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Review article

Critical Determinants of Extracellular Vesicle Stability for Enhanced Therapeutic Applications in Biotechnological Production

Victoria Shestakova[1,2]*, Ekaterina Smirnova[1,2], Lyudmila Komarova[2], Andrey Kotlyarov[2] and Elena Yatsenko[1]

[1]A. Tsyb Medical Radiological Research Center - Branch of the National Medical Research Radiological Centre of the Ministry of Health of the Russian Federation, Obninsk, Russia
[2]Obninsk Institute for Nuclear Power Engineering, National Research Nuclear University MEPhI, Obninsk, Russia

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Abstract

Paracrine factors play a central role in the regulation of cellular communication and function and serve as the primary agents in cell-based therapeutic interventions. These factors facilitate a wide range of critical physiological and pathological processes through diverse mechanisms, including secretion of soluble molecules, release of extracellular vesicles, and direct intercellular channel transfer. The study highlights the significant impact of stress-induced changes in extracellular vesicles on cellular responses. Several stressors, including temperature, and oxidative, mechanical, osmotic, and transportation stress, have been shown to alter the release and composition of extracellular vesicles in affected cells. Extracellular vesicle modifications can undermine the success of cell therapies by altering the paracrine activity of the delivered cells, although these cells retain their phenotypic characteristics. Therefore, a deeper understanding of the mechanisms that govern paracrine signaling and strategic modifications of this signaling are critical for improving the efficacy of cell therapy.

Keywords: cellular stress; extracellular vesicles; mesenchymal stromal cells; mesenchymal stem cells; regenerative medicine; tissue engineering

1. Introduction

The field of biotechnology has made tremendous progress in recent years, with the development of innovative therapeutic approaches that harness the potential of extracellular vesicles (EVs) (Wiklander et al., 2019). EVs are spherically shaped particles comprising a variety of cellular components, including polynucleotides, proteins, and metabolites. They are formed through the separation of intracellular compartments or the plasma membrane. EVs are synthesized by almost all cells and fulfill the transport role of highly active biological molecules on a variety of biological systems (Uldry et al., 2022).

*Corresponding author: E-mail: schestakova.vika2017@yandex.ru
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EVs have shown great promise in the treatment of various diseases, including cancer, neurodegenerative disorders, and cardiovascular disease (Khawar et al., 2019; Huang et al., 2021; Palanisamy et al., 2023). However, as the field moves towards the translation of EV-based therapies from the laboratory to the clinic, several challenges have emerged (Melling et al., 2019; Li et al., 2020; Rai et al., 2024). One of the major hurdles is the development of scalable and cost-effective manufacturing processes that can produce high-quality EVs with consistent properties and potency (Ng et al., 2019).

Biotechnology manufacturing of EVs for therapeutic applications is a complex process that requires the integration of multiple disciplines, including cell biology, bioengineering, and pharmaceutical sciences (Paganini et al., 2019; Wang et al., 2024). The production of EVs involves the cultivation of cells, isolation and purification of EVs, and characterization of their physical and biological properties (Gardiner et al., 2016; Du et al., 2023). However, the lack of standardized protocols, limited understanding of EV biology, and the need for robust and reproducible manufacturing processes are some of the key challenges that need to be addressed (Buschmann et al., 2021).

This means that EVs can be exposed to different types of stress during the biotech manufacturing process, which are unrelated to their physiologically natural production and release by cells in the body (Thompson & Papoutsakis, 2023). These types of stress can result in changes in EV composition, structure, and function, which may influence their therapeutic efficacy and safety (Colao et al., 2018; Grangier et al., 2021). For instance, the use of harsh chemicals, extreme temperatures, and mechanical forces during the manufacturing process can cause damage to the EVs, leading to loss of their natural properties and the introduction of impurities (Alexandre et al., 2024; Tang et al., 2024).

This review focuses on current knowledge of the composition, properties, quality and quantity of EVs released by cells subjected to temperature, oxidative, mechanical, osmotic, and transportation stress.

2. Paracrine Signaling: A Key Mechanism in Transplanted Cell Therapy

Paracrine signaling represents a form of intercellular interaction whereby paracrine factors are produced, inducing changes in neighboring cells. These changes may include the modulation of reparative and inflammatory processes, and the survival and differentiation of cells into mature cell lineages (Ferraris, 2016). This form of intercellular communication involves the release of specific biologically active molecules, particularly growth factors, cytokines, and chemokines that influence neighboring target cells. In cell therapy, paracrine factors have demonstrated a wide range of therapeutic potential, including promoting tissue regeneration (Liu et al., 2016; Kumar et al., 2019), reducing inflammation (Flower et al., 2015), and modulating immune responses (Fontaine et al., 2016). These factors, such as growth factors, cytokines and chemokines, are instrumental in driving angiogenesis and attracting endogenous stem cells (Ridiandries et al., 2018). They have been observed to have significant positive effects in the treatment of a wide variety of conditions, including cardiovascular disease (Tachibana et al., 2017; Mabotuwana et al., 2022), neurological disorders (Al-Kaabi & AL-Rubai, 2021), autoimmune diseases (Yoo et al., 2015), and tissue injury (Legrand & Martino, 2022). Therefore, paracrine factors are important signaling molecules secreted by cells that exert their effects on neighboring cells and play a central role in a variety of physiological and pathological processes. These processes include cell growth, differentiation, migration and survival (Mannerström et al., 2019; Arabi et al., 2020) (Figure 1).

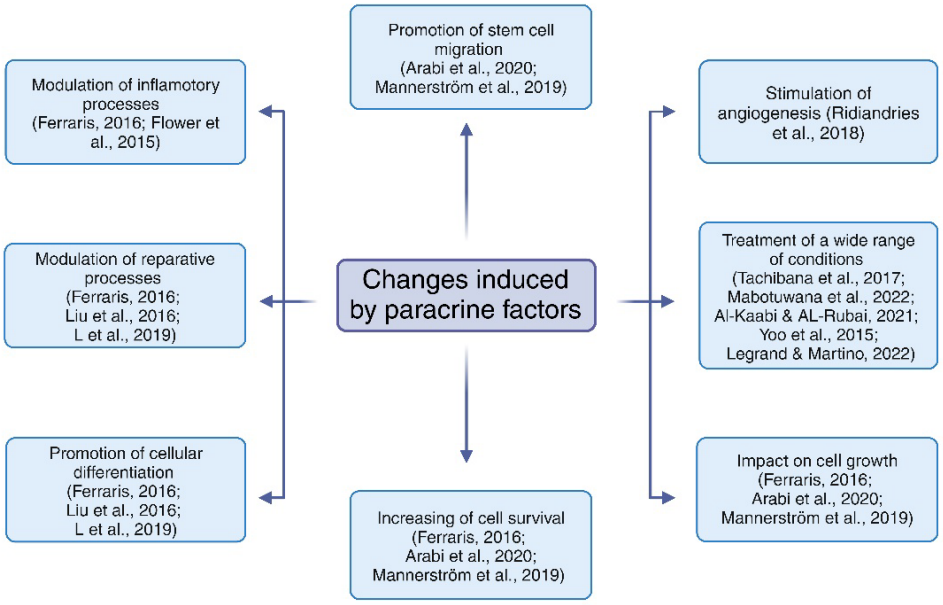

Figure 1. Effects of paracrine factors

Paracrine factors can be classified into several distinct types, based on their molecular structure and functional properties (Figure 2).

The first type includes soluble proteins and RNA-protein complexes. These molecules are released by cells and have the ability to influence neighboring cells, thereby regulating a wide range of physiological processes (Glembotski, 2017; Lopatina et al., 2022). Soluble proteins, which include cytokines, growth factors, and chemokines, are involved in cell signaling, immune responses, and tissue repair mechanisms (Kefaloyianni, 2022). Conversely, RNA-protein complexes are primarily involved in the post-transcriptional regulation of gene expression, thereby modulating the behavior of cells that receive these signals (Rissland, 2017). Proteins and RNA-protein complexes are stable and remain active in the blood and interstitial fluids for a long period (Etheridge et al., 2013).

The second type consists of RNA- and protein-loaded EVs, including exosomes. These vesicles are released by cells into the extracellular milieu and can be taken up by proximal or distal cells (Zaborowski et al., 2015). Upon uptake, EVs release their cargo (Joshi et al., 2020). Exosomes, a specialized subtype of EVs, play important roles in cell-to-cell communication, immune regulation, and intercellular transfer of genetic information (Greening et al., 2015). The RNA and proteins carried by these vesicles can significantly alter the behavior and functionality of recipient cells, thereby influencing a range of physiological and pathological processes, for example, inflammation (Lyamina et al., 2023), cellular senescence (Wijesinghe et al., 2022), or inducing senescent drift in affected tissues (Smirnova et al., 2023). Currently, the most commonly used source of EVs is mesenchymal stem cells (MSCs) (Baek et al., 2019; Weng et al., 2021; Rezaie et al., 2022). This is due to the fact that the focus of current research has shifted from the direct differentiation and engraftment of MSCs to their paracrine signalling, which is now regarded as the primary mechanism of tissue repair and immunomodulation. MSCs secrete a diverse

array of bioactive molecules, including cytokines, chemokines and extracellular vesicles. (Baranovskii et al., 2022; Krasilnikova et al., 2022a). These molecules regulate the immune microenvironment, as well as promote tissue regeneration (Liang et al., 2014). MSC-derived EVs contain bioactive molecules such as RNA and proteins, which can be transferred to target cells (Cai et al., 2020; Ha et al., 2020). These EVs have shown therapeutic potential in various disease models, offering advantages over cell-based therapies due to their lower immunogenicity, ability to cross biological barriers, and fewer safety concerns (Keshtkar et al., 2018; Zhao et al., 2020). MSC-derived EVs can mediate cellular communication, induce cell differentiation, and promote tissue repair through paracrine effects (Yuan et al., 2020). EVs can pass through biological barriers without rejection and shuttle genetic information, reprogramming recipient cells. This paracrine activity of MSC-derived EVs is now considered a predominant mechanism in tissue repair, offering a promising cell-free therapy option that may overcome obstacles associated with native stem cell use (Nooshabadi et al., 2018).

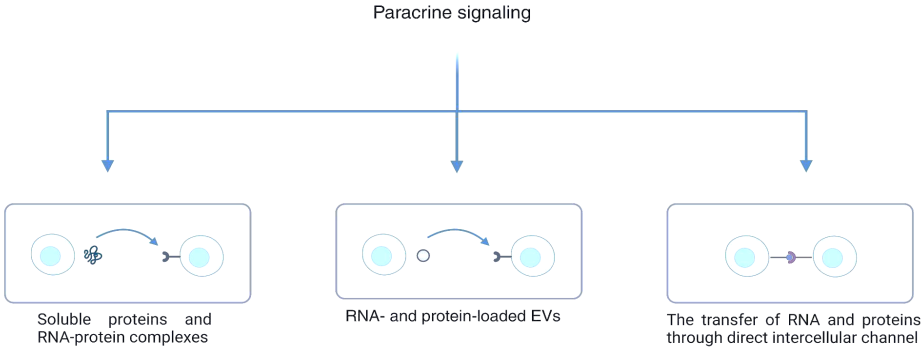

Figure 2. Three types of paracrine signaling

The third type of paracrine factors includes mechanisms that underlie the transfer of RNA and proteins through direct intercellular channels. This type of transfer allows a direct and efficient exchange of molecular signals between cells, thereby promoting the coordination of cellular activities (Plotnikov et al., 2017). Direct cell-cell communication is essential for the regulation of various physiological processes in organism, ensuring the maintenance of homeostasis and the proper functioning of biological systems (Vinken, 2015).

In contrast to soluble proteins and RNA-protein complexes and the transfer of RNA and proteins through direct intercellular channels, EVs provide a stable environment for small RNA molecules, protecting them from degradation in biological fluids and enabling efficient signaling across species (Lefebvre & Lécuyer, 2017). The capacity of EVs to transport a diverse array of molecular structures enables them to engage with numerous target cells, eliciting a spectrum of responses (Edelmann & Kima, 2022; Ginini et al., 2022). Their stability in the extracellular environment contributes to their long-term functioning and efficient signal transduction (Maas et al., 2017). Furthermore, EVs are capable of transporting a diverse range of cargo over relatively long distances within the body, thereby acting as effective signal carriers between cells that are distant from one another (Cesi et al., 2016).

However, in the process of biotechnological production, cells and EVs obtained from them are subjected to various intracellular and extracellular stresses. This, in turn, has a strong impact on EV biogenesis, secretion, and content, as well as their impact on EV morphology, size, and function (Figure 3). Therefore, it is necessary to understand how specific types of stresses affect changes in the properties of cells and their secreted EVs in order to take into account the specifics of further development of new advanced methods for the production of EVs for subsequent therapeutic applications.

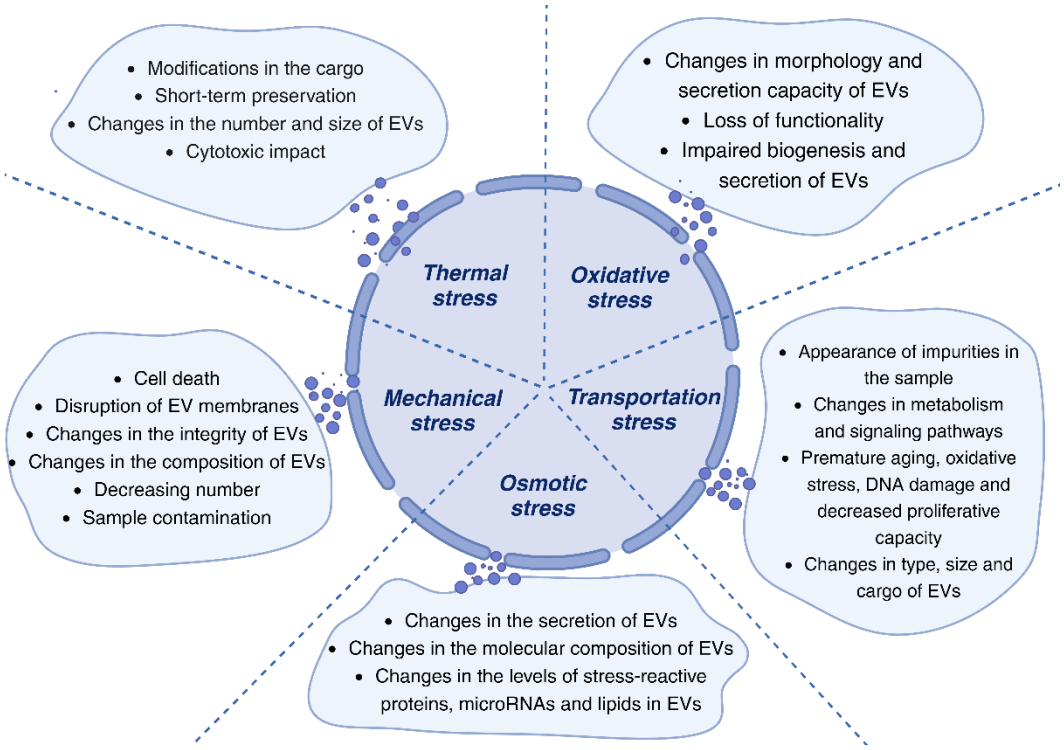

Figure 3. Potential effects of stress exposure on cell-released extracellular vesicles (EVs)

3. Challenges of biotechnological production of extracellular vesicles: how different types of stress affect extracellular vesicles

The biotechnological production of extracellular vesicles (EVs) has garnered significant interest due to their potential applications in drug delivery, diagnostics, and therapeutics (Figure 4).

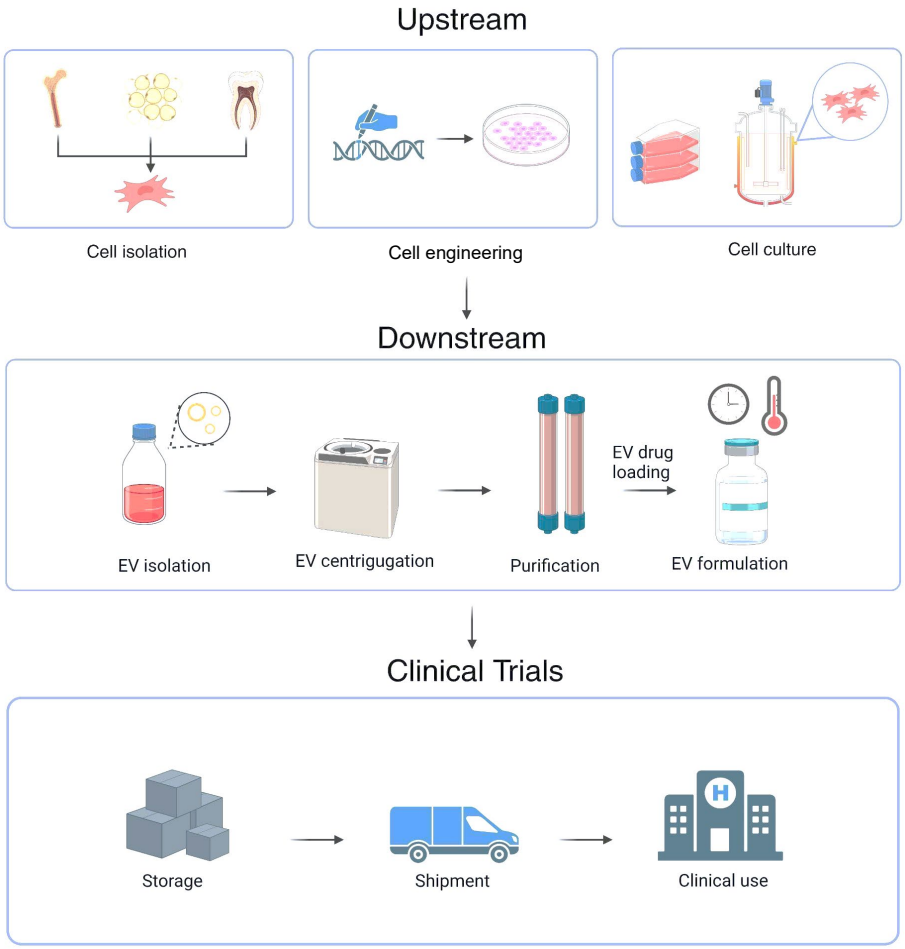

Figure 4. Biotechnology manufacturing of extracellular vesicles

However, the industrial manufacture of EVs necessitates the implementation of specific biotechnological methodologies for the large-scale production of EVs and the maintenance of their quality and efficacy. Researchers are exploring various strategies to enhance EV production, including optimizing cell culture conditions, developing large-scale bioreactor systems, and employing cell stimulation techniques (Syromiatnikova et al., 2022). In recent years, there has been a notable emergence of companies, such as Aposcience AG (Austria), ZenBio (USA) and Paracrine Therapeutics (Singapore), that are exploring the therapeutic potential of EVs in the treatment of various diseases. Aposcience AG has already developed a commercially available peripheral blood mononuclear cell secretome product "APOSEC™", which is currently undergoing clinical research for the treatment of wound healing disorders in skin wounds. ZenBio offers a range of products derived from placental MSCs, cord blood serum and pre-adipocytes. These are utilized for the treatment of cutaneous lesions. Paracrine Therapeutics is an organization that specializes in the use of human mesenchymal stem cell (hMSC) exosome products for the treatment of a range of medical conditions, including osteoarthritis, liver fibrosis and

eczema (Gimona *et* al., 2017). While progress has been made, the scalable production of EVs with stable quality is a major obstacle to the clinical application of EVs, due to their heterogeneity, lack of standardized methods of isolation, purification and transport, and optimal storage conditions (Nelson et al., 2020; Zeng et al., 2022).

3.1. Thermal stress: exposure of cells to low or high temperatures, including cryopreservation

Temperature stress is one of the main environmental factors affecting both individual cells and their molecular composition, and the organism as a whole. In addition, temperature variations can affect the cargo and amount of EVs secreted by cells (Rayamajhi et al., 2023). Studies have shown that lower temperatures may lead to a decrease in EV release, while higher temperatures could enhance vesicle secretion (Mahmood et al., 2023).

3.1.1 Cold stress

The composition of EVs can also be influenced by negative temperatures during cell transportation of cell products. Cold-induced stress can trigger modifications in the cargo of EVs, potentially altering the types and amounts of biomolecules encapsulated within these vesicles (Sivanantham & Jin, 2022). Proteomic and lipidomic analyses have revealed shifts in the protein and lipid profiles of EVs released under cold conditions, reflecting cellular adaptations to low temperatures (Abramowicz et al., 2019; Magnadóttir et al., 2020).

Short-term storage at temperatures below -70°C has been found to be favorable for cells and allows for short-term preservation, e.g., during transportation, of EVs for further clinical use and research (Lee et al., 2016). However, long-term storage at low temperatures can significantly affect the composition and structure of EVs. Thus, storage of EVs at -70°C for 25 days resulted in a decrease in the number and size of EVs, changes in their morphology, increased vesicle aggregation, and a decrease in the amount of proteins in exosomes (Park et al., 2018). Interestingly, EVs isolated from human serum retained their morphological features and remained stable at -80°C for one year following repeated cycles of thawing and freezing (Lacroix et al., 2012; Jin et al., 2016).

3.1.2 Heat stress

Elevated temperatures can lead to protein denaturation, unfolding, and aggregation, resulting in inactivation and loss of functionality (Wilkening et al., 2018). Consequently, proteins are particularly susceptible to the detrimental effects of hyperthermia which makes heat shock a classic example of proteotoxic cellular stress. When proteins function improperly, it disrupts cellular homeostasis and triggers the activation of the heat shock response (HSR) (Neves-da-Rocha et al., 2023; Singh et al., 2024). The primary goal of the HSR is to minimize damage to essential cellular components and halt processes related to proliferation and growth until the stress factor is eliminated and any damage is repaired (Guisbert et al., 2013).

It was shown that cells exposed to a temperature of 42°C produced a much higher number of EVs enriched in HSP70, HSP90 and SOD1 and contained higher amounts of GRP78 and GRP94 compared to the control (37°C) (Gebremedhn et al., 2020). A similar situation was observed in EVs of peripheral blood mononuclear cells; an increase in the level of stress-inducible HSPA1 was observed (Lancaster & Febbraio, 2005). It was found

that after incubation at 45°C for 1 h, cells exhibited increased HSP70 levels, increased DNA damage and decreased viability. Also, these cells produced EVs of smaller size and diameter than control cells and induced a witness effect in native cells, which led to higher levels of DNA damage, induced apoptosis and significantly reduced the overall survival of native cells (Bewicke-Copley et al., 2017). Interestingly, heat stress (40°C) caused an increase in CD63+ EV secretion by T- and B- leukemia/lymphoma T cells (Hedlund et al., 2011), and also resulted in a 1.25-fold increase in EV secretion by B-lymphoblastoid cells (Clayton et al., 2005).

Understanding how temperatures impact EV secretion and composition is essential for uncovering novel insights into cellular responses. Cold-induced changes in EV secretion and characteristics could have implications for physiological processes, including immune modulation, tissue repair, and disease progression. Moreover, exploring the role of EVs in temperature-stress adaptation may offer new avenues for therapeutic interventions in conditions associated with hypothermia or cryopreservation and hyperthermia. In summary, temperatures exert notable effects on the secretion and composition of EVs, highlighting the intricate interplay between environmental factors and cellular communication mechanisms. Further investigations into the molecular mechanisms underlying these effects could unveil potential applications in biomedicine and biotechnology, advancing our understanding of EV biology in diverse contexts of temperature stress.

3.2 Oxidative stress

Oxidative stress is a condition that occurs when there is an imbalance between the production of reactive oxygen species (ROS) and the body's ability to simultaneously detoxify these reactive intermediates or repair the resulting damage (Ragusa, 2023). This imbalance can significantly impact the secretion and composition of EVs, leading to both favorable and negative effects. One of the negative effects of oxidative stress on EVs is the impairment of their secretion. ROS can interfere with the mechanisms that regulate EV release, resulting in a decrease in the amount of EVs released by cells (Chiaradia et al., 2021). This may affect the ability of cells to interact with each other and coordinate their activities. (Kulakov et al., 2021; Maksimova et al., 2022; Krasilnikova et al., 2022b; Klabukov et al., 2023a,b,c,d; Krasilnikova et al., 2023).

Ketamine exposure has been shown to induce oxidative stress and apoptosis through activation of the nuclear factor erythroid 2 (Nrf2) pathway in various animal models (Félix et al., 2016; Tannich et al., 2020). It was found that ketamine-induced toxicity and oxidative stress altered the morphology and secretion capacity of EVs. Ketamine's impact on EVs was observed in ketamine-induced cystitis, where it altered EV secretion and enhanced oxidative stress via the P38/NF-κB pathway (Xi et al., 2020).

Ultraviolet radiation (UVR) exposure, particularly ultraviolet A (UVA), induces oxidative stress. UVA exposure generates ROS in various tissues, affecting cellular redox balance, mitochondrial membrane potential, and DNA integrity. UVR can cause oxidative stress through direct cellular damage, photosensitization mechanisms, and by affecting enzymes like catalase and nitric oxide synthase (de Jager et al., 2017). It was shown that exposure of EVs to ultraviolet light caused them to lose their ability to transmit a protective signal to recipient cells after 12 h, which in turn affected the viability of recipient cells under oxidative stress (Eldh et al., 2010).

Polycyclic aromatic hydrocarbons (PAHs) have been associated with increased oxidative stress and inflammation markers in various studies (Zhang et al., 2020a; Wu et

al., 2022a). PAHs stimulated increased secretion by hepatocytes of EVs with altered structure and composition, including enrichment of EVs with proteins associated with oxidative stress and increased iron content in EVs, causing oxidative stress leading to mitochondrial and lysosomal changes, and ultimately leading to cell death by apoptosis (van Meteren et al., 2019).

Oxidative stress has been shown to disrupt the intricate machinery involved in EV biogenesis and secretion, leading to aberrant release. This disruption is linked to the impairment of endosomal sorting complexes required for transport (ESCRT) by elevated levels of ROS. The redox state of protein thiols, which can be modulated by oxidative stress, is also implicated in the regulation of EV release. Furthermore, oxidative stress-induced alterations in lipid metabolism and membrane integrity compromise the budding and release of EVs from donor cells (Benedikter et al., 2018; Chiaradia et al., 2021).

Beyond impairing EV secretion, oxidative stress exerts profound effects on the molecular composition of EVs. ROS-mediated modifications of lipids, proteins, and nucleic acids within EVs not only compromise their stability but also alter their cargo content and functional properties (Nathan & Cunningham-Bussel, 2013). For instance, oxidative stress-induced lipid peroxidation generates lipid-derived reactive aldehydes, such as 4-hydroxynonenal (4-HNE), which can covalently modify proteins and nucleic acids within EVs, thereby influencing their biological activities (Zhong & Yin, 2015). Moreover, ROS-mediated oxidation of EV-associated proteins and nucleic acids may impair their functionality and alter signaling pathways in recipient cells, contributing to the propagation of oxidative damage and cellular dysfunction (Chiaradia et al., 2021).

Several studies have demonstrated that oxidative stress can stimulate the release of EVs from cells (Takasugi et al., 2017). This may be due to the fact that oxidative stress can induce cellular damage and trigger a protective response that involves the release of EVs. Additionally, oxidative stress has been shown to alter the composition of EVs, leading to changes in their cargo of bioactive molecules (Biasutto et al., 2013; Chiaradia et al., 2021). Interestingly, utilization of EVs derived from oxidative stress increased viability and exerted anti-apoptotic effects on target cells (Mas-Bargues et al., 2023).

In this analysis, we present findings indicating the significant involvement of EVs in cellular responses to oxidative stress. The secretion and content of EVs are notably influenced by the redox status of the parent cell. However, there is no consistent set of biochemical characteristics defining EVs released during oxidative stress, as their molecular compositions vary based on specific oxidative stressors, cell types, and EV subtypes examined. Nonetheless, the majority of reviewed studies demonstrate that EVs released under oxidative stress conditions exhibit heightened levels of oxidatively modified molecules, as well as an enrichment of transcription factors, RNAs, and enzymes involved in regulating cellular responses to oxidative stress.

3.3 Mechanical stress during isolation and separation

Obtaining and isolating EVs from biological samples is an important step in studying their functions and potential applications. However, mechanical forces can occur during the isolation process that negatively affect the secretion and composition of EVs, as well as cell survival, growth and differentiation.

Various isolation techniques, such as ultracentrifugation, size exclusion chromatography, and precipitation methods, involve mechanical steps that can impact EV integrity. High centrifugal forces or shear stress during ultracentrifugation, for example, may lead to EV damage or aggregation, altering their properties (Linares et al., 2015;

Konoshenko et al., 2018), resulting in the release of intravesical contents or changes in the surface marker expression. Similarly, microfluidic-based techniques involving shear forces can lead to the disruption of EV membranes or the aggregation of EVs, affecting their functional characteristics (Wu et al., 2022b). Exclusion chromatography has been shown to result in low purity and yield of EVs and lack of specificity (Gámez-Valero et al., 2016).

In addition, the choice of isolation method may affect the composition of the isolated EVs. Different methods may preferentially isolate certain subtypes of EVs based on size, density, or surface markers, which may skew the results of subsequent analyses of EV cargo and function (Taylor & Shah, 2015; Veerman et al., 2021; Abyadeh et al., 2024). Moreover, the composition of EVs, including their cargo of proteins, nucleic acids, and lipids, can be altered due to mechanical stress during isolation and separation. Changes in the content and distribution of specific molecular species within EVs may influence their biological activity and their potential as biomarkers or therapeutic agents. For example, the use of ultrafiltration method results in non-specific binding of EVs to the membranes and hence a reduction in the number of EVs extracted from the cells (Vergauwen et al., 2017; Zhang et al., 2020b). It has been reported that the use of density gradient ultracentrifugation results in significant losses in the amount of EVs (Van Deun et al., 2014). The method of precipitation of EVs involves the use of hydrophilic polymers such as polyethylene glycol. However, the separation of such polymers after precipitation from EVs can strongly distort the results of further studies. And even if the EVs are separated from the polymers, they may be contaminated with other precipitated substances such as plasma lipoproteins, vesicles other than EVs, protein impurities, and non- EV-associated extracellular nucleic acids, which would also prevent their use for further studies and clinical applications (Martins et al., 2018; Clos-Sansalvador et al., 2022). Similarly, ultrafiltration of EVs leads to a decrease in their number, filter clogging and protein contamination of the sample (Clos-Sansalvador et al., 2022).

Understanding the effects of mechanical forces on the secretion and composition of EVs during isolation and separation is a critical area of research of importance for both basic research and clinical applications and is crucial for the development of standardized protocols that preserve the integrity and functionality of EVs. Strategies to minimize mechanical effects, such as optimizing centrifugation parameters, adjusting flow rates in microfluidic devices, and using gentle filtration techniques, are necessary to mitigate negative effects on EVs (Thompson & Papoutsakis, 2023). This knowledge may lead to the development of novel isolation methods that minimize mechanical disruption or even exploit mechanotransduction mechanisms to promote the release of EVs with desired characteristics. This, in turn, will allow the potential of EVs in biomedicine to be fully explored.

3.4 Osmotic stress

Osmotic stress disrupts the cellular homeostasis by altering solute concentrations inside and outside of cells, leading to changes in water movement across cell membranes. This phenomenon can significantly affect cellular functions, including modulation of EV secretion and composition.

Upon encountering osmotic stress, such as hyperosmotic or hypoosmotic conditions, cells initiate complex adaptive mechanisms to maintain homeostasis. These mechanisms often involve adjustments in vesicle trafficking and secretion (Abramowicz et al., 2019). Consequently, osmotic stress can regulate the release of EVs, affecting both their abundance and their molecular cargo. For example, under hypertonic conditions,

renal epithelial cells have been observed to increase the secretion of EVs enriched in proteins critical for stress response pathways (Rasmussen et al., 2019). This response can occur through a variety of pathways, including activation of ion channels, changes in membrane fluidity, and cytoskeletal rearrangements. EVs produced under these conditions can act as vehicles for signaling molecules, facilitate cellular adaptation to osmotic challenges, and mediate intercellular communication. Conversely, hypoosmotic stress, characterized by reduced extracellular osmolarity, has been associated with increased release of small EVs (sEVs) and changes in their cargo, potentially altering their biological effects (Walbrecq et al., 2020).

In addition, osmotic stress can affect the molecular composition of EVs, thereby modulating their biological functions. Variations in osmotic pressure can lead to selective packaging of biomolecules within EVs, altering their signaling capabilities and effects on target cells. Research suggests that osmotic stress can alter the levels of stress-responsive proteins, microRNAs, and lipids in EVs, reflecting the cellular stress response and potentially mediating adaptive mechanisms in recipient cells (Abramowicz et al., 2019, Wu et al., 2021). Osmotic stress can alter protein expression and localization, resulting in the enrichment or depletion of specific proteins in secreted EVs (Barron et al., 1986; Clark & Parker, 1984). In addition, osmotic stress can alter the lipid composition of EVs, including the ratio of saturated to unsaturated fatty acids and the presence of certain lipid classes, affecting their membrane properties and functionality (Aziz & Larher, 1998). Moreover, an increase in the RNA content of EVs, including mRNAs and non-coding RNAs such as microRNAs, has been observed under osmotic stress, potentially modifying gene expression and cellular regulation in recipient cells (Belliveau & Papoutsakis, 2023).

Modulation of EV secretion and composition by osmotic stress has important implications for several physiological and pathological processes. It can affect the secretion and composition of immune-related EVs, thereby influencing immune cell functions (Nolte-'t Hoen, 2018). In addition, osmotic stress has been implicated in several diseases, including cancer (Sheta et al., 2023), neurodegenerative disorders (Niedzielska et al., 2016), obesity-related diseases (Matsuda & Shimomura, 2013), and cardiovascular disease (Cheng et al., 2022). The alterations in EV secretion under osmotic stress conditions may contribute to the pathogenesis of these diseases.

Understanding how osmotic stress affects EVs sheds light on the regulation and function of these vesicles in health and disease. However, further studies are required to elucidate the underlying mechanisms of these effects and their potential therapeutic applications.

3.5 Transportation stress: timing of EV delivery from the incubator to the patient in a physiologically non-relevant gas environment

The successful translation of EV-based therapies from the laboratory to clinical settings hinges on the effective transport of these delicate entities. One critical aspect of this process is the transportation of EVs from the incubator, where they are cultured and harvested, to the patient, often requiring transit through environments that may not mimic physiological conditions (Gimona et al., 2017).

During transportation, EVs are immediately subjected to several types of stress, which can have profound implications for their viability and functionality. These stressors include mechanical, thermal, and chemical factors, each of which can independently compromise the integrity of EVs. However, the real challenge arises from the synergistic effects of these stressors acting together, leading to a compounded impact that is often

greater than the sum of their individual effects (Qin et al., 2020). As noted earlier, one of the most significant stressors is temperature fluctuation. EVs are typically cultured and stored at specific temperatures to maintain their stability and bioactivity, often around 4°C for short-term storage or in controlled incubators for optimal growth. When these vesicles are transported, any deviation from these ideal temperature conditions can lead to detrimental effects (Sivanantham & Jin, 2022). For instance, exposure to elevated temperatures can accelerate lipid oxidation and protein denaturation, destabilizing the lipid bilayer that encases the EVs. This destabilization can result in the leakage of vesicle content, including proteins, RNA, and other bioactive molecules, thereby altering their intended therapeutic effects (Schulz et al., 2020).

Pressure changes during transport can present substantial challenges to the stability and functionality of EVs. One of the primary concerns is physical deformation, where the vesicles may undergo shape changes due to external forces acting on them. This deformation can compromise the integrity of the lipid bilayer, potentially leading to the rupture of the vesicles (Thompson & Papoutsakis, 2023). When the lipid bilayer is disrupted, the carefully encapsulated cargo—comprising proteins, lipids, and nucleic acids—may be released prematurely or become inaccessible for therapeutic delivery. This loss of cargo not only diminishes the biological efficacy of the EVs but can also lead to unintended consequences if the released molecules have biological activity that could interfere with surrounding tissues (Melling et al., 2019; O'Brien et al., 2022). Moreover, the mechanical forces exerted during transport can promote aggregation among individual EVs. Under normal physiological conditions, EVs maintain a stable dispersion, allowing them to function effectively in cellular communication and therapeutic delivery (Thompson & Papoutsakis, 2023). However, when subjected to mechanical stress, EVs can clump together, forming larger aggregates. This aggregation can hinder their ability to interact with target cells, as larger complexes may not efficiently bind to specific receptors or may be cleared more rapidly from circulation by the immune system (Wang et al., 2019). Consequently, the distinct biological activity of individual EVs is compromised, and their therapeutic potential is significantly reduced. Additionally, the aggregation of EVs can lead to a decrease in their overall bioavailability. When EVs aggregate, they may become trapped in the microenvironment of the transport medium or within the delivery system, preventing them from reaching their intended target cells. This is particularly concerning in therapeutic applications, where the precise delivery of EVs to specific tissues or cells is crucial for achieving the desired therapeutic outcomes (Linares et al., 2015). The inability to effectively deliver the EVs can result in suboptimal treatment responses and limit the overall effectiveness of EV-based therapies.

Gas composition is another critical factor that can impact EV stability during transport. EVs are typically surrounded by a carefully regulated mixture of gases, which includes lower levels of oxygen, often existing in a more hypoxic state. This physiological context is crucial because it helps maintain the integrity and functionality of the EVs, allowing them to perform their roles in cellular communication and cargo delivery effectively (Bister et al., 2020). When EVs are transported in a physiologically irrelevant gas environment, such as ambient air, they encounter significantly higher concentrations of oxygen than they would in their natural surroundings. This exposure can lead to several adverse effects. Elevated oxygen levels can induce oxidative stress, a condition characterized by an imbalance between the production of ROS and the body’s ability to detoxify these harmful byproducts. In addition to these biochemical changes, the higher oxygen levels in ambient air can also affect the biophysical properties of EVs. The stability of the vesicles may be compromised, leading to changes in their size, charge, and surface characteristics. These alterations can impact the EVs' interactions with target cells, as the

ability of EVs to bind to specific receptors is often dependent on their surface composition. If the vesicles aggregate or change in size due to oxidative stress, their capacity to effectively deliver therapeutic payloads can be significantly hindered (Chiaradia et al., 2021).

Consequently, transport stress represents a serious problem that cannot be avoided by simply changing the conditions and methods of transport. The various stresses that EVs experience during transportation do not act independently; instead, they can have synergistic effects that exacerbate the overall stress experienced. For example, mechanical stress may increase the susceptibility of EVs to oxidative damage, while thermal stress can impair their ability to recover from mechanical injuries. This interconnectedness of stresses means that addressing one type of stress may not be sufficient to ensure the integrity and functionality of the EVs. Moreover, the combined effects of these stresses can lead to alterations in the cargo of EVs. Changes in the protein or RNA content can directly impact the therapeutic potential of EVs, potentially leading to reduced efficacy or unintended biological responses once administered to the patient.

To mitigate transport stress, it is essential to establish optimized protocols that preserve the stability and functionality of EVs during transit. This may involve using specialized transport media that maintain a controlled environment, minimizing exposure to oxygen and other harmful elements (Kusuma et al., 2018). Additionally, employing temperature-controlled transport systems can help maintain the optimal conditions for EV preservation. Furthermore, it is crucial to consider the timing of transport. Delays in delivery can exacerbate stress on EVs, leading to decreased efficacy once administered to patients. Therefore, streamlining logistics to ensure rapid and efficient transport is vital to the success of EV-based therapies (Aubertin et al., 2021).

4. Quality Control Problems of EVs in the Process of Biotechnological Production

As potential therapeutic agents and diagnostic tools, EVs carry significant promise; however, their successful translation into clinical practice hinges on addressing these quality control issues comprehensively (Stawarska et al., 2024). Key considerations include establishing clear characterization practices, developing methods for mass production, and ensuring reproducibility and safety (Ingato et al., 2016). The complexity of EV production encompasses various stages, from upstream and downstream manufacturing processes to post-processing technologies such as surface bioengineering. Regulatory affairs pose additional challenges, as specific guidelines for EV-based medicines are lacking (Stawarska et al., 2024). To advance EVs towards clinical applications, stringent quality control measures, standardization of manufacturing processes, and compliance with FDA requirements are essential. Addressing these challenges comprehensively is crucial for the successful translation of EVs into safe, effective, and reproducible therapeutic and diagnostic tools (Davies & Rafiq, 2017; Cheng & Kalluri, 2023).

4.1 Complexity of production systems

EVs can be derived from a variety of biological sources, including cell cultures, tissues, and body fluids. Each source presents its own set of challenges regarding the consistency and quality of the EVs produced (Gudbergsson et al., 2016). For instance, the type of cells used, their growth conditions, and the methods employed for EV isolation can all influence

the yield and characteristics of the EVs (Mas-Bargues & Borrás, 2021). Variability in these factors can lead to differences in the size, composition, and biological activity of the EVs, complicating efforts to achieve a standardized product (Gandham et al., 2020). Therefore, establishing a controlled production environment and optimizing culture conditions are essential for minimizing variability and ensuring reproducibility.

4.2 Characterization techniques

Effective quality control of EVs requires the implementation of robust characterization techniques to assess their physical, chemical, and biological properties (Bağcı et al., 2022). However, the current landscape lacks standardized methods for EV characterization, leading to discrepancies in data interpretation across different laboratories. Common techniques such as nanoparticle tracking analysis, electron microscopy, and western blotting can provide insights into EV size, morphology, and protein content, but their application must be harmonized to ensure consistent results (Gardiner et al., 2016; Camacho et al., 2017; Royo et al., 2020). Developing and adopting standardized protocols will be crucial for accurately characterizing EVs and confirming their identity and purity.

4.3 Isolation and purification processes

The isolation and purification of EVs are critical steps that can significantly impact their quality. Various methods, including ultracentrifugation, filtration, and affinity-based techniques, are employed to isolate EVs from complex biological mixtures (Liangsupree et al., 2021). However, these processes can inadvertently co-isolate contaminants such as proteins, lipids, and nucleic acids, which may compromise the integrity and biological functionality of the EVs (Simonsen et al., 2017; Brennan et al., 2020). Quality control measures must include thorough validation of isolation protocols to ensure that the final EV product is free from impurities that could affect its safety and efficacy (Patel et al., 2024).

4.4 Stability and storage conditions

Once produced, the stability of EVs during storage and transport is a significant quality control concern. EVs are sensitive to environmental factors such as temperature, pH, and exposure to light, which can lead to degradation or loss of bioactivity (Yuan et al., 2021). For example, prolonged exposure to ambient air can increase oxidative stress, resulting in lipid peroxidation and protein modifications that impair the functionality of EVs (Kusuma et al., 2018). To mitigate these risks, it is essential to establish optimal storage conditions and shelf-life parameters through rigorous stability testing. This may involve determining the best storage temperature, the use of cryoprotectants, and the development of appropriate packaging solutions that minimize exposure to adverse conditions (Sivanantham & Jin, 2022). Understanding and standardizing EV storage methods are crucial for maintaining EV quality and stability, ultimately facilitating their widespread clinical application.

4.5 Biological activity assessment

Evaluating the biological activity of EVs is critical for quality control, as their therapeutic efficacy is directly linked to their functional properties. However, the lack of standardized functional assays poses a challenge in consistently assessing the bioactivity of EVs. Quality control protocols should incorporate a variety of assays that measure the ability of

EVs to deliver their molecular cargo, modulate target cell behavior, and elicit desired biological responses (Nguyen et al., 2020; Bonsergent et al., 2022). To address this, researchers have developed various approaches. The detectEV assay, an enzymatic-based method, enables quantitative measurement of EV bioactivity and integrity in small samples. For clinical applications, EV assays must meet ISO 15189 standards, requiring thorough validation of performance characteristics (Adamo et al., 2025). These efforts aim to establish reliable methods for assessing EV therapeutic efficacy, ensuring batch-to-batch reproducibility, and facilitating their transition into clinical use (Ayers et al., 2019; Nguyen et al., 2020; Chua et al., 2022).

4.6 Secretome analysis

The manufacturing of EVs for clinical applications requires standardization of bioprocessing parameters, as their composition and functionality can be influenced by various factors including cell source, culture conditions, and isolation methods (Phelps et al, 2018). Changes in the secretome do not necessarily result in alterations to the phenotypic characteristics of EVs. Consequently, the integration of advanced technologies for the qualitative and quantitative analysis of EVs is imperative for clinical applications (Shao et al., 2018). Secretome analysis is crucial for monitoring batch-to-batch variability in EV production, ensuring regulatory compliance and therapeutic effectiveness. Studies have demonstrated that Good Manufacturing Practice (GMP)-compliant production can achieve high batch-to-batch consistency in cellular secretomes (Laggner et al., 2020). Secretome analysis facilitates the assessment of the purity and quality of EVs, thereby enabling confirmation of their composition, function and therapeutic potential during production (Gualerzi et al., 2019). Furthermore, the analysis of EVs` secretomes has the potential to facilitate their engineering to carry specific cargo molecules. This, in turn, could enhance the stability and efficacy of drugs and minimize off-target effects (Rafieezadeh & Rafieezadeh, 2024). Developing standardized methods for assessing batch-to-batch consistency is crucial for advancing EV products towards clinical applications (Korchak et al., 2023). These findings underscore the importance of rigorous quality control measures in secretome-based therapeutic development.

4.7 Regulatory compliance

As EVs move closer to clinical use, compliance with regulatory standards becomes increasingly important. Regulatory agencies require thorough documentation of the production process, including detailed descriptions of quality control measures, validation of analytical methods, and adherence to GMP (Ayers et al., 2019; Stawarska et al., 2024). Meeting these requirements is essential for ensuring that EV products are safe for human use and have consistent therapeutic efficacy. Therefore, establishing a robust quality management system that aligns with regulatory guidelines is vital for the successful commercialization of EV-based therapies (Cheng & Kalluri, 2023).

5. Discussion

The biotechnology of producing EVs for therapeutic applications is a rapidly growing field driven by the promising potential of EVs to revolutionize the treatment of various diseases, including cancer, neurodegenerative disorders, and cardiovascular diseases. These nanoscale vesicles, which are naturally released by cells, play critical roles in intercellular

communication and can carry a diverse array of bioactive molecules, including proteins, lipids, and nucleic acids (Salmond & Williams, 2021). However, the development of EV-based therapeutics is hindered by a number of challenges that need to be addressed to ensure scalability, consistency, and safety of EVs production.

One of the major challenges of EV production is the lack of standardized methods for EV isolation, storage, purification, characterization, and transportation (Nelson et al., 2020). Currently, various techniques are employed to isolate EVs, such as ultracentrifugation, filtration, and precipitation, but these methods often yield inconsistent results and can impact the integrity and functionality of the EVs (Gardiner et al., 2016). Furthermore, stress factors significantly alter cellular secretion, influencing the cargo and composition of EVs. For instance, environmental stressors like hypoxia, nutrient deprivation, and oxidative stress can lead to the secretion of EVs with altered molecular profiles, which may result in inadequate therapeutic effects, adverse events, and side effects in cell therapy applications (O'Neill et al., 2019). The administration of a cellular product produced by stressed cells can consequently alter the cellular niche, potentially leading to unintended consequences.

It is believed that alterations in the secretome— the collection of proteins and other molecules secreted by cells—can cause these side effects, even if the cellular phenotype remains unchanged according to traditional markers (Wallis et al., 2020). This highlights a critical gap in current manufacturing practices, as secretome analysis is not typically performed in the production of cellular products due to its high cost and complexity. As a result, manufacturers may overlook significant changes in the EV profile that could compromise the therapeutic efficacy and safety of the final product.

Therefore, it is imperative to be attentive to the various factors that may induce changes in EVs during production. Understanding the mechanisms underlying these changes provides a basis for developing strategies to mitigate their negative effects. For example, implementing rigorous quality control measures and standard operating procedures that account for environmental and cellular stressors can help ensure the production of high-quality EVs. Additionally, investing in advanced analytical techniques to assess the secretome and EV cargo could lead to a more comprehensive understanding of how these factors influence therapeutic outcomes. By addressing these challenges, the field can move closer to realizing the full potential of EVs as a transformative therapeutic modality.

6. Conclusions

Paracrine factors are integral to the orchestration of cellular communication and function. The importance of changes in extracellular vesicles following the cellular response to stress has been emphasized. Various stressors, in particular temperature, oxidative, mechanical, osmotic, and birth stress, occurring during the biotechnological production of extracellular vesicles, alter the release and content of extracellular vesicles in stressed cells. Biomechanical forces during production and isolation can impact EV biogenesis, cargo, and biological activity. Preservation and storage methods are critical for maintaining EV stability, with -80°C storage showing promise, although effects may vary depending on the sample source. Environmental stressors can also influence EV composition and function, with stem cell-derived EVs showing the ability to adapt their cargo, particularly heat-shock proteins, in response to different microenvironments. This, in turn, can lead to failure of cell therapy due to altered paracrine activity of the cells used, even if the cells retain their

phenotypic characteristics. Therefore, understanding the underlying mechanisms and modification of paracrine signaling is important in improving the efficacy of cell therapy.

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8. Conflicts of Interest

The authors declare no conflicts of interest.

ORCID

Victoria Shestakova https://orcid.org/0000-0002-4129-8750

Ekaterina Smirnova https://orcid.org/0000-0001-7533-760X

Lyudmila Komarova https://orcid.org/0000-0003-0491-4486

Andrey Kotlyarov https://orcid.org/0000-0003-2766-7895

Elena Yatsenko https://orcid.org/0000-0003-0869-0133

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