling of blood samples specifically for EV research are far from elucidated, although comprehensive and detailed EV studies are quickly emerging. Ideally, samples should be freshly obtained and processed in order to achieve maximal EV recovery and stability. A majority of times, not only is EV isolation not feasible but also minimal blood processing (cells removal) is delayed, thus recording of technical details is critical. Technical factors which may have impact on EVs include: blood collection volume, first-tube discard, type of container(s), time to processing, choice of anticoagulant (in the case of blood plasma), use of mixing or agitation, temperature of storage and processing, description of transport, whether tubes remained upright before processing, exact centrifugation or filtration procedures to remove cells, degree of haemolysis. Inter-laboratory studies must consider the potential influence of differences in their protocols. In this way, using biobank samples, a smart and integrated interpretation of pre-analytical treatments is required for meaningful comparisons.

Blood plasma or serum are normally the preferred sources of EVs, however platelet-derived EVs may have substantial biological significance, therefore removal of platelets and its derivates prior to processing, isolation and storage should be considered carefully if these are not of research interest. Blood derived EVs are a highly complex and heterogeneous population, with presence of all types of EVs and multiple cellular origins.

General considerations:

- Historically, the anticoagulant citrate has been recommended for microEV stability, however recent studies have provided with more information of effects on other EV populations. The choice of blood collection tube (or anticoagulants in it) can have significant effects not only in EVs but also in down-stream analyses, as for example, heparin and EDTA residues may linger in the sample and interfere with PCR reactions[4], a common end-point molecular assay. Thus, thoughtful experimental planning must be considered throughout.

- Studies have shown that microEVs significantly increase within 3 hours in unprocessed whole blood collected in citrate or heparin tubes[5], suggesting to use EDTA tubes instead, if immediate cell removal is not possible, to avoid generation of microEVs after blood collection.

- If platelet-derived EVs are not of interest, quick and full platelet removal is critical. As the name states, platelet poor plasma (e.g.1550 x g for 20 minutes at RT) does not remove platelets completely[6]. Studies have shown that "classic" platelet-free protocols (twice at 2,500 x g for 15 min at RT)[7] can be quickened by a single-step centrifugation protocol (5,000 x g for 20 minutes at RT) without impact in EV recovery[8].

- In line with platelet-EV free plasma samples, platelet activation should also be minimised to avoid their EVs released into sample[9] by using appropriate blood collection tubes and acknowledging that there are differences regarding the affected EV sub-populations. For example, studies have shown that both citrate phosphate dextrose adenine (CPDA) and EDTA tubes have significant reduction on small EVs (sEV, exosome-sized EVs) recovery if cells are not removed within an hour in comparison to heparin tubes (Figure 1)[10]. This outcome may be explained by surface rearrangements in platelets which might drag sEVs down during platelet removal by centrifugation. Conversely, for microEV there are divergent results. Some studies show microEVs are stable up to 4 hours at RT without blood processing in sodium citrate and heparin tubes[11] while others show microEV significantly increase in citrate or CTAD tubes within 3 hours[12]. It is worth noting that significant higher microEV counts found in EDTA tubes in both aforementioned studies, could be explained by specific generation of myeloid EVs[11,12].

- When analysing all types of EVs, overall EV concentration in plasma has been unaffected by the type of blood collection tube when processed immediately, however compositional-wise, there are still more platelet-EVs in citrate tubes compared to acid citrate dextrose (ACD) and EDTA tubes[13].

- Awareness that plasma and serum EV composition differ substantially is essential. The absence of anticoagulant in the blood collection tube, such as the case for serum samples, provides important numbers of platelet EVs within all serum EVs[13].

- Studies on the effect of storage temperature on EVs may not be currently conclusive for all type of EVs. sEVs are reported to maintain their concentration in long time (months) storage from -20°C to -160°C (Figure 2), however, sEV do increase their concentration during days storage at RT or 4°C[10]. In turn, microEVs increase their concentration after platelet poor plasma is stored for one month at -80°C, suggesting that complete removal of platelets avoids artificial production of microEVs[6].

- Repeated freeze/thaw cycles of serum samples have been shown to significantly decrease the DNA content in sEVs, but not their EV concentration[14]. Therefore, preparation of aliquots of serum samples intended for different DNA-based assays is recommended prior to storage.

- Long-term storage of cell-free plasma (e.g. 1 year or up to 7 years) at –80°C should be avoided as aggregation of sEV, proteins and nucleic acids can occur [15], as well as a significant increase of microEVs[6].

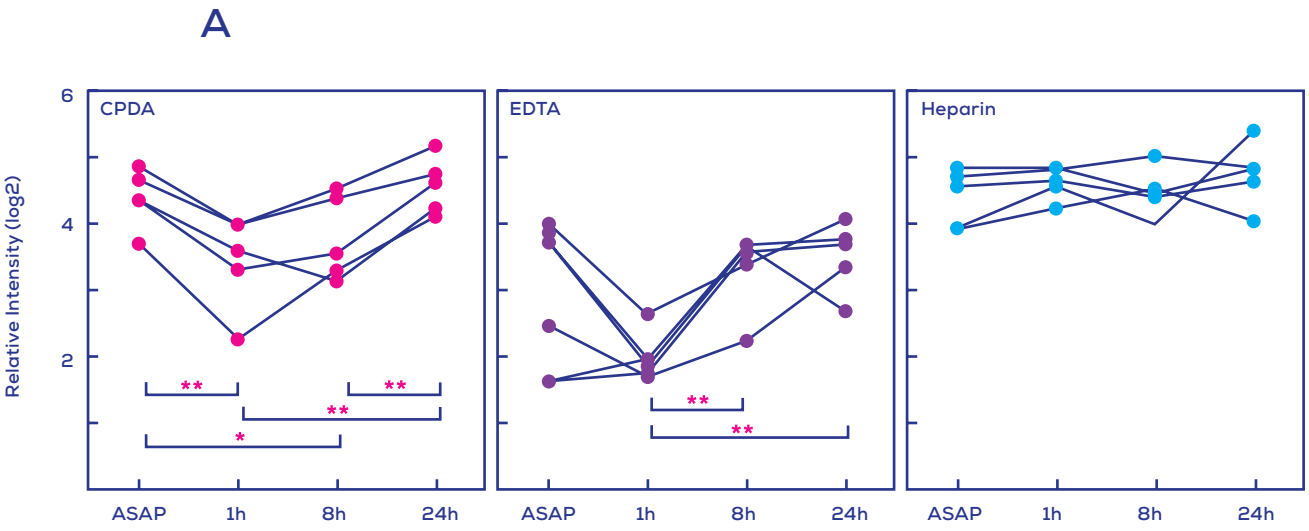

Figure 1. Effect of RT incubation time of blood sample before initial centrifugation. Exosome-sized EV concentration is presented as transformed relative intensities from protein array against CD9, CD63, and/or CD81 markers. Adapted from[10].

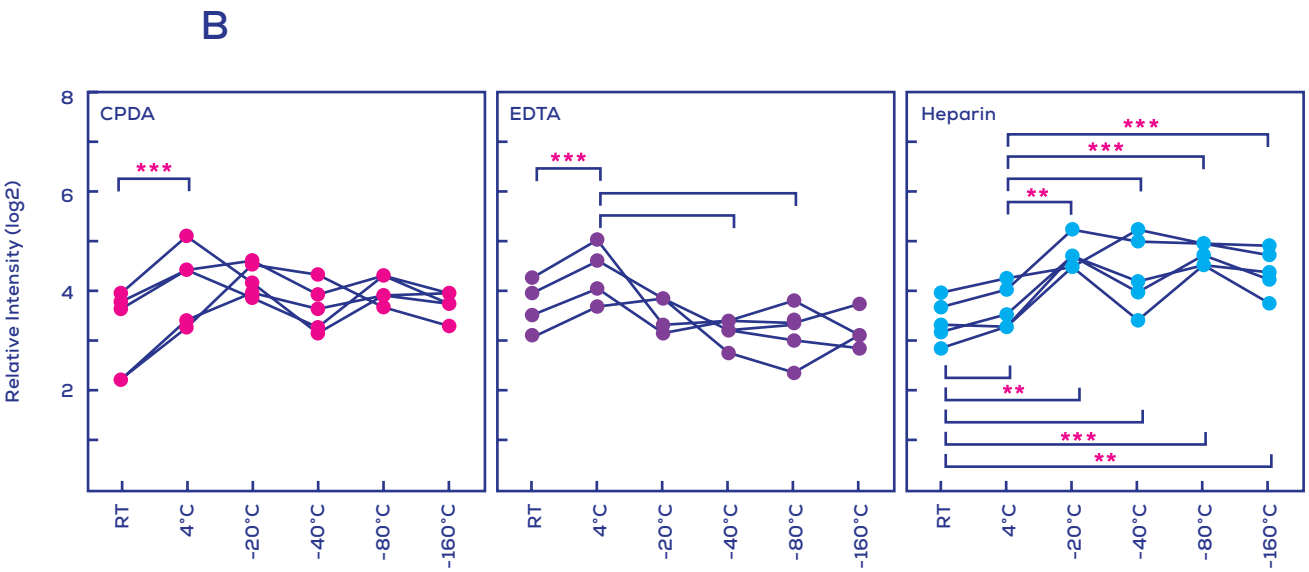

Figure 2. Effect of storage temperature (months) on plasma samples. Exosome-sized EV concentration is presented as transformed relative intensities from protein array against CD9, CD63, and/or CD81 markers. Adapted from[10].

2.2 Urine

The popularity of urine for EV research has increased, since samples can be provided recurrently in large quantities and with minimal disturbance for the individual. Nevertheless, urine samples are not sterile biofluids, therefore influence of bacterial contamination is a significant factor for urinary EV analysis. Also, urine contains significant amounts of aggregating glycoprotein uromodulin (UMOD, or Tamm Horsfall) which can entrap EVs[16], affecting its recovery.

General considerations:

- First and second morning urine samples[17] and early or midmorning urine samples[16] have been reported to yield similar amounts of urine sEVs, therefore either can be used for collection. Nevertheless, there are no studies analysing urine EV composition under these variables.
- Protease inhibitors (PI) should be added immediately to whole urine after collection, since lack of PI triggers significant degradation of membrane-bound proteins and sEVs (exosome-like)[17], even when urine is stored frozen[18].
- Urinary EVs degrade rapidly following collection (within 2 hours)[18], therefore samples should be analysed or frozen as soon as possible after collection.
- Room temperature or 37°C should be maintained for fresh urine processing, as cold temperature (4°C or ice) can promote uromodulin aggregation and loss of EV recover (Figure 3A)[19]. Otherwise, previously frozen urine samples should be maintained for processing at 4°C.
- An initial 17,000 x g spin for 10-15 minutes at RT removes cells, large debris and large EVs[17,19,20] However, if microEVs are of interest, then a 2,000 x g spin is enough to remove cells and debris.
- Researchers have used different approaches to eliminate uromodulin. For example, addition of DTT (dithiothreitol) for 5–10 minutes at 37°C to the SN after the low-speed centrifugation, depolymerises uromodulin, allowing it to be removed by another 17,000 x g spin, improving EV recovery, regardless of the storage temperature of unprocessed urine (Figure 3B-C)[20]. A treatment of ZnSO4 (1 to 4 mM) in fresh urine samples, will effectively reduce uromodulin contamination in EV preparations[16], while other researchers have shown that adding 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic (CHAPS) to the high-speed resuspended EVs is able to reduce uromodulin whilst maintaining EV functionality[21].
- A single freeze-thaw cycle in cell-free urine has been shown to significantly decrease EV concentration[22], therefore freeze-thaw cycles should be minimised and pre- and post-freezing analysis carried out to evaluate the effect on individual samples.
- Urine should be stored at -80°C as storage at higher temperatures (even -20°C) can result in increased loss of EVs[17,22].
- Recovery of EVs from frozen (-80°C) urine samples can be boosted by vigorous vortexing of the sample to detach EVs that are adhered to the tube[17].

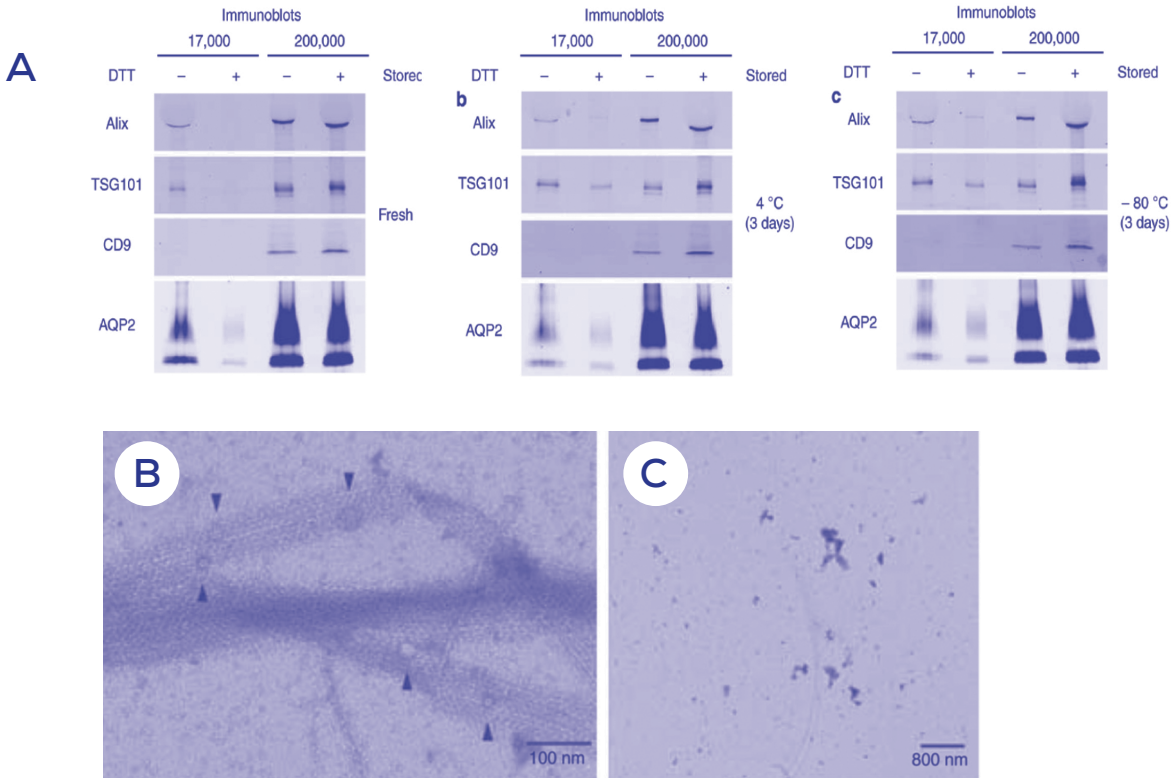

Figure 3. Effect of DTT treatment on storage temperature and removal of Tamm-Horsfall protein in EVs from unprocessed urine samples. (A) Immunoblots of 17,000 and 200,000 × g pellets showing only fresh processing of urine samples returns no exosomal markers in treated 17,000 × g pellets, and presence of exosomal markers in untreated 17,000 × g pellets, regardless of storage temperature of urine. Electron microscope images of the 17,000 × g pellets (B) without DTT and (C) with DTT showing short convoluted THP oligomers in untreated samples. Adapted from[20].

2.3 Milk

EVs have been investigated and detected in milk from humans, cows, donkey, sheep, goat, pig[23–26]. Due to their potential of mass production for therapeutic use, it is of particular importance that storage-induced effects are minimised in the case of milk EVs. For human breast milk, subject specific factors that may have impact on EVs and need recording include: stage of lactation, parity, volume of milk production, infant feeding, maternal diet and energy status, maternal health (infectious or non-infectious diseases), illness, and stress influence the composition and EV populations of milk[27]. For farming animals' milk EVs, there is substantially more research needed to analyse subject factors influencing EV composition. Milk, as other biofluids, has its own complexities and challenges for EV isolation, containing abundant other nanostructures of similar size such as fat globules, casein micelles and aggregated proteins, as well as cells, antibodies, oligosaccharides, fatty acids, and bacterial cells[28].

General considerations:

- It is recommended to remove cells and cream layer before milk storage at -80°C if EV isolation is not possible immediately[29]. Storage temperature of unprocessed milk samples has shown significant effects on the EV content; storage at -80°C, 4°C, RT and 37°C leads to cell death and the release of stress-induced EVs due to apoptosis[29].
- The initial cell/cream removing low-speed centrifugation (3000 x g) is mostly performed at RT, whereas following centrifugation steps can be performed at 4°C[25,29].

- Caution must be taken to remove fat layer or fat globules (positioned in the upper phase after centrifugations) before every transfer of SN for storage or to continue processing[25].

- In high-casein milk samples (e.g. cow, goat), EV isolation can become difficult, thus some treatments have been suggested to help remove casein. EDTA[30], acetic acid or hydrochloric acid treatments[31] to the low-speed SN have been reported to help casein aggregation and subsequent removal by centrifugation (5000 x g). However, caution must be paid on the latter low-pH treatments, since they can affect the EV surface markers[31].

- Freezing can disrupt the fat/water emulsion of milk which may affect EV isolation and purity[3], however, removal of potential sources of stress-induced EV prior to freezing appears to be a suitable approach.

2.4 Bronchoalveolar lavage fluid

Appropriate handling and storage of bronchoalveolar lavage fluid (BALF) is critical for assessment of EVs for potential diagnostic purposes of any airway conditions. Mouse models have identified that BALF contains predominantly microEVs, yet exosome-sized and apoptotic bodies-sized EVs are also present[32]. As BALF collection is a medical procedure mostly done on ill human patients, there are no studies reporting baseline EV composition in healthy human individuals. In patients, BALF samples are normally processed immediately or briefly stored at 4°C, aiming to not disturb microbial load and growth, important for the identification of aetiology of infectious diseases[33]. Nonetheless, for EV studies, it is unclear whether and how BALF storage prior to EV isolation may affect the EV population. BALF does not have the high protein content of plasma, thus we speculate that it is unlikely that EVs in BALF will be more prone to degradation when stored. However, we recommend that laboratories use consistent storage protocols and document all details of storage conditions.

General considerations:

- Cell removal by low-speed centrifugation is recommended as it may also remove any mucus plugs present in BALF samples.

- Frozen BALF samples have been reported to generate significant aggregates, therefore immediate EV isolation is recommended[34].

- Storage at -80°C has been shown to inhibit bacterial growth in BALF[33] and so BALF should be stored at this temperature to avoid increasing bacterial-derived EVs during storage and minimise degradation of EVs of interest.

2.5 Semen

Seminal EVs are gaining relevance as they can be used as cancer biomarkers or as potential therapeutics as they have demonstrated, for example, anti-HIV-1 activity[35]. Clearly, either of the above-mentioned applications require stability of EV biophysical properties through time, especially important when access to samples from biobanks is possible for research. In general, seminal EVs appear to be more robust than those found in other biological fluids, however there is a knowledge gap understanding the specific storage effects on seminal EV sub-populations with different origins, e.g. epididymosomes and prostasomes[36]. As with other EVs, fresh analysis is preferable where possible, but this is often not practical for such samples.

General considerations:

- Very long-term (30 years) storage of semen at -80°C has shown stability of EVs in morphology, concentration and size, however, it does not maintain full EV-associated enzymatic activity[37].
- If possible, it is recommended to remove cells before storage: fresh semen samples are left to liquefy at RT for 20-30 min after collection, to then centrifuge for 10 minutes at 1000 × g at 4°C to pellet spermatozoa and large pieces of undissolved seminal gel, allowing to store seminal plasma in SN at -80°C until EV isolation[35].
- It's been reported that visible light affects spermatozoa of animals and humans[38, 39]. Thus, as precaution, all semen samples are maintained in the dark prior to analysis[26].

2.6 Saliva

Recently, saliva EVs caught the attention of researchers due to their potential for unknown disease biomarkers[39]. Saliva is not only complex biological fluid, containing antimicrobial compounds, antibodies and glycoproteins, but also, like urine, it contains significant microbial cells releasing their own EVs. Therefore, sample preservation is a balance between stabilizing EV population and preventing uncontrolled microbial growth. Saliva composition can vary greatly with many factors which can have impact in EVs, therefore technical details that should be recorded include: food and drink intake, smoking and physical activities prior to collection, brush of teeth (releasing blood into saliva), presence of oral diseases, saliva collection method (unstimulated or stimulated). MicroEVs-sized and exosome-sized EVs have been isolated form saliva[40], however there are no rigorous studies investigating effects of storage conditions on different EV subtypes.

In general, considerations for this biofluid:

- Addition of protease inhibitors cocktail has been used to prevent protein degradation in general[41]; however, no studies have demonstrated the specific effects on EV recovery.
- Due to its viscosity, dilution of saliva 1:1 with PBS or Tris-buffered saline[42] is recommended before continuing with processing (e.g. filtration, centrifugation)[41,43].
- Cell removal by low-speed centrifugation is recommended before EV isolation or storing.
- Storing cell-free whole saliva at 4°C, ensures sEV integrity and bioactivity for up to months[44].
- Maintaining the sample in ice or centrifugations at 4°C is commonly used by researchers, since the possibility of generation of artificial cell-derived EV is negligible[40,41,43].
- If EV isolation is not performed immediately, freezing at -80°C is recommended.

2.7 Cell Culture Conditioned Media (CCM)

In vitro culture of cells is the most effective way of producing significant amounts of EVs for down-stream analyses, as it can be more easily scaled up to necessary requirements. Although culturing cells can be a relatively controlled environment (e.g. single cellular origin), there are technical factors, and subtle variations to them, known to have impact on EVs that should be precisely recorded, including: culture volume, capacity and type of culture vessel (flasks, bioreactors), culture conditions (temperature, gas concentrations, time), full media description, complete cell line information, cell passage number, seeding density at EV harvest time[45].

Unfortunately, there very few studies evaluating the stability of different EVs populations in cell-free conditioned media under different storage conditions. A study has reported less than 5% of microEV are recovered after freezing cell-free CCM, although, the freezing-EV damaging effect could be neutralized by addition of 5% DMSO to CCM before storage[46]. This opens an opportunity to investigate these effects, since mass production of sEVs, for example, for biomedical applications might require temporary storage or transportation of large volumes of CCM prior processing or full EV isolation.

General considerations:

- As fetal bovine serum (FBS) is a commonly used supplement in cell culture, attention should be paid to FBS containing significant amount of EVs which will be isolated along with EVs of interest as well. Using EV-depleted FBS (lab-made or commercially available) is recommended instead of no FBS at all, as lack of serum may have effects on cell growth and released EVs.

- A consideration prior to EV isolation from CCM is cell death; if this occurs, the sample will become contaminated with stress-induced cells and/or dead-cell-derived EVs, which will co-isolate with other EVs of interest. Therefore, laboratories should analyse the extent of cell death prior to isolation and decide upon an acceptable limit according to the specifications of their cells[3,45].

- Although most microbial contamination might quickly become apparent in cell culture, as bacterial growth is notorious after 4-6 hours of incubation, other contaminating microbes, such as Mycoplasma have dormant stages resulting in days or weeks of cell culture without noticing[47]. Therefore, appropriate molecular-based checks for Mycoplasma contamination should be performed.

- As with the majority of the other biofluids, it is recommended to remove cells and large debris before storage, to avoid artificial cell-derived EVs or degradation of existing EVs.

3 / POST-EV ISOLATION CONSIDERATIONS

In general, EVs should initially be characterised fresh after isolation and prior to storage using single particle analyses, surface markers to confirm their origin, microscopic visualisation and/or other approaches recommended by MISEV[45]. Also, a standardized protocol must always rule out microbial contamination during the EV isolation process and storage, therefore EV preparations should be tested for the presence of bacteria or fungi (the most common contaminants) by available microbiological techniques (e.g. growth on liquid or solid culture media). If microbes are detected, the EV preparation must be discarded, as bacteria-derived EVs or fungi-derived EVs may be contaminating the EVs sample. While isolated EVs are often considered to be fairly stable, the handling, storage conditions, choice of buffers, and storage time can have dramatic effects on the results of analyses[3]. In order to achieve reproducible results, laboratories must use consistent protocols for the storage of isolated EVs. Lastly, caution must be taken when evaluating storage effects and correlating EV stability, EV cargo and EV bioactivity altogether, as these variables can flow independently.

Generally, the following considerations are made:

- Siliconized vessels should be used to prevent adherence of EV preparations to vessel walls[3].
- Phosphate-buffered saline (PBS) is most commonly used for EV resuspension and storage[41], however, other buffers such as HBSS (Hank's Balanced Salt Solution)[47] or Tris-buffered saline[42] are also useful.
- Freezing at -80°C is a reliable method of storage in buffer-resuspended EVs; storage at higher temperatures (even -70°C) has been shown to have significant effects on vesicle properties[49,50].
- Fast freezing instead of slow freezing is recommended although the benefits for all types of EVs is yet to be investigated[48]. Individual samples should be tested to identify the optimal freezing method.
- Addition of cryoprotectants to EV storage buffers may result beneficial. It has been shown that trehalose increases stability of sEVs and its cargo (protein, RNA), with repeated freeze/thaw cycles in comparison to PBS (Figure 4)[51]. Addition of DMSO to EV preparations has shown to reduce damage to EV morphology or alteration to size distribution with storage at -80°C compared to PBS. However, DMSO did not improve EV-RNA degradation with time[49].
- Lyophilisation of sEVs along with storage at -80°C has been reported, with maintenance of sEV bioactivity compared to non-lyophilized sEVs)[50]. However, sEV number/integrity was not assessed in this, with speculations that bioactivity might be linked non-EV components.

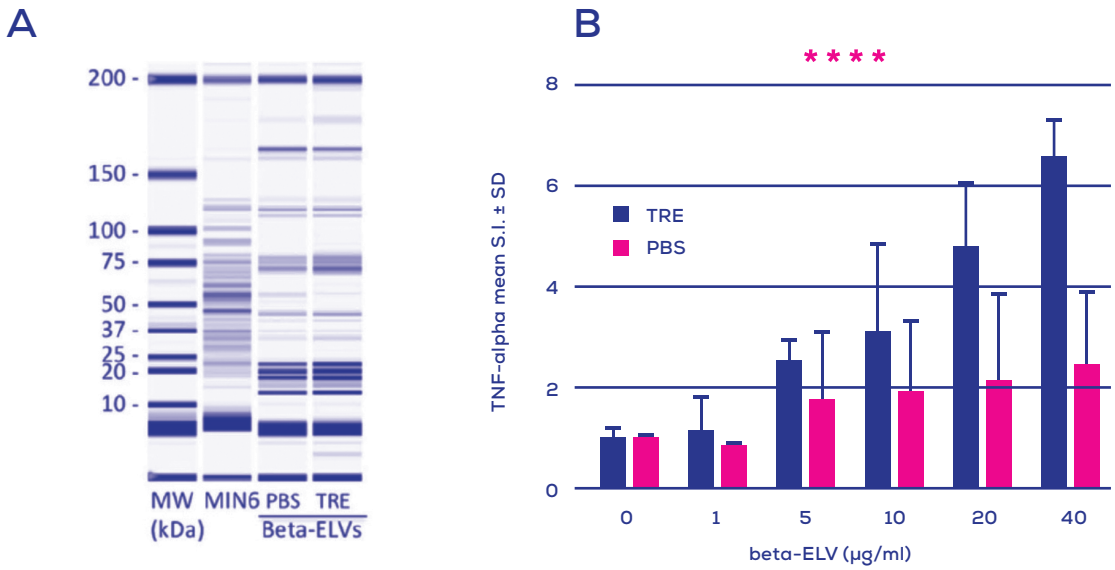

Figure 4. Effect of Trehalose on storage of purified EVs. (A) Automated electrophoresis analysis of exosome-sized EV protein profiles under reducing conditions and stored with and without Trehalose, (B) TNF-alpha cytokine secretion of macrophages stimulated with Exosome-sized EVs stored with and without Trehalose. Adapted from[51].

3.1 Blood

Awareness that EVs isolated from blood present an enormous heterogeneity in their cellular origin, with different EV subtypes presenting specific effects by different EV isolation methods is paramount to adjust protocols to the area of interest. For example, platelet derived EVs have shown high aggregation with ultracentrifugation-based isolations, significantly affecting EV recovery, while other cell EVs are unaffected[22]. This has resulted in whole plasma EVs also presenting aggregation in some studies where platelet EVs are present, overall suggesting this method should be strongly discouraged for this type of biofluid[23] and usage of other EV-friendlier methods, such as size-exclusion chromatography being encouraged.

Keeping in mind, the array of EV isolation methods and their strengths and limitations with impacts on EV populations, once blood derived EVs are isolated, they appear to be relatively stable for long term storage and at temperatures below zero. Since there are significant knowledge gaps in the effects of storage conditions in particular EV molecules or down-stream analyses, researchers should perform their own EV, EV-content or EV-based activity characterisation when storage conditions are decided.

- Purified platelet and erythrocyte EV have shown enumeration stability with storage at -20°C, -80°C and -196°C for up to one year[22].
- The integrity of purified plasma exosome-sized EVs is unaffected by a single freeze/thaw cycle [15].
- When isolated plasma EVs are stored at -80°C, RNA may be stable for up to 12 years[49].
- Plasma exosome-sized EVs isolated immediately after collection, maintain morphology and function when stored at -80°C[15].

3.2 Urine

The most critical optimisation is the storage conditions of unprocessed urine. As fully isolated EVs is a relatively robust means of storage, there are few studies on the storage of isolated urine-derived EVs.

- Buffer-resuspended urine-derived EVs appear to have normal morphology, surface markers, and size distribution when stored at -80°C[17].
- Most isolated urine-derived EVs are stored in PBS and recommended be thawed on ice and handled on ice where possible throughout analyses.

3.3 Milk

For therapeutic applications, storage of large supplies of milk with the guarantee of keeping EV integrity is the priority. Once pure milk-derived EV preparations are obtained, they are subject to storage effects in a similar manner as other biofluid EV preparations.

- Bovine milk-derived exosome-sized EVs have been shown to remain unaltered with storage at -80°C, from 4 weeks to 18 months, without any changes in their physical properties or biological activities[24,52].
- Storage of human milk derived sEVs at 4°C for up to 10 days increases the average size, suggesting some swelling and/or or aggregation of particles[53].

3.4 Bronchoalveolar Lavage Fluid

In the case of exosome-sized EVs, even with careful handling and conditions, BALF EVs appear to be particularly prone to destabilizing and even short-term storage can lead to changes in the surface properties, morphological characteristics, and protein content of the vesicles[54]. Therefore, it is suggested BALF sEVs should be analysed immediately; where this is not possible, preliminary measurements should be taken immediately after chosen specific EV isolation method, to evaluate and account for the effects of storage.

- PBS-resuspended BALF sEVs have shown a significant increase in diameter, number of sEVs and decrease in zeta potential with storage at -80°C, whereas storage at 4°C causes substantially less effects on sEVs[54]. In light of these results, if BALF-derived sEVs must be stored, it is recommended storing at 4°C.
- Gentle sonication (water-bath) in purified BALF EV preparations has been suggested by some groups, to help dispersing aggregates that might have arisen from freeze storage[34].
- Isolated EVs should be kept on ice during analyses; handling and storage at room temperature significantly affects the zeta potential of the vesicles[54].

3.5 Semen

Studies have focused on the pre-analytical storage of semen, and currently there are no studies addressing different storage conditions and their effects on purified seminal EVs.

- Semen-derived exosome-sized EVs stored in PBS buffer at -80°C show a good/normal integrity [35].

3.6 Saliva

Unlike other sturdy pure EV preparations, saliva-derived EVs appear to behave sensitively to freezing conditions.

- Exosome-sized EVs have shown a stable EV structure with storage at -20°C or -80°C, however they are significantly impaired in their inherent bioactivity, suggesting non-freezing storage (4°C) conditions should be used (Figure 5)[44].
- PBS is a common buffer to resuspend and store saliva-derived EVs[40,41,43].

3.7 Cell Culture Conditioned Media

EV or EV-cargo stability is partially determined by isolation methods which grant different degrees of EV purity. In this aspect, there has been a lot advancement in investigating EVs from cell CCM, but also substantial standardisation of protocols is still needed.

- Exosome-sized EVs storage at -80°C, -20°C or 4°C for a week has demonstrated maintenance of EV numbers[50].
- Storage at -80°C has been reported to stabilise sEV RNA cargo (miRNAs), which is particularly sensitive to temperature, whereas storage at -20°C or 4°C grants significant reductions[50].
- Storage of EVs in medium at -80°C for 2-3 months has shown negligible effects on sEV-RNA cargo , however longer storage times, over 2 years, causes significant sEV-RNA degradation. Storing isolated EVs in medium supplemented with 10% DMSO showed protection of EV size and integrity, however it did not rescue EVs from RNA degradation (Figure 5)[49].
- Larger EVs from CCM, such as microEVs, have also been evaluated under different storage conditions. These seem to be more sensitive, as a week or a month at 4°C gradually reduces EV numbers. Although storage of microEVs at -20°C and -80°C maintained EV numbers, their bioactivity was compromised[48].

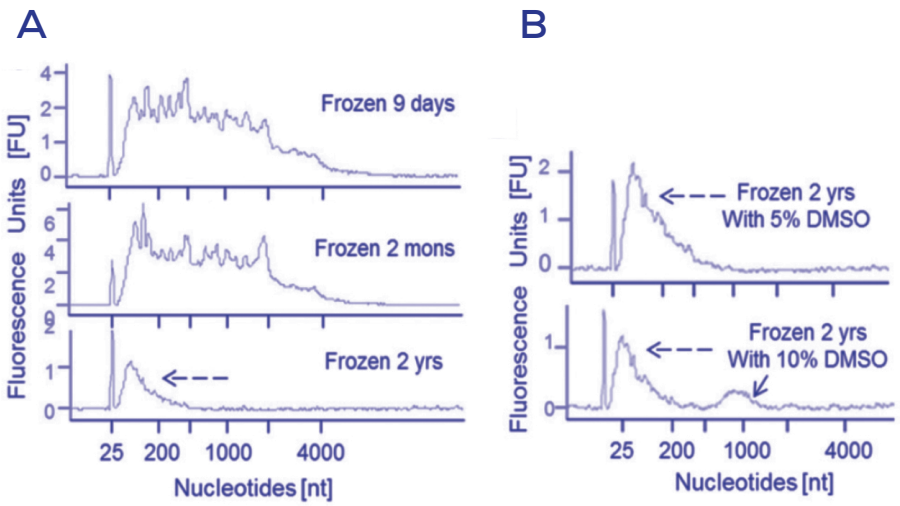

Figure 5. Effect of freezing storage conditions of EVs on EV-RNA cargo. Results of capillary electrophoresis of exosome-sized EV RNA isolated from frozen EVs stored at -80 °C in media alone (A) or stored at -196 °C with 5–10% DMSO (B), show a left shift in nucleotide size towards small, degraded RNA at 2 years pf storage. Adapted from[49].

4 / CONCLUDING REMARKS

EVs are becoming increasingly recognised and exploited specifically in the biomedical field, where their functional properties and composition can provide insights to understanding many human and animal diseases. Nevertheless, as sources of EV are incredibly varied and complex, it becomes imperative to fully understand them, so handling and storage conditions can guarantee and maintain their original information and intended functionality. Research is quickly advancing on comprehensive studies on storage of specific biofluids, however new technologies are also needed to improve current conditions which may restrict the full potential utility of EVs.

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