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PERSPECTIVE

Cell culture-derived extracellular vesicles: Considerations for reporting cell culturing parameters

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Abstract

Cell culture-conditioned medium (CCM) is a valuable source of extracellular vesicles (EVs) for basic scientific, therapeutic and diagnostic applications. Cell culturing parameters affect the biochemical composition, release and possibly the function of CCM-derived EVs (CCM-EV). The CCM-EV task force of the Rigor and Standardization Subcommittee of the International Society for Extracellular Vesicles aims to identify relevant cell culturing parameters, describe their effects based on current knowledge, recommend reporting parameters and identify outstanding questions. While some recommendations are valid for all cell types, cell-specific recommendations may need to be established for non-mammalian sources, such as bacteria, yeast and plant cells. Current progress towards these goals is summarized in this perspective paper, along with a checklist to facilitate transparent reporting of cell culturing parameters to improve the reproducibility of CCM-EV research.

KEYWORDS
cell culture-conditioned medium, cellular therapy, ectosomes, exosomes, extracellular vesicles, in vitro, reproducibility, rigor, standardization

1 | INTRODUCTION

Extracellular vesicles (EVs) are heterogenous membrane-delimited particles that are released from cells in physiological and pathological states (Buzas, 2023; György et al., 2011; Witwer & Théry, 2019) and include endosome-origin exosomes and plasma membrane-shed ectosomes or microvesicles. EVs are composed of luminal cargo, a surrounding phospholipid membrane and membrane-embedded and -attached macromolecular entities. A more loosely associated bio-corona may also be of critical importance (Buzas, 2022; Wolf et al., 2022; Yerneni et al., 2022). In vivo, EVs are present in biological fluids such as blood, cerebrospinal fluid, saliva, tears and urine and in solid tissues. In vitro, EVs can be prepared from cell culture-conditioned media (CCM; Figure 1a). EVs in CCM are released by the cultured cells but may also originate from supplements used for cell growth and/or differentiation. Since cells and medium supplements also release or contain non-EV particles, it is often necessary to separate EVs from non-EV components to identify EV-specific contents and functions (Figure 1b).

Among the different sources of EVs, CCM is especially important because CCM-derived EVs (CCM-EV), whether native or engineered, can exert therapeutic functions. CCM-EV have been administered to animals and patients to assess their therapeutic potential for several pathological conditions (Elahi et al., 2020; Kordelas et al., 2014; Kwon et al., 2020; Morse et al., 2005; Nassar et al., 2016; Park et al., 2021; Sengupta et al., 2020; Shekari et al., 2021; Warnecke et al., 2021). Table S1 gives an overview of clinical trials which have used or are currently using CCM-EV from cellular sources such as dendritic cells, mesenchymal stromal cells (MSCs), tumor cells, T cells and plant cells. Producing cells, cell culture components, culture conditions and CCM collection and processing will impact the biochemical composition of EVs, association with other entities such as a co-purifying bio-corona, and biological function. However, there are currently no reporting guidelines for CCM-derived EV.

To maximize the reliability and reproducibility of CCM-EV research, transparent reporting is needed for the production and characterization of CCM-EV. There are several guidelines on good practices for general cell and tissue culture for cellular therapy (Coecke et al., 2005; Pamies, 2018; Pamies et al., 2017). Furthermore, minimal criteria for reporting EV studies were proposed in MISEV2014 (Lötvall et al., 2014) and MISEV2018 (Théry et al., 2018), and a systematic assessment of articles published from 2012 to 2020 showed a clear association of study quality with citation of MISEV (Poupardin et al., 2021). In addition, working groups including members of the International Society for Extracellular Vesicles (ISEV), the International Society for Cell and Gene Therapy (ISCT), and the Society for Clinical, Research and Translation of Extracellular Vesicles Singapore (SOCRATES) have published best practice recommendations to produce MSC-EVs and assays for therapeutic applications (Gimona et al., 2021; Pachler et al., 2017; Rohde et al., 2019; Witwer et al., 2019).

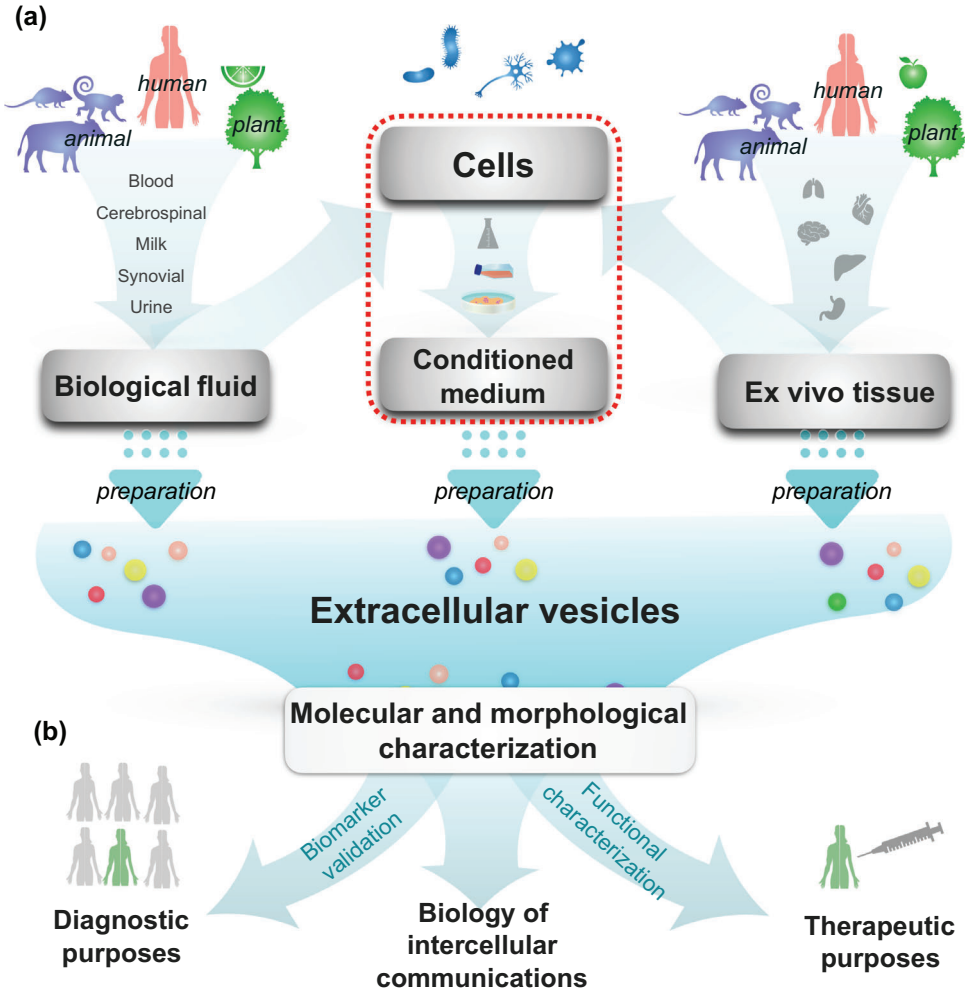


FIGURE 1 Source and application of extracellular vesicles. (a) Extracellular vesicles (EVs) can be prepared from biological fluids, cell culture-conditioned medium (CCM), or ex vivo tissue. (b) Following molecular and morphological characterization, EVs can be used to study intercellular communication biology, to diagnose diseases (biomarker research), or as therapeutic agents (directly or as drug delivery vehicles). The dotted box indicates the focus of the CCM-derived EV (CCM-EV) task force on cell culturing parameters and their effects on EV release, content, and biological function.

The ISEV Rigor and Standardization (R&S) Subcommittee (Nieuwland et al., 2020) aims to improve reproducibility of EV research (Börger et al., 2020; Clayton et al., 2019; Erdbrügger et al., 2021; Welsh et al., 2020). Although MISEV2018 provides mandatory reporting regarding CCM (Théry et al., 2018), and the importance of culturing parameters for the production of EVs is commonly recognized (Hill et al., 2013; Gudbergsson et al., 2016; Gurunathan et al., 2021; Patel et al., 2017, 2018; Staubach et al., 2021), standardized reporting of culturing parameters has not been implemented in most publications. Therefore, the R&S subcommittee established a task force on CCM-EV to focus on cell culturing parameters and their effects on EVs (Figure 1a, red dotted box). Here, the CCM-EV task force focuses on selected cell monoculture parameters that may affect EV characteristics, gives recommendations for transparent reporting, and identifies open questions. Our goals are to increase awareness and promote reproducibility in EV research when CCM is the starting material.

2 | CELL CULTURE PARAMETERS

2.1 | Cells (source cells)

2.1.1 | Cell identity

The identity of the donor/parental cell is a major determinant of EV yield, phenotype and biological function. Different cells may have different basal rates of EV production. For example, EV yield and function may differ between MSCs derived from different

donors and tissue sources (Almeria et al., 2022; Fafián-Labora et al., 2017; Kang et al., 2016; Komaki et al., 2017; Mendt et al., 2018; Merckx et al., 2020; Nakamura et al., 2015; Rosenberger et al., 2019; Teng et al., 2015; Tracy et al., 2019). Also, different cancer cell lines produce different numbers of EVs (Charoenviriyakul et al., 2017; Hurwitz et al., 2016; Salomon et al., 2014). For cell lines, regular short tandem repeat (STR) profiling can confirm identity of cells and detect cellular cross-contamination (American Type Culture Collection Standards Development Organization Workgroup ASN-000, 2010; Barallon et al., 2010; Masters et al., 2001; Reid et al., 2004).

Recommendations

- Report the identity of EV-producing cells according to consensus in the research communities that regularly use such cells. For example, ISCT criteria define multipotent MSCs by positive and negative marker expression and differentiation potential (Dominici et al., 2006; Viswanathan et al., 2019; Witwer et al., 2019). Criteria for clinical-grade human induced pluripotent stem cell lines have also been established (Sullivan et al., 2018). Additional information regarding specific cell lines can be found at https://www.atcc.org/, and cell-line specific molecular information is available at https://depmap.org/portal.
- If downstream EV analysis focuses on a particular marker, confirm that the EV marker of interest is also present in/on the cultured cells and thus likely originates from those cells and not from another source.

Outstanding questions

- In some cell monocultures, multiple populations of cells are nevertheless present (Costa et al., 2021; Sato et al., 2016; Wang et al., 2021) (i.e., cells with genetic, epigenetic, morphologic or functional differences), which contributes to the heterogeneity of EVs and makes it more difficult to interpret from which cell types the detected EVs are derived. How can we reduce or control the heterogeneity of source cells?

2.1.2 | Characteristics of the cell/tissue donor

The potency of primary cells may be affected by characteristics of the cell donor, including overall health status, age (Choudhery et al., 2014; Dorronsoro et al., 2021; Gruber et al., 2012; Katsara et al., 2011; Payr et al., 2020; Sarkar et al., 2018; Siegel et al., 2013), biological sex (Katsara et al., 2011; Sammour et al., 2016; Siegel et al., 2013), pregnancy (Li et al., 2017; Mathew & Bhonde, 2017; Xia et al., 2007; Zhu et al., 2020), medication intake, nutritional status, infection and microbiota. In turn, these factors may influence the release, composition and function of EVs. For example, functional comparison of EVs from young and old donors revealed donor age dependencies (Agarwal et al., 2017; Sun et al., 2020; Umezu et al., 2017). The biochemical composition of EVs derived from different human donors also varies, possibly due to donor characteristics (Kim et al., 2016; Kordelas et al., 2014).

Recommendations

- Report (if available) known donor characteristics, including but not limited to biological sex, age, chronic diseases, infection status, medication and pregnancy/complications.
- Screen cells for the presence of infectious agents, especially if clinical applications are expected. Note that some viruses can integrate into the genome.
- Report pre-culture processing of the cells.

2.1.3 | Initial seeding density

The initial concentration, or the number of cells per volume or surface area in the culture flask or plate, affects cell growth and differentiation, as well as the release of EVs. This dependence was shown for MSCs and cancer cells (Ludwig et al., 2019; Patel et al., 2017).

Recommendations

- Report the initial seeding density of cells, ideally as cells per cm^2 (for adherent cells) or cells per mL (for suspension cells).
- Reduce variation by using the same seeding concentration and cell splitting intervals across experiments.
- Optimal plating density has been reported for specific cells and culturing conditions (Sotiropoulou et al., 2006); otherwise, optimize seeding density.

2.1.4 | Cell density or confluency of cells at the time of CCM collection

The density of cells affects the cell physiology (Balint et al., 2015; Bereiter-Hahn et al., 1998; Jacobs et al., 2016; Ren et al., 2015; Sekiya et al., 2002; Trajkovic et al., 2019), thereby affecting the production rate, biochemical composition and function of released

EVs (Cao et al., 2020; Gao et al., 2020; Guerreiro et al., 2018; Gurunathan et al., 2021; Haraszti et al., 2018; Hayes et al., 2005; Mitchell et al., 2008; Palviainen et al., 2019; Rocha et al., 2019; Sekiya et al., 2002; Steinman et al., 2003; Thippabhotla et al., 2019; Yan & Wu, 2020; Yang et al., 2020). In over-confluent cell cultures, the therapeutic potential of MSCs may change due to contact-inhibited growth or contact-induced senescence (Balint et al., 2015; Ho et al., 2011; Sekiya et al., 2002). For 2D cultures, the percentage of surface area covered can be reported, but this metric may be inadequate, as cells are not equally large.

Recommendations

- Count cell numbers in a standardized procedure (e.g., with technical replicates) before seeding and after final harvesting at a given passage.
- Report cell density at the time of CCM collection (cells/cm^2 or cells/mL). For 3D cultures of spheroids or organoids, the reporting parameter might be the diameter of the spheroids or another appropriate measure.

Outstanding questions

- In 2D cultures, the term 'confluency' is often used. However, this remains a largely 'qualitative' parameter, and measurements may differ between individual researchers and laboratories due to different equipment and procedures. More objective measurements may be achieved by preparing and following detailed standard operating procedures that help to improve reproducibility.

2.1.5 | Cell sub-culturing, population doubling and passage number

Transferring cultured cells from high density to low density for propagation ('sub-culturing', 'passaging', 'splitting' or 're-plating') can influence cellular properties including the expression of cell surface markers, senescence and genetic stability (Kassem et al., 1997; Meza-Zepeda et al., 2008; Wagner et al., 2008; Yang et al., 2018). Suspension cells are either simply diluted into fresh culture medium, or, in some cases, the whole medium is replaced by fresh medium, following a low-speed centrifugation step. However, adherent cells regularly must be detached from vessel of carrier surfaces with the help of enzymes and/or mechanical intervention before passaging, and detachment methods may affect the cells and their membrane. For example, human embryonic stem cells experienced genetic instability based on the sub-culturing method (Bai et al., 2015; Garitaonandia et al., 2015). The passage time is directly related to the population doubling time of a given cell culture. If primary cells are used, senescence is dictated by the total achievable population doubling number (Hayflick limit) (Shay & Wright, 2000) and depends on cell type, donors and culture conditions (Meza-Zepeda et al., 2008). EVs from different passage numbers of the same cells may differ in size, concentration and functions (Beer et al., 2015; Boulestreau et al., 2020; Dorronsoro et al., 2021; Fafián-Labora et al., 2017; Lehmann et al., 2008; Lei et al., 2017; Patel et al., 2017, 2018; Sarkar et al., 2018; Takahashi et al., 2017; Takasugi et al., 2017; Venugopal et al., 2017).

Recommendations

- Report passage number, starting from the original stock and consider Master and Working Cell Bank passage numbers. It is recommended to consider the maximum number of passages that primary cells or cancer cells can undergo without changing their genotype and/or phenotype.
- For adherent cells, indicate the method of passaging with details necessary for exact replication, including but not limited to the type of treatment (e.g., trypsin or EDTA with any commercial name, mechanical, or any other methods), the concentration of any reagents, time of treatment (in minutes), temperature of incubation and if/how any enzymatic treatments are stopped.
- Report recovery time, if any, between sub-culturing and the start of EV collection.
- Consider avoiding the application of undefined animal-derived products during sub-culturing processes, the use of recombinant enzymes is preferable.

Outstanding questions

- To what extent does the effect of passage number and senescence differ between culture conditions, including static versus bioreactor, and adherent versus free-floating?

2.1.6 | Cell viability

In contrast to healthy cells, dying cells may preferentially release EV subtypes such as apoptotic bodies and necroptotic bodies, although these may also have therapeutic activity (Atkin-Smith et al., 2015; Battistelli & Falcieri, 2020; Baxter et al., 2019; Brock et al., 2019; Caruso & Poon, 2018; Crescitelli et al., 2013; Dieudé et al., 2015; Galluzzi et al., 2018; Gregory & Dransfield, 2018; Kakarla et al., 2020; Lázaro-Ibáñez et al., 2014; Li et al., 2020; Liu et al., 2020; Park et al., 2018; Phan et al., 2020; Poon et al., 2019; Shlomovitz et al., 2021; Théry et al., 2001; Zheng et al., 2021). Since the proportion of live and dying/dead cells affects the

proportion of EV subtypes in CCM, it is important to assess the viability of the cells as well as the contribution of dying or dead cells at the time of CCM collection. Cell death is commonly estimated using membrane permeability-based stains or metabolic assays. In bioreactors, metabolic readouts such as glucose and lactate provide insight throughout the cell culture (Mendt et al., 2018). However, only living cells contribute to metabolism, and these methods do not indicate the percentage of apoptotic or dying cells. An early ISEV position paper recommended that cell death percentage in culture should be less than 5% (Witwer et al., 2013); however, achieving 95% viability is not always possible—for example, in drug response studies—and assessing viability is also not always immediately possible, such as with some types of bioreactors and in multiple- or continuous-harvest 2D systems.

Recommendations

- Wherever possible, report the percentage of viable cells at the time of CCM collection.
- Wherever possible, document cellular morphology by taking representative images when harvesting.

Outstanding questions

- Can we understand the contribution of dying cell-derived EVs to the overall biological and potential therapeutic activity in a given EV preparation?
- Can we develop monitoring systems, for example, glucose consumption or lactate production, for 2D and 3D cell expansion systems?

3 | EV PRODUCTION MEDIUM

Basal cell culture medium contains nutrients, especially glucose (as the main source of energy), and amino acids, and can be supplemented with serum or other components. The EV production medium (the medium used for EV isolation) may be different from the basal cell culture medium.

3.1 | Basal medium, glucose and amino acids

Commonly used basal media for mammalian cell cultures, such as Roswell Park Memorial Institute (RPMI), Dulbecco's Modified Eagle Medium (DMEM), and alpha-modified Minimal Essential Medium (MEM), contain inorganic salts (sodium, ferric, magnesium and potassium salts), and micronutrients (vitamins and minerals). Any of these components may affect cell growth and differentiation, and EVs (Arigony et al., 2013; Arodin Selenius et al., 2019; Bhat et al., 2021; Kawakami et al., 2016; Watchrarat et al., 2017; Wu et al., 2009; Zhu et al., 2021).

D-glucose is the major carbon source in the cell culture growth medium. Increased release of EVs has been observed in the presence of both elevated and reduced glucose levels, and this effect seems cell type-dependent (Burger et al., 2017; Garcia et al., 2015; Rice et al., 2015; Thom et al., 2017). Several reports have shown a change in the biochemical composition or biological function of EVs when producing cells cultured in a medium containing high concentrations of glucose (Davidson et al., 2018; De Jong et al., 2012; Huang et al., 2020; Lin et al., 2019; Thom et al., 2017; Wu et al., 2017; Zhu et al., 2019; Zhou et al., 2021).

Amino acids and proteins are required for cell growth. The effects of glutamine and leucine on cell proliferation and EV biogenesis have been documented (Dai et al., 2015; Fan et al., 2020; Kim et al., 2017; Rubin, 2019; Zhao et al., 2021). If stabilized versions of amino acids are used as supplements, a different concentration of this amino acid in the medium over time will be maintained than when using the native version. Moreover, free amino acids present in the CM may become incorporated into EVs.

3.1.1 | Recommendations

- Report the basal medium used, including the catalog number.
- Report the nature and concentration of additives added during cell culture.

3.2 | Antibiotics and antimycotics

Antibiotics affect cell characteristics (marker expression) and function, and may also change the biochemical composition and function of EVs (Cohen et al., 2006; Llobet et al., 2015; Ryu et al., 2017; Skubis et al., 2017). Similarly, antimycotics may also change the production and composition of EVs (Németh et al., 2017).

3.2.1 | Recommendations

- Report the use of antibiotics and antimycotics, including if they are used during pre-conditioning and/or conditioning steps.

3.2.2 | Outstanding questions

- What are the effects of antibiotics and antimycotics on the biochemical composition and function of EVs?
- If packaged into or associated with EVs, how do antibiotics and antimycotics affect the therapeutic function of EVs?

3.3 | Complex biological supplements

The most commonly used complex biological supplements are serum and platelet lysate (PL). Serum, often fetal bovine serum (FBS), also known as fetal calf serum (FCS), provides essential components such as growth factors for cell proliferation and differentiation. However, FBS also contains (bovine-derived) EVs and non-EV particles such as lipoproteins (Brunner, 2010; Fisher et al., 1958; Gstraunthaler, 2003; Palviainen et al., 2020; Subbiahanadar Chelladurai et al., 2021).

Platelet lysate (PL) has effects comparable or superior to serum in some cell cultures (Hemeda et al., 2014), and, if harvested from donors of the same species as the cultured cells, PL may be used as a xenogeneic-free serum substitute (Becherucci et al., 2018; Bianchetti et al., 2021; Capelli et al., 2007; De Almeida Fuzeta et al., 2020; Gottipamula et al., 2012; Griffiths et al., 2013; Hemeda et al., 2014; Johansson et al., 2003; Juhl et al., 2016; Menard et al., 2013; Oeller et al., 2021). Like serum, also PL is rich in growth factors and may contain pathogens like viruses, and may exhibit donor-to-donor variability (Burnouf, 2018; Guiotto et al., 2020; Hawkes, 2015; Henschler et al., 2019; Hemeda et al., 2014; Schallmoser et al., 2020). Pathogen inactivation or depletion by irradiation, with or without the addition of photoactive chemicals or filtration (Oeller et al., 2021; Organization, 2004; Schallmoser et al., 2020; Viau et al., 2019) is required for regulatory compliance. PL also contains components secreted from platelets such as EVs, coagulation proteins and growth factors, which may contribute to its proliferation-supporting activity (Torreggiani et al., 2014) (reviewed in Antich-Rosselló et al., 2021; Kerris et al., 2020; Melki et al., 2017).

A position statement from the working group on cellular therapies of the International Society of Blood Transfusion (ISBT) discussed human PL (hPL) production, manufacturing, and quality management (Schallmoser et al., 2020), and the barriers to the translational use of hPL have been discussed in a joint publication of the Association for the Advancement of Blood and Biotherapies (AABB) and ISCT (Bieback et al., 2019). The use of hPL reduces the problem of animal components, although the use of PL for the manufacturing of therapeutic EVs requires specific precautions and pre-processing steps.

The presence of coagulation factors and fibrinogen in PL can lead to fibrin precipitates during the cell expansion process, which may be incompatible with processes such as filtration. Addition of heparin to the growth medium inhibits fibrin formation. Alternatively, addition of calcium chloride to PL may trigger coagulation and fibrin formation (Staubach et al., 2021), and the fibrin clot that is formed can be removed by centrifugation prior to use.

3.3.1 | Recommendations

- Report the percentage, producing company (city, country), and catalog number of complex biological additives including sera.
- Report the source and percentage of PL used at the various steps of cell expansion and CCM production.
- Report the process of PL production, including fibrin depletion methods and pathogen inactivation.
- Report the concentration of heparin present in the growth medium, and consider the potential adverse effects of heparin on separation and downstream analysis of EVs (Atai et al., 2013; Beutler et al., 1990).

3.4 | EV depletion

Serum and PL contain EVs, DNA fragments, non-EV particles such as protein aggregates and lipoproteins, and micronutrients (Arigony et al., 2013; Lehrich et al., 2021; Urzì et al., 2022), which may complicate the isolation, assessment, and interpretation of the biochemical composition and function of CCM EVs. For example, FBS-derived EVs or particles may co-isolate with EVs produced by cultured cells (Lehrich et al., 2021). Various methods have been described to deplete EVs from the serum (Driedonks et al., 2019; Kim et al., 2021; Kornilov et al., 2018; Lehrich et al., 2018; Liao et al., 2017, 2019; Mannerstrom et al., 2019). However, these methods do not remove all or exclusively EVs (Kornilov et al., 2018; Lehrich et al., 2018; Shelke et al., 2014; Tosar et al., 2017), and most studies do not report the degree of depletion. The biochemical composition and function of EVs released by cells cultured in the presence of EV-depleted serum may not be identical to those released in the presence of EV-containing

serum. This is possibly due to the fact that serum-derived EVs themselves have multiple effects on cultured cells (Beninson & Fleshner, 2015; Cavallari et al., 2017; Gu et al., 2018; Ochieng et al., 2009; Urzì et al., 2022).

3.4.1 | Recommendations

- When serum is diluted prior to removal of EVs, indicate whether, how (fold dilution), and with what (buffer, medium) the serum was diluted.
- For ultracentrifugation-based depletion of EVs, report the centrifugation speed and time, rotor specifications (K value, angle, tube volume), and temperature.
- For tangential flow filtration-based depletion of EVs, report the details including membrane/device manufacturer, material type, pore size, filtration surface area, flow rate, and temperature.
- Monitor and report the level of EV depletion by comparing pre-and post-depletion material

3.5 | Serum-free culturing

Considering the complex nature and important functions of serum in cell culture media, any manipulation including replacing the medium with a serum-free medium, or heat treatment to inactivate the complement system, may alter the physiology of the cultured cells and derivative EVs (Alcolea et al., 2016; Angelini et al., 2016; Aswad et al., 2016; Bhat et al., 2021; Cavallari et al., 2017; Clabaut et al., 2015; Eitan et al., 2015; Frigerio et al., 2021; Lehrich et al., 2018, 2021; Li et al., 2015; Liao et al., 2017; Mehta et al., 2010; Obrochta et al., 2015; Pham et al., 2021; Urzì et al., 2023). Some reports showed that serum-free or chemically defined medium enhance EV production (Biadglegne et al., 2021; Bost et al., 2022; Faruqu et al., 2021; Figuero, 2021).

3.5.1 | Recommendations

- In the case of serum-free media, report if no replacement supplement was added (e.g., in 'starvation' experiments), or if replacement supplements were added that are nutritionally complete.
- Report the time duration of cell culture without serum or other complex additives.
- Document any observed change in cellular morphology or characteristics.
- Quantify the number of particles in non-conditioned medium using the same process as for conditioned medium at least once.

3.5.2 | Outstanding questions

- EV depletion from serum may result in the removal of non-EV components such as lipids and proteins. Does EV-depleted growth medium support cell proliferation to the same extent as a non-EV depleted serum-containing medium?
- The efficiency of EV depletion methods varies. Monitoring the efficiency of EV depletion is difficult because none of the currently available EV detection methods can detect all EVs. Performing a procedural control, that is, comparing the negative control of the same volume of complete, unconditioned medium processed the same way as CCM, is a functional alternative, but will not fully answer the question of depletion efficiency. How can we best report the efficiency of EV depletion?

3.6 | Cell (pre)conditioning

Other factors used to promote proliferation, differentiation, silencing, or priming of cells, and/or to otherwise manipulate cells and their functions, may influence the release of EVs (Bai et al., 2020; Emam et al., 2018; Martens-Uzunova et al., 2021; Pecan et al., 2020; Savina et al., 2003; Segura et al., 2005; Soekmadji et al., 2017; Taylor et al., 2017; Wang et al., 2020) or their therapeutic potential (Akbar et al., 2017; Cossetti et al., 2014; De Jong et al., 2012; Hosseinkhani et al., 2017, 2020; Lopatina et al., 2014; Patel et al., 2019; Peltzer et al., 2020; Sutter et al., 2021; Varkouhi et al., 2019; Yang et al., 2020; Zhang et al., 2018, 2020).

3.6.1 | Recommendations

- Report the concentration, company (city, country), and catalog number of small molecules, cytokines/chemokines, or other RNA/protein/lipid-based cell-modulatory reagents. Also indicate if they are used during pre-conditioning and/or medium-conditioning periods.

3.6.2 | Outstanding questions

- Do cell-modifying factors associate with EVs or non-EV particles, and if so, what are their possible effects?

4 | CULTURE CONDITIONS

Changes in biophysical and biochemical cell culture conditions may affect cell growth and the release and biochemical composition of EVs. External culturing conditions include the concentration of gasses, 2D or 3D culturing, pH, temperature, physical stimulus, the composition of culture vessels/substrates, and in-experiment manipulations of the cell culture medium.

4.1 | Gas conditioning

The effect of oxygen concentration on the biochemical composition, function, and release of EVs is well studied (Agarwal et al., 2017; Alharbi et al., 2021; Almeria et al., 2019; Davidson et al., 2018; De Jong et al., 2012; Dougherty et al., 2020; Dutta et al., 2020; King et al., 2012; Kucharzewska et al., 2013; Kumar & Deep, 2020; Salomon, Kobayashi et al., 2013; Salomon, Ryan et al., 2013; Xue et al., 2018; Zhang et al., 2017). So-called 'normoxia', that is, 21% O_2 or 'atmospheric' oxygen, is in fact hyperoxia for most mammalian cell types. Even in lung alveoli, the oxygen level is 14.5%, and at the venous end of circulation, oxygenation is around 6.5% (Mckeown, 2014). Depending on the exact tissue type and anatomy, physiological hypoxia may be in the range of 2%−6% oxygen, and pathological hypoxia may be even lower, ranging from 0.3% to 4.2% oxygen (Mckeown, 2014). Cells cultured under atmospheric conditions have thus been exposed or even adapted to higher-than-physiologic oxygen concentrations. To reach true normoxia, specialized culture chambers are required.

4.1.1 | Recommendation

- Report gas concentrations used during cell culture, especially of oxygen, but where applicable also of CO_2, and nitrogen, as well as any interruptions in the gas conditions during the culture process.

4.2 | 2D or 3D cultures: vessels, coatings, matrices

Some reports have shown that cells cultured in 3D systems typically produce more EVs compared with conventional 2D cultures or adherent cultures (Cao et al., 2020; De Almeida Fuzeta et al., 2020; Grangier et al., 2021; Guo et al., 2021; Haraszti et al., 2018; Kim et al., 2021; Patel et al., 2018; Phan et al., 2018, 2019; Rocha et al., 2019; Watson et al., 2016; Yan & Wu, 2019; Yan et al., 2018; Yang et al., 2020; Zhang et al., 2021). On the other hand, 3D system of cultures can lead to different biochemical compositions and functions of EVs compared with those obtained with 2D systems (Cao et al., 2020; Gao et al., 2020; Haraszti et al., 2018; Jarmalavičiūtė et al., 2015; Kim et al., 2021; Miceli et al., 2019; Rocha et al., 2019; Thippabhotla et al., 2019; Yan & Wu, 2019, 2020; Yang et al., 2019). The materials from which cell culture vessels (e.g., flasks or plates) are, as well as any support matrices, such as bead substrates and vessel coatings, may affect EV characteristics and should be reported accordingly (Jangamreddy et al., 2018; Park et al., 2020; Sutter et al., 2021; Szvicsek et al., 2019; Wu et al., 2021).

4.2.1 | Recommendations

- Report all culturing conditions, including, but not limited to, surface area, volume, and preconditioning with buffers.
- Report the composition of any support matrix, including, for example, substrate beads and vessel coatings.
- Indicate the procedure used to prepare and apply any support matrix.

4.3 | pH

The pH of the medium affects release, uptake and biochemical composition of EVs (Ban et al., 2015; Boussadia et al., 2018; Cheng et al., 2019; Logozzi et al., 2018; Parolini et al., 2009; Perut et al., 2019; Nakase et al., 2021). Medium pH affects the stability of

released EVs (Cheng et al., 2019). Although we are not aware of reports on the effects of pH on generation of therapeutic EVs, changes in pH influence stem cell reprogramming or differentiation (Fliefel et al., 2016; Kim, 2021; Massa et al., 2017).

4.3.1 | Recommendations

- Monitor and report the pH of medium.
- Report if cells were grown using a pH buffering agent, and, if so, its concentration.

4.3.2 | Outstanding questions

- How does pH affect the production and composition of EVs?

4.4 | Physical stimulation

Applying physical stimuli such as electrical stimulation, acoustic irradiation, and mechanical forces (including shear and stretching of the scaffold) may increase the production and/or change the biochemical composition of EVs (Ambattu et al., 2020; Eichholz et al., 2020; Fukuta et al., 2020; Guo et al., 2021; Hergenreider et al., 2012; Letsiou et al., 2015; Maeshige et al., 2021; Morrell et al., 2018; Ridger et al., 2017; Vion et al., 2013; Xia et al., 2020; Yang et al., 2017; Zeng et al., 2019; Zhao et al., 2020). Moreover, for some cell types, temperature may affect EV release and uptake dynamics and may also change EV function (Mahmood et al., 2023; Otsuka et al., 2020).

4.4.1 | Recommendation

- Report all applied physical stimuli in detail, including, but not limited to, intensity, voltage, time and temperature.

4.5 | Replacement of medium

Medium replacement during cell culturing keeps the cells healthy by providing fresh nutrients and eliminating waste products. However, during medium replacement, the secretome of cells including EVs is removed, and cells start to secrete new EVs (Patel et al., 2017; Vis et al., 2020).

4.5.1 | Recommendations

- Report the medium replacement protocol, including what percentage of the volume is replaced, at what interval (in hours) the medium is changed or replenished, intermediate washing steps (if any), changes to the medium composition, and continuous-flow feeding (with recirculation or not).
- In some cases, different media formulations are used at various stages of an experiment. If so, indicate when and how, as well as the details of each formulation and why each specific formulation was used.

4.5.2 | Outstanding questions

- What are the effects of maintaining cells in the same medium for extended durations and of medium replacement on EV release, biochemical composition, and function?

5 | COLLECTION (HARVEST) OF CCM

Duration and frequency of CCM collection may affect the yield and surface protein expression of EVs (Mendt et al., 2018; Patel et al., 2017). There are three main approaches to CCM harvest: single harvest, multiple harvests, and continuous harvest. The duration of conditioning differs between studies and assays from hours to days. During conditioning, cells continuously release

and uptake EVs; therefore, the time interval during which cells are cultured affects the biochemical composition, function, and release of EVs due to changes in the release and possible re-uptake of EVs, along with cell growth and differentiation (; Davidson et al., 2018; Flores-Bellver et al., 2021; Kim et al., 2016; Li et al., 2015).

5.1 | Recommendations

- For a single harvest: report the total time (duration) of conditioning.
- For multiple harvests: report the number of harvests, duration of conditioning for each, and whether all or some fraction of medium is collected (i.e., complete or partial medium replacement), specifying the fraction where applicable.
- Report how many (if any) of the multiple harvests are pooled.
- Report on any pre-pooling analyses and inclusion criteria.
- For continuous harvest, report the duration of continuous collection and harvest rate (i.e., volume per unit time).
- Report the duration of conditioning and keep it constant across experimental repeats.

5.2 | Outstanding questions

- How do continuous harvest and multiple harvests affect EV release, biochemical composition and function?
- Since cells release EVs into and simultaneously uptake EVs from CCM, cultures may reach an equilibrium between EV release and uptake, and this may occur at different times for different cultures and conditions. What are the effects of conditioning time on EV release, biochemical composition and function?

6 | MICROBIAL CONTAMINANTS OF CCM

CCM may be contaminated with viruses, bacteria, fungi, and other unwanted bioactive components such as endotoxins. Active bacterial and fungal contaminations are often apparent in culture and in stored EV samples by visual examination or aided by light microscopy, in which case such materials can be discarded. However, many contaminations are not easily detected. The presence of Mycoplasma, for example, may not be readily apparent, and Mycoplasma species can release EVs into CCM (Gaurivaud et al., 2018). Mycoplasma-infected cells release EVs that differ in function from EVs released by non-infected cells (Cronemberger-Andrade et al., 2020; Quah & O'neill, 2007; Yang et al., 2012). Bacterial or fungal-derived EVs and other bioactive components such as endotoxins may also be introduced into CCM from raw materials or culture vessels even if actively replicating organisms are not present. Viruses and viral components may also be present in cells and/or culture components and may cause unintended effects (Barone et al., 2020; Merten, 2002). Contaminants such as Mycoplasma and viruses may not be eliminated by 0.22-micron filtration of culture medium, conventionally used to eliminate active bacterial contamination.

6.1 | Recommendations

- Report if any screening of Mycoplasma or other contaminants in parental cell lines/cultures was done and the results thereof. Report details of any antimicrobial treatment.
- Especially if EVs are administered to animals or humans, measure and report the level of endotoxins in prepared EVs according to international pharmacopoeia (USP<85>, EP 2.6.14, JP 4.01) (Commission, E.P., E.D.f.t.Q.o 2010; Revision, U.S.P.C.C.o 2008; The Japanese pharmacopoeia 1996), and provide evidence of sterility of prepared EVs according to international pharmacopoeia (USP<71>, EP 2.6.1, JP 4.06) (Commission, E.P., E.D.f.t.Q.o 2010; Revision, U.S.P.C.C.o 2008; The Japanese pharmacopoeia 1996).

7 | DISCUSSION

In this perspective paper, we aim to raise awareness of the effects of cell culturing parameters (Figure 2a) that may affect EV release (Figure 2b), biochemical composition, and/or function (Figure 2c). Cells can ingest components that are present in the media and re-package them into or onto EVs (Figure 2d). Moreover, cell culturing parameters can influence cellular processes other than EV biogenesis that indirectly affect EVs (Figure 2e). We have compiled a checklist of these parameters in Table 1. The aim of the checklist is to collect as much information as possible to improve reproducibility. The information can be summarized in the Methods section, and the checklist can be added as supplementary information to the manuscript. We are aware that some

TABLE 1 Cell culturing parameter checklist.

Cell culturing parameters		Items	
1. Producing cells (source cells)	**1.1 Cell identity**	Name of cell	
		Origin (e.g. donor or cell bank)	
		Cell type identification	
	1.2 Cell donor characteristics	Biological sex	
		Age	
		Health status	
		Pre-culture processing of the cells	
	1.3 Initial seeding density	Initial seeding number per surface area or volume	.....
	1.4 Cell density or confluency of cells at the time of CCM collection	Percentage of surface area covered (2D cultures)	..... %
		Estimated number of cells per volume (suspension cultures)	.....
		The diameter of the spheroids (3D cultures)	.....
	1.5 Cell sub-culturing, population doubling, and passage number	Passage number	.....
		Passaging method	.....
		Recovery time	.....
	1.6 Cell viability	Percentage of viable cells	..... %
		Method of viability assessment	.....
2. EV production medium	**2.1 Basal medium, glucose, and amino acids**	Name of basal culturing medium	.....
		Company, and catalog number	.....
		Additions	..... , ... mM
		Salts (name, concentration)	..... , ... mM
		Glucose (D/L glucose, concentration)	..... , ... mM
		Amino acids (name, concentration)	
	2.2 Antibiotic	Name	....
		Concentration	..... mM ..... mM ..... mM
		During conditioning or pre-conditioning or both	.....
	2.3 Complex biological supplements	If the EV production medium contains serum/PL	.....
		Percentage	.....
		Producing company, and catalog number of sera	.....
		Process of PL production, including fibrin depletion methods and pathogen inactivation	.....
		Concentration of heparin present in the growth medium	.....
		If the EV production medium contains EV-depleted serum/PL	.....
		Indicate whether, how (fold dilution), and with what (buffer, medium) the serum was diluted before EV depletion	.....
		Method of EV depletion	
		Checking the efficiency of EV depletion	
		Result of checking the efficiency of EV depletion	
		If there are washing steps (before changing to EV depleted medium), write down the details	
		Source, and catalog number of commercial EV-depleted serum	
		If serum/PL free media is used indicate time duration	
		If using a replacement supplement	
		Percentage	
		Producing company, and catalog number of replacement supplement	

(Continues)

TABLE 1 (Continued)

Cell culturing parameters		Items	
	2.4 Cell (pre) conditioning	Name of factor Company, and catalog number Concentration Conditioning or pre-conditioning	…… …… …… …… …… mM …… mM …… mM ……
3. Culturing conditions	**3.1 Gas conditioning**	Name (e.g oxygen, nitrogen, etc) Percentage	…… …… %
	3.2 2D or 3D cultures: vessels, coatings, matrices	Type (2D, 3D, or spheroid) Vessels (e.g. flasks or plates) Coating materials 3D support matrix (type, details) Preconditioning with buffers Volume of medium (at the time of CCM harvest for EV preparation) Details of spheroid formation	…… …… …… …… …… …… ……
	3.3 pH	pH of medium If a pH buffering agent is used indicate its concentration	…… ……
	3.4 Physical stimulus	Type (such as acoustic irradiation, and mechanical forces including shear and stretching of the scaffold) Details Culture temperature	…… …… …… °C
	3.5 Replacement of medium	What percentage of the volume is replaced At what interval (in hours) the medium is changed or replenished Intermediate washing steps (if any) Continuous-flow feeding (with recirculation or not) Are different media formulations used at various stages of an experiment?	…… …… …… …… ……
4. Collection (harvest) of CCM	**Duration and frequency of CCM collection**	Single harvest: Duration of conditioning (in hours) Multiple harvests: Number of harvests Duration of conditioning (in hours) Whether all or some fraction of medium is collected (i.e., complete or partial medium replacement), specifying the fraction where applicable. How many of the multiple harvests are pooled (if any) Continuous harvest: Duration of collection (in hours) Harvest rate (i.e. volume per unit time)	…… …… …… …… …… …… ……
5. Microbial contaminants of CCM	**Viruses, bacteria, fungi, and any unwanted microbial components**	Presence of viruses Measurement method When is checked? Outcome Presence of bacteria (e.g. mycoplasma) Measurement method When is checked? Outcome Presence of fungi Measurement method When is checked? Outcome Presence of other unwanted microbial components (e.g. endotoxin) Measurement method When is checked? Outcome	yes no …… in producing cells in CCM in both …… yes no……in producing cells in CCM in both …… yes no …… in producing cells in CCM in both …… yes no…… in producing cells in CCM in both ……

Abbreviations: CCM, cell culture-conditioned medium; EV, extracellular vesicle; PL, platelet lysate.

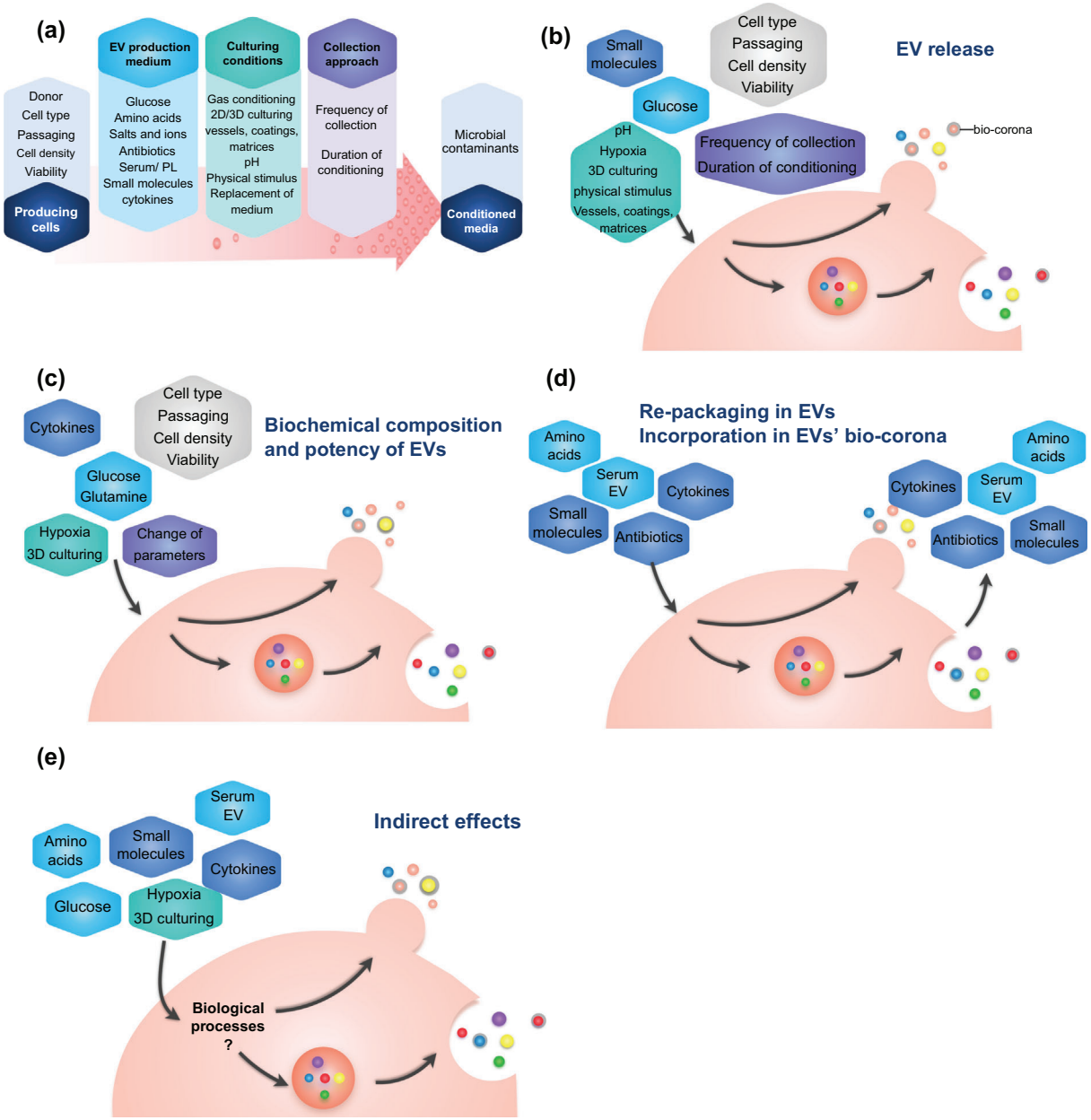


FIGURE 2 Cell culture parameters and their effects on production of extracellular vesicles. (a) All parameters affect cells during conditioning and release of extracellular vesicles (EVs). (b) Parameters influencing release of EVs, and (c) biochemical composition of EVs. (d) Cells might also re-package cell culture medium supplement components into/onto released EVs. (e) Cell culturing parameters may affect processes that indirectly affect EVs.

recommendations may be harder to implement than others. For instance, measuring pH of a culture medium at different stages of the culture is not a routine process; thus, it may not become readily or routinely implemented. At present, multiple task forces and working groups have developed or are developing checklists, and the goal of ISEV is to develop on online repository, either as a stand-alone or combined and integrated with an already existing platform such as EV-TRACK. Such an online repository will be useful to re-evaluate collected data and can be used to update future recommendations accordingly.

There is hardly any discussion on the mechanisms by which changes in cell culture impact EVs, but these should no longer be overlooked. Cellular processes such as autophagy can affect EV production both directly (Xing et al., 2021; Xu et al., 2018) and indirectly (Moruno et al., 2012; Wang et al., 2021), influencing the number and type of released EVs. Comparing autophagy levels in cultures used for preparing EV batches may explain inter-experiment / batch differences in EVs. Since hypotonic dialysis can be used to load drugs into EVs (Mehryab et al., 2020), allowing therapeutic agents to cross the membrane (Xie et al., 2021), the osmolarity of the culture medium may affect EV cargo. Given the effects of amino acids on the mammalian target of rapamycin (mTOR) signaling (Jewell et al., 2013) and the connection between mTOR and EV release (Zou et al., 2019), it is reasonable to

assume that amino acid concentrations will impact EV production. Finally, oxygen concentration modulates cellular senescence, which may impact EVs, as mentioned in the cellular sub-culturing section (Seno et al., 2018; Welford & Giaccia, 2011; You et al., 2019).

Of all cell culture supplements, serum and PL are among the most ubiquitous and challenging. They are rich in EVs, their EVs support cell growth and function in ways that cannot easily be achieved with a 'defined' medium, and EV depletion from these supplements is time-consuming and/or inefficient (Lehrich et al., 2021). In addition, they may indirectly influence cells and provide cells with materials that can be re-packaged into newly produced EVs or incorporated as a bio-corona (Figure 2d). Hence, more investigations are needed to critically evaluate the consequences of serum/PL removal or EV depletion on each cell type.

In summary, the effects of cell culture parameters on EVs are complex, and our current understanding of these influences is far from complete. A distinction should be made between culturing cells for basic EV research versus therapeutic applications since the latter involved additional considerations around safety and regulation. Missing and vague information about manufacturing and processing parameters can lead to misinterpretations and false conclusions and hampers study reproduction. We therefore emphasize the importance of rigorous reporting of all cell culture parameters to enable researchers to better compare experimental in vitro and in vivo data and pre-clinical and clinical outcomes. Only with full reporting can we achieve our common goal of comprehensively understanding the biological functions of EVs. We hope that these suggestions (Table 1) will be a useful starting point for further discussions and that they will promote good reporting practices in CCM-EV research.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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