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REVIEW

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Engineered extracellular vesicles: a breakthrough approach to overcoming sperm cryopreservation challenges

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Abstract

Freezing sperm for artificial insemination (AI) has been common for decades, but this method causes damage to sperm, which affects its viability and fertility. Various strategies have been used to treat sperm cryopreservation complications, but their results are still not satisfactory. The latest approach in this field is using extracellular vesicles (EVs). The role of EVs in reproduction, such as spermatogenesis, sperm capacitation, and fertility has been proven. EVs can deliver proteins, lipids, nucleic acids, and other molecules to the sperm for repair. The EVs carry proteins, lipids, nucleic acids, and other molecules, which could be involved in sperm quality, functionality or fertility. The application of EV derived from animal and human cell sources for cryoinjury treatment indicates the improvement of sperm quality after freeze-thawing. In addition, different EV engineering methods regarding various EV cargos could be more influential for cryopreserved sperm treatment because they could provide EV customized content for delivering to cryoinjured sperm, according to their unique needs to enhance viability and fertility. In this review, first, we reminded the sperm cryopreservation complications, and next explained the conventional and modern strategies for overcoming them. Then, we have pointed out the role of EV in sperm development and the following mentioned the study results of using EV from different cell sources in sperm cryoinjuries repair. Also, we suggested several predisposing molecules (including microRNAs and proteins) for EV engineering to treat sperm cryopreservation complications by indirect engineering procedure, including genetic manipulation and incubation with therapeutic molecules, and direct engineering procedure, including electroporation, sonication, incubation, saponin permeabilization, extrusion, CaCl2-heat shock, and freeze/thawing. Finally, we discussed the limitations of EV application and ethical considerations in this context. In the meantime, despite these limitations, we pointed out the promising potential of the EV engineering strategies to reduce infertility rates by helping to overcome sperm cryopreservation challenges.

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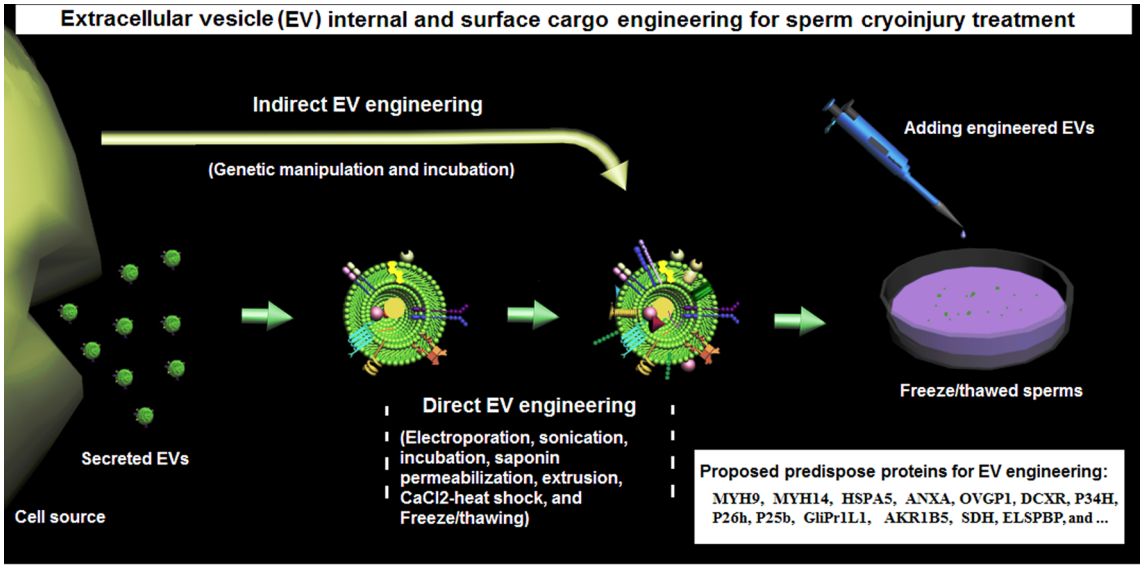
Graphical abstract



Keywords Extracellular vesicle engineering, EV therapy, Fertility preservation, Cryopreservation, Sperm cryoinjury

Background

Artificial insemination (AI) is an important technique for addressing infertility in humans under specific circumstances, such as treatment for cancer, vasectomy, or surgical interventions for infertility, and in animals for ex-situ wildlife conservation [1, 2]. However, using AI is hampered by poor cryopreserved sperm [2]. Sperm cryodamage is attributed to factors such as oxidative stress, osmotic stress and temperature fluctuations [3, 4]. Cryopreservation could include various structural damage to sperm, such as the plasma membrane, acrosome damage, nucleus, mitochondria and vital molecules including proteins, lipids and nucleic acids (mRNAs, microRNAs, and DNA) damage [3, 5]. These freeze-thawing (FT) injuries decrease the quality, fertility of sperm and pregnancy rates [6–8]. Consequently, researchers have been striving for years to find solutions to enhance the viability and fertility of sperm post-cryopreservation. These approaches can be categorized into three procedures: before and during cryopreservation for prevention of sperm cryoinjury, and after cryopreservation for healing cryoinjured sperm. Currently, a range of methods are employed to prevent sperm cryoinjury. These include traditional approaches like cryoprotectants and membrane stabilizers as well as innovative techniques such as applying mild sub-lethal stress and inducing a magnetic field prior to freezing [5]. Although these strategies are effective in improving the results, they don't seem to be sufficient for achieving better post-thaw sperm quality and there is a need for new strategies [4]. In recent years, the biological roles of extracellular vesicles (EVs) have become better understood, particularly their functions in cell communication and their therapeutic effects [9, 10] and engineering potential [11]. EVs are nano-sized membrane vesicles that are released by a wide variety of cells into the extracellular space and body fluid. They are abundant in biological fluids such as blood and semen. EVs have three main subtypes including exosome, microvesicle, and apoptotic body that have different sizes, contents and formation mechanisms [12]. However, the International Society for Extracellular Vesicles (ISEV) has recommended using the general term extracellular vesicle [13]. Exosomes have a diameter of approximately < 100 nm and are released by the fusion of endosomal compartments to the plasma membrane (exocytosis), while microvesicles are around 100–1000 nm and they are secreted by cell membrane budding. Apoptotic bodies are released by apoptotic cells of approximately 500–5000 nm diameter. The roles of EVs play a crucial role in cell-to-cell communication the regulation of biological processes in both physiological and pathological conditions. For EV characterization transmission electron microscope (TEM), dynamic light scattering (DLS) and internal markers expression such as Alix, TSG101 and surface markers expression such as CD63 and CD81, are used. Additionally, methods like differential ultra-centrifugation, density gradients, precipitation, filtration, size exclusion chromatography and immunoisolation are used for the separation and concentration of EVs [13, 14]. New studies show that EVs are emerging significant players in next-generation

diagnostic and therapeutic platforms [15, 16]. EVs contain various superficial and internal cargo, including proteins, lipids and nucleic acids, which can be delivered to recipient cells to influence their functions [3, 5]. EVs can create biological responses by contact and binding to the membrane receptor on the surface of the recipient cell for triggering intercellular signaling pathways, direct membrane fusion with target cells or internalization via endocytosis by the recipient cell [17]. Today, the role of EVs in various biological processes has been significantly elucidated, for example, in reproduction including spermatogenesis, capacitation, fertility, pregnancy, and even childbirth, and also in reproductive regulation and pregnancy-related diseases [18, 19]. It has been proved that exosomes can interact with sperm and transfer their contents into the sperm cells [20]. In recent years, EVs have been employed to enhance sperm vitality [21] and fertilizing ability [22]. In this section, we begin by discussing the cryoinjuries in sperm sustain during cryopreservation, followed by an overview of traditional and innovative approaches to mitigate these effects. Finally, we explore the potential of EV engineering to address sperm cryopreservation challenges and propose some recommendations.

Sperm cryopreservation injuries (sperm cryoinjury)

In humans, freezing of sperm has led to thermal shock with the appearance of intracellular and extracellular ice crystals, cellular dehydration and osmotic shock [23]. Cryopreservation can harm essential sperm components, affecting various aspects such as the plasma membrane (integrity, ion channel alterations, lipid composition changes and membrane fluidity) [2, 5]. Plasma membrane damage can result not only from the equilibration process with cryoprotectant media but also from events that occur during cooling [24, 25]. On the other hand, high levels of DNA fragmentation have been observed following sperm cryopreservation [26], due to increased oxidative stress on DNA, therefore, further research is needed to fully understand the underlying mechanisms [27]. However, recently, oxidative stress has emerged as the most likely suspect in sperm DNA damage caused by freezing [28]. Mitochondrial dysfunction following cryopreservation is caused by the generation of reactive oxygen species (ROS). This is likely due to the disruption of oxidative phosphorylation and the inactivation of antioxidant enzymes [29], although it may be linked to damage to the inner and outer mitochondrial membranes as well as its DNA [30]. After freezing, protein degradation, phosphorylation and carbonylation have also been observed [31]. For example, cryopreservation has been found to induce abnormal modifications in protein phosphorylation in ram sperm [32]. Furthermore, cryopreservation can modify sperm function by influencing the many protein expression levels in human, such as glucose-6-phosphate isomerase (GPI), lactate dehydrogenase B (LDHB), alcohol dehydrogenase 5 (ADH5) and phosphoglycerate mutase 1 (PGAM1) [33, 34]. These proteins are involved in major sperm characteristics, including motility, plasma membrane integrity, energy metabolism, capacitation and sperm–oocyte fusion after cryopreservation [33, 35]. Besides, the cryopreservation can lead to the release of associated proteins with the nucleus, mitochondrial structure or metabolism, the cytoskeleton and the cytosol into the extracellular fluid [36]. Cryopreservation also increases lipid peroxidation, triggers premature acrosome exocytosis, degrades mRNAs and microRNAs (miRs) [2, 5].

It has been proved that using different cryopreservation media has adversely impacted sperm capacitation, motility and morphology after thawing [37, 38]. Also, studies have demonstrated that bovine, ovine and goat sperm cryopreservation significantly reduces sperm parameters such as acrosome integrity, enzyme activity, motility and fertility [39].

Studies have clearly shown that oxidative stress is increased in cryopreserved sperm, leading to epigenetic changes (including histone modifications, DNA methylation, and non-coding RNAs) [40]. These modifications, in addition to reducing sperm motility and fertilization potential, affect embryo development and can be passed down to offspring [41–43].

Conventional and novel strategies for sperm cryoinjury prevention and treatment

Strategies for preventing sperm cryoinjury have been categorized into conventional and novel approaches, which have recently been thoroughly reviewed [5]. These strategies encompass a range of methods that will be reviewed below. A sperm extender requires suitable osmolarity and buffering capacity at an appropriate pH [42]. Also, cryopreservation extenders for sperm may contain penetrating cryoprotectants like glycerol or dimethyl sulfoxide (Me2SO) as well as non-permeating cryoprotectants such as milk, egg yolk, soybean lecithin/raffinose or combinations of these [5, 44, 45].

Conventional strategies encompass a variety of methods [5]. For instance, glycerol and ethylene glycol serve as traditional penetrating cryoprotective agents (CPAs) that help protect sperm during cryopreservation. However, high doses of glycerol can be detrimental to the plasma membrane and the overall function of the cells. Toxic levels of glycerol can alter sperm tail movement by affecting the polymerization and depolymerization of α and β tubulins [46]. Additionally, non-permeating cryoprotectants like sugars can provide colligative protection outside the cells [47]. In contrast, permeating CPAs cause changes in water volume during equilibration, whereas

non-permeating solutes lead to significant long-term volume changes that can damage membranes and cytoskeletal structure [47]. Notably, egg yolk and soybean lecithin have been successfully used as low-density lipoproteins to protect against sperm freeze-thaw injury [48–51]. During semen cryopreservation, high amounts of polyunsaturated fatty acids (PUFAs) in sperm are exposed to lipid peroxidation because of the tremendous production of ROS. This oxidative stress is controlled by diverse intrinsic antioxidant protective systems in sperm and seminal plasma (SP) containing enzymatic antioxidants, and non-enzymatic antioxidants [52]. Besides, some studies have indicated that vitamin E or C supplements may have beneficial effects on sperm cryopreservation [53, 54].

In addition to providing energy resources, sugars regulate osmotic pressure, decrease relative ion concentrations, stabilize proteins and phospholipid bilayers during sperm cryopreservation [55]. Trehalose and its combinations with other additives are commonly used as a non-permeating cryoprotectant. This sugar can help stabilize cell plasma membranes [56]. Furthermore, it is reported that other sugars are also successfully applied for sperm cryopreservation. Some sugars in cryopreservation media recipes have shown different results in some species. For instance, while raffinose is beneficial for mice, it negatively affects chicken sperm [57]. Additionally, Chanapiwat et al. (2012) found that lactose is more effective than fructose, glucose and sorbitol in enhancing sperm recovery in boars when using an egg yolk-based medium [58]. In recent years, cholesterol-loaded cyclodextrins or cholesterol-cyclodextrin complexes (CLC) have been used before cryopreservation to improve sperm plasma membrane and acrosomal integrity, and increase osmotic tolerance [59–61]. In addition, CLC pretreatment Addra gazelle sperm before freezing prevented the loss of metabolism and proteins associated with sperm motility and after thawing motility [62]. Although these effects were not observed in some species, such as sheep [38].

It is acceptable that diet can affect reproductive enhancement. For example, it has been shown that the dietary content of fish oil (3%) alters the semen lipid composition, while not enhancing the post-thaw semen quality of ram spermatozoa [63], while another study found that including fish oil (3% of the diet's dry matter) significantly improved several post-thaw ram sperm parameters [64]. Furthermore, the warming and thawing process can influence sperm quality. To avoid harmful recrystallization during warming, samples that were cooled quickly should also be warmed rapidly. For example, it has been demonstrated that faster warming leads to improved motility parameters in boar sperm, with the effects being influenced by the interaction between warming rates and glycerol concentrations [65].

To enhance the outcomes of sperm cryopreservation, alongside traditional methods, innovative techniques have been developed. These approaches include various strategies [5]. Research indicates that inducing mild, non-lethal stresses before freezing, such as osmotic [66], hydrostatic [67] or oxidative stress [68] improves sperm resistance to cryo-damage, likely by inhibiting the apoptotic pathway [5]. Additionally, it has been shown that in certain species, magnetizing extenders using a high magnetic field can reduce hexagonal ice crystal formation and protect sperm from damage [69, 70]. Moreover, increasing attention is being given to freeze-drying (lyophilization) for sperm preservation [71]. Even though freeze-dried sperm may not remain viable, the chromatin remains intact, making them suitable for use in intracytoplasmic sperm injection [72]. Besides, single-layer centrifugation (SLC) application as a colloid-based procedure can separate strong sperm from the rest, thus, it can select viable sperm with higher persistence against post-thawing cryo-damages [73]. Recently, studies have demonstrated that adding some nanoparticles (NPs) to cryopreservation media can improve fertility results [74]. Applied nanoparticles in this field can be divided into two groups, including inorganic (such as cerium oxide (CeO_2) and selenium nanoparticles (SeNPs) and organic nanoparticles (such as nanoliposome and extracellular vesicles). It has been shown that CeO2 NPs conserve the integrity of DNA and plasma membranes in ram sperm by acting as ROS scavengers against oxidative stress [75]. Additionally, SeNPs also act as a scavenger of ROS, by the addition of vitamin E into rooster semen extender, which could lead to enhancing total antioxidant capacity (TAC) level and decreasing malondialdehyde (MDA) concentration [76]. Furthermore, it has been proved that lecithin nanoliposome extender, in addition to decreasing apoptotic sperm percentage, could enhance post-thawing ram sperm quality [77]. In addition, it has been demonstrated that the combination of vitamin E with 1.0 glutathione peroxidase (GPX) in a lecithin-nanoliposome-based extender enhanced post-thawing bull sperm viability and blastocyst formation rate [78]. In recent decades, EV as a natural nanoparticle group that attracted much attention due to their important role in intracellular communication. Exosomes, microvesicles and apoptotic bodies are the main subtypes of EVs. They could have many different cargoes such as proteins, lipids and nucleic acids [12]. The male genital tract has shown particulate location diversity [79] and cryo-electron microscopy has revealed a variety of EVs in human semen [80]. Studies confirmed that EVs play a role in regulating reproductive tract activity [18, 19]. Recently, many studies have highlighted the role of EVs in regulating mammalian reproduction, including follicular and early embryonic development, the implantation process of embryos and

male reproductive function [19, 81, 82]. In addition, it has also been proven that EVs can be used in the diagnosis and treatment of pregnancy-related diseases [19]. The authors have emphasized the role of proteins and miRNAs among the various compounds in EVs [83, 84], demonstrating their importance in regulating biological functions within the mammalian reproductive system. However, the role of other biomolecules in EVs still requires further investigation. Du et al. showed that exosomes from boar SP help maintain sperm function by integrating into the sperm membrane [85]. Another study has demonstrated that co-incubation of spermatozoa with porcine oviductal EVs increased frozen sperm survival [86]. In addition, it has been shown that canine adipose-derived MSCs can effectively improve the viability and fertility of cryopreserved sperm [87]. Altogether, these studies indicate that EVs derived from various cell sources have significant potential to improve the quality and fertility of cryopreserved sperm.

Mechanism of extracellular vesicles (EV) effect in sperm maturation and function

Sertoli cells, the epididymis and the prostate play a crucial role in spermatogenesis, sperm maturation, and functioning. New findings show the involvement of EVs in the testis in the communication between the Sertoli cell, germ cells and spermatozoa [88]. It is observed that Sertoli-derived exosomes (SC-EXO) as guardians of germ cells ameliorate the negative impact of electromagnetic field-induced oxidative stress in mouse spermatogonial stem cells (SSCs) [89]. However, their effects go beyond this, as exosomes appear to mediate the interaction of Sertoli cells with SSCs and Leydig cells. Exosomes as a part of Sertoli cell paracrine secretion, besides hormones and growth factors play an important role in spermatogenesis. Tian et al. confirmed that SC-EXO participates in the regulation of the spermatogenesis process. They revealed that exposure to SC-EXOs increases promyelocytic leukaemia zinc finger protein (PLZF), mouse vasa homologue (MVH) and Stimulated by retinoic acid 8 (STRA8) expression in SSCs cultures, simultaneous with a reduction of inhibitor of DNA binding4 (ID4) and GDNF family receptor α1 (GFRA1) levels [90]. Recent studies have shown that exosomes released by Sertoli cells not only potentially promote the survival of Leydig cells by crossing the blood-testis barrier but also regulate the differentiation of SSCs through miR-486–5p, which binds to PTEN in mice [91, 92]. In addition, delivering miR-24-3p inhibitor into germ cells enhances sperm mobility by Sertoli-derived EVs crossing the blood-testis barrier [93]. These findings suggest that miRs carried by Sertoli-derived EVs effectively mediate the regulation of spermatogenesis and sperm mobility. Given the important role of Sertoli cells in spermatogenesis regulation, further research is needed to more accurately elucidate the mechanism of action of Sertoli-derived EVs components, specifically miRs in the spermatogenesis process regulation.

The epididymis is a tube tightly coiled on the testis surface, carrying sperm from the seminiferous tubules to the ductus deferens. One of its important roles is to help sperm post-testicular maturation [18, 94]. The principal cells (PCs) of epididymal epithelium secrete the EVs via apocrine secretion [95]. After the release of EVs into the fluid, they interact with passing sperms in the epididymis [96]. The EVs secreted by the epididymis are called epididymosomes. It has been proved that epididymosomes interact with sperm in vitro. In addition, about 12–36% of the epididymosomes carry the targeted sperm protein [97]. Epididymosomes can be divided into two groups, including smaller CD9-positive EVs, and larger CD9-negative EVs. The small EVs fuse with sperm via their tetraspanin domains, and the larger ones contain higher levels of epididymal sperm-binding protein 1 (ELSPBP1) [98, 99]. In several species, the presence of ELSPBP1 has been proved in epididymosomes [100]. These proteins bind to sperm after ejaculation and facilitate multiple functions such as capacitation [101]. A study of comparative proteome and lipid profiles of bovine epididymosomes shows the cargo differs between the epididymal fluid, caudal and distal epididymosomes [102]. It has also been shown that the miRNA cargo differs between EVs released from the proximal and distal epididymis [103]. Some of the molecules transferred between epididymosomes and sperm such as sperm adhesion molecule 1 (SPAM1), play a role in fertilization [104], glioma pathogenesis-related 1-like protein 1 (GliPr1L1) [105], and metallo-proteases [106]. Furthermore, it seems that the exchange of proteins such as the proto-oncogene C-terminal tyrosine-protein Src (cSrc) kinase [107] and macrophage migration inhibitory factor (MIF) [108] via epididymosomes to sperm have roles in sperm capacitation and motility. Furthermore, the transfer of non-conventional glutathione peroxidase 5 (GPX5) can protect sperm DNA from oxidative stress [109] and Liprin α3 transferring is necessary for acrosome reaction [110]. Epididymosomes from various segments of the epididymis exhibit distinct interactions with spermatozoa. The transfer of proteins from epididymosomes to spermatozoa is influenced by factors such as time, temperature, and pH with zinc enhancing the efficiency of this process [84]. Additionally, sperm migration inhibitory factor is delivered to the flagellar fibers, which are essential for sperm motility [111], while P26h and P25b, which regulate binding to the zona pellucida, are transported to the plasma membrane of the acrosome [112].

The prostate, a small accessory gland in the male reproductive system, produces a crucial fluid that supports

sperm transport and protection. The EVs released by the prostate epithelium are called prostasomes [113]. These EVs are heterogeneous in size and appearance and have one exosome entity. They contain prostate-specific proteins such as PAP, PSA, type 2 transmembrane serine protease (TMPRSS2), prostate-specific transglutaminase, prostate stem cell antigen (PSCA) and chromosomal DNA [113, 114]. The presence of prostasomes is positively influenced by sperm motility and they play a role in preventing premature capacitation and acrosome reaction of sperm [115].

Research indicates that the presence of SP exosomes is linked to the maintenance of boar sperm motility, membrane integrity, antioxidant capacity and inhibition of premature capacitation [85]. Recently, exosomes have been candidates as biomarkers for male infertility identification such as in patients with azoospermia, hypospermia and teratozoospermia [16]. In addition, it has been observed that oviductosomes, uterosomes, vaginosomes, endometriosomes and oocytosomes can interact with sperm [116]. These EVs are naturally absorbed by sperm within the female reproductive system to exert their effects. These findings highlight the physiological and transformative roles of EVs in the male reproductive system [100] and propose a potential strategy to more effectively address the challenges of sperm cryopreservation through the application of EVs. Furthermore, EVs are not only crucial in the male reproductive system but have also been shown to contribute to oocyte maturation, fertilization, prevention of polyspermy and embryo implantation [117].

Extracellular vesicles (EV) as a new strategy for prevention and treatment of sperm cryopreservation complications

The therapeutic potential of EVs has been a topic of interest for over twenty years [118]. EVs transport a range of surface and internal materials, including proteins, lipids and nucleic acids (Fig. 1). They have the ability to interact with spermatozoa and deliver these bioactive molecules to them in mammals such as porcine [19, 100, 119]. This results in the acquisition of essential molecules for sperm

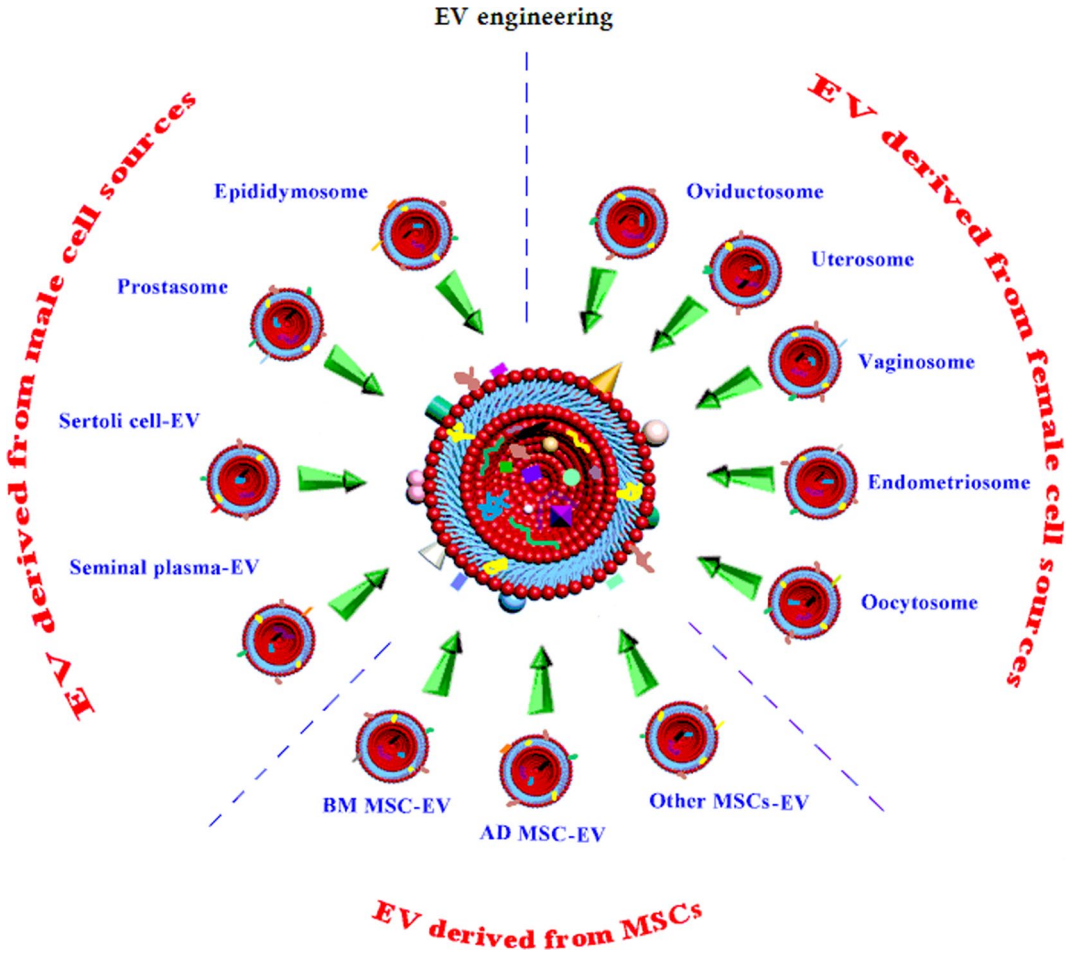


Fig. 1 Schematic view of extracellular vesicles (EVs) from cell sources (including male and female genital tract and MSCs) that could be engineered about superficial and internal cargo for cryoinjury sperm treatment (in the center: engineered EV)

maturation, motility, activation, protection, capacitation, acrosome reaction and fertilization [87]. Research shows that exosomes derived from HEK293T cells were taken up by boar sperm within 10 min of co-incubation [120]. It has been noted that sperm-derived extracellular vesicles (SP-EVs) carry delicate molecules associated with sperm fertility in pigs [121]. Accordingly, it is proved that EVs from seminal vesicles interact with spermatozoa in farm animals and pets [122]. Shamsi et al. (2024) have indicated that supplementing the freezing diluent with exosome-derived SP could preserve the quality parameters of the post-thawed bull sperm [123]. Furthermore, treatment with follicular fluid (FF) vesicles remarkably alters the trajectory of human sperm motion, which can certify an enhancement in their hyperactivation and, assumable, fertilizing capacity [124]. It has also shown that the minimum of 5×10^5 EVs/mL of bovine FF was sufficient to support sperm viability and also induce bull spermatozoa capacitation and acrosome reaction [125]. Chen et al. (2024) have demonstrated that exosomes secreted from epithelial cells and follicular layer cells regulate sperm motility during female long-term storage sperm in black rockfish (*Sebastes schlegelii*) ovary [20]. Based on findings, it is expected that EVs could also be effective in improving sperm cryoinjury treatment.

EVs of many types of MSCs have indicated anti-inflammatory and immunomodulatory effects [126–128]. These EVs can mitigate inflammation, modulate the immune response and lead to a conducive environment for cell survival. For example, it has proved that adipocyte-derived microvesicles play a role in maintaining cell survival [129]. Accordingly, Mokarizadeh et al. (2013) have revealed that incubation with mesenchymal stem cell (MSC)-derived microvesicles (MVs) improved cryopreserved sperm quality parameters (viability, motility and antioxidant capacity) and their adhesive properties (CD29, CD44, ICAM-I and VCAM-I). Qamar et al. (2020) have used adipose-derived MSCs for the treatment of frozen-thawed dog sperm and observed improving their viability and fertility. They have also found enhancement in the post-thaw quality of canine semen after incubation with exosomes from a conditioned medium harvested from adipose-derived MSCs. Alcantara-Neto et al. (2020) proved that porcine oviductosomes interact with gametes and regulate sperm mobility and survivance. It has been shown exposure to epididymal EVs improves the function of immature sperm and maintains the vitality of cryopreserved spermatozoa in the domestic cat model [21]. Mahdavinezhad et al. (2022) have proved that exosomes and microvesicles from sperm play a protective role during human sperm cryopreservation. So far, there are no suitable options for preserving fertility in boys who undergo chemotherapy before puberty. Because controlled vitrification of testicular tissue leads to potential structural damage to the SSCs niche during cryopreservation. In this regard, Liakath et al. (2023) have indicated the protective effect of human umbilical cord-derived MSC exosomes by preserving the SSCs niche and preventing testicular damage in mice, against chemotherapy-induced testicular cytotoxicity. They observed that mice receiving multiple injections of exosomes exhibited remarkably higher fertility rates and serum testosterone levels [130]. These findings highlight the significant potential of EVs in engineering solutions to repair cryoinjured sperm (Table 1). Additionally, research indicates that the proteomes of large and small EVs in pig sperm

Table 1 Extracellular vesicles (EV) treatment effects on cryopreserved sperm

EV cell sources	EV donor	EV recipient	During/After cryopreservation	Main Results	Some important predispose proteins for EV engineering	Reference(s)
Oviductosomes	Porcine	Porcine	After	Regulating sperm mobility and survivance	MYH9, MYH14, HSPA5, ANXA and OVGP1	[86, 200–202]
Epididymal EVs	Domestic cat	Domestic cat	After	Improves the function of immature sperm and maintains the vitality	DCXR, P34H, P26h, P25b, GliPr1L1, AKR1B5, SDH and ELSPBP1	[21, 105, 203–207]
Exosomes and MVs of SP	Human	Human	During	Cryoprotective properties	CRISPs, MSMB and MIF	[135, 136, 144, 208, 209]
Adipose-derived MSCs exosomes	Canine	Dog	After	Viability and fertility	ANX1, H3, and HMGB	[87]
Bone marrow MSC-derived MVs	Wistar rat	Wistar rat	After	Viability, motility and antioxidant capacity, and adhesive properties	IGF1, HGF, LIF, FGF, and HO1	[210–217]

Abbreviations in Table 1: MYH9, Myosin Heavy Chain 9; MYH14, Myosin heavy chain 14; HSPA5, Heat Shock Protein Family A (Hsp70) Member 5; ANXA, Annexin A1; OVGP1, Oviductal Glycoprotein 1; DCXR, Dicarbonyl/l-xylulose reductase; P34H, Epididymal sperm protein; P26h, Hamster sperm protein; P25b, Bull sperm fertility marker protein; Glipr1l1, GLIPR1 (GLI Pathogenesis Related 1) like 1; AKR1B5, Aldoketoreductase 1B5; SDH, Succinate dehydrogenase; ELSPBP1, Epididymal sperm binding protein 1; CRISPs, Cysteine Rich Secretory Proteins; MSMB, Microseminoprotein Beta; MIF, Macrophage Migration Inhibitory Factor; ANX1, Annexin A1; H3, Histone H3; HMGB, High Mobility Group Box; IGF-1, Insulin-like Growth Factor-1; HGF, Hepatocyte Growth Factor; LIF, Leukemia Inhibitory Factor; FGF, Fibroblast growth factors; HO1, Heme oxygenase 1

differ [131]. Consequently, comparing the cargo of different EV subgroups could enhance research on the treatment of cryopreserved sperm.

Possible mechanisms of action for extracellular vesicles (EV) in cryopreserved sperm repair

Recent studies have demonstrated that EVs possess significant therapeutic properties that can aid in the repair of damaged cells [132, 133]. These properties may address various aspects of sperm cryoinjuries. These effects are linked to the delivery of contents from EVs such as mitochondria, microRNAs and antioxidants, which can be taken up by cells through mechanisms like endocytosis or membrane fusion [134] (Fig. 2). We will explore the potential mechanisms by which EVs may contribute to the treatment of cryopreserved sperm in the following discussion.

Bioenergy

Access to adequate energy can aid in repairing cellular damage and enhancing sperm motility. Additionally, extracellular ATP has been shown to trigger the acrosome reaction in human spermatozoa. Ronquist et al. (2013) proposed that prostasomes produce high levels of extracellular ATP which interacts with sperm surface receptors to facilitate the acrosome reaction [135, 136]. It has been demonstrated that mitochondria lacking MSC-derived EVs significantly lost their ATP content, whereas those treated with MSC-EVs retained at least 90% of their ATP after two days of cold storage, compared to fresh mitochondria [137]. Furthermore, another study indicated that EVs from a human brain endothelial cell line can enhance ATP levels in recipient cells [138]. It has been suggested that damaged mitochondria in recipient cells can be converted into functional ones through the delivery of medium-to-large EVs (m/lEVs) containing mitochondria, as healthy mitochondria can fuse with and repair damaged ones [139]. Moreover, research has found that EVs can transfer polarized mitochondria (intact mitochondria with a highly negative charge) and improve cellular energetics in ischemic endothelial cells [140]. Additionally, studies indicate that mitochondrial-derived vesicles (mitovesicles) maintain membrane potential and contain functional ATP synthase, which could potentially restore ATP-deficient mitochondria [141]. Recently, Kowalczyk et al. (2024) have shown that isolated exosomes from the bull SP and supplemented with a concentrated dose in the extender for sperm freezing were demonstrated to greatly improve cell cryostability by keeping the potentials of the mitochondrial membrane of the frozen/thawed spermatozoa [142].

Sperm motility

Sperm motility is crucial for normal fertility, but it can be compromised by cryopreservation. Research has proved that when prostasomes fuse with sperm, a calcium ion (Ca2+) surge occurs in the sperm cytoplasm. Since ionic signals and particularly calcium ions are essential for stimulating sperm motility in the midpiece, prostasomes can sustain prolonged Ca^{2+} signals in spermatozoa through pH-dependent vesicle fusion [115]. Therefore, using prostasomes after cryoinjury may enhance sperm motility.

Delivering membrane compartments

The side effects of glycerol during sperm cryopreservation arise from protein denaturation, alterations in actin interactions and the induction of plasma membrane

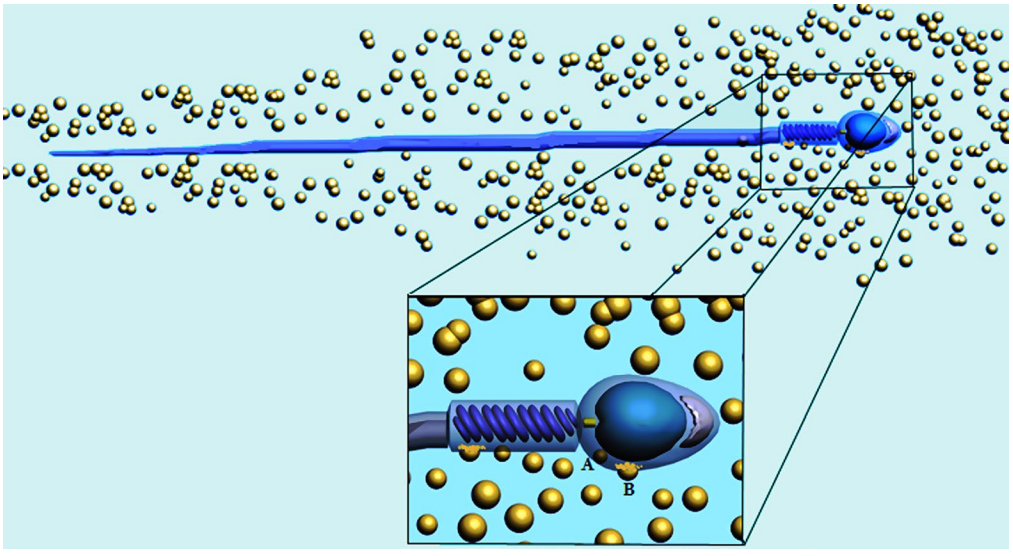


Fig. 2 Schematic view of natural/engineered extracellular vesicles (EVs) uptake by sperm (**A**: internalized EV, **B**: cell surface membrane fusion EV)

fragility [3]. EVs have significant potential for delivering membrane-associated proteins for therapeutic purposes [143]. Microvesicles (MVs) and exosomes are rapidly absorbed by the sperm plasma membrane [144]. Additionally, it has been noted that exosomes secreted by epithelial and follicular layer cells attach to the mid-piece of sperm stored in the ovaries of black rockfish (*Sebastes schlegelii*) [20]. It is expected that using EV during and/or after cryopreservation can help to plasma membrane damage repair of sperm. Measurements of sperm quality indicators have proven that treatment with exosomal proteins has protective and regenerative effects, including preserving the integrity of the plasma membrane as well as the chromatin material of canine sperm during cryopreservation [145]. Furthermore, research has indicated that oviductosomes from dogs and cats contain proteins capable of restoring sperm function after cryopreservation. Thawing red wolf and cheetah sperm in the presence of these oviductosomes improves post-thaw motility in red wolf sperm and prevents premature acrosome exocytosis (AE) in both red wolf and cheetah sperm [2]. It has been noted that oviductosomes contain lipids with protective properties. Research indicates that these lipids can aid in safe guarding sperm during thawing by altering the plasma membrane's permeability and fluidity. The bilayer membrane of EVs is abundant in lipids like cholesterol, sphingomyelin, glycosphingolipids and phosphatidylserine, which sperm can acquire through EV uptake. By delivering cholesterol to sperm via oviductosomes, membrane stability could be enhanced, offering protection against membrane damage and premature acrosomal exocytosis during the thawing process [2].

MiRNAs

Studies have confirmed that exosomal cargoes and particularly miRNAs are involved in countless physiological functions such as reproduction [19] by playing a role in the post-transcriptional regulation of gene expression [146]. It has been demonstrated that EVs can deliver miRNAs to sperm. For instance, murine oviductosomes are capable of transferring miR-34c-5p which localizes to the centrosome of sperm [147]. A comparison of miRNA profiles between fertile and subfertile individuals reveals distinct differences. miRNAs from SP-EVs are linked to spermatogenesis and sperm quality as they can regulate transcription and translation processes [148]. Whole transcriptome analysis of Yorkshire boar SP-EVs has identified several key miRNAs related to sperm motility [149]. Recent research suggests that miR-222 released by semen EVs plays an important role in maintaining sperm viability and reducing apoptosis [150]. Additionally, miRNAs within epididymosomes, secreted by the proximal epididymis, have been shown to alter gene expression in distal epididymal cells [151, 152]. The impact of this on the subsequent release of epididymal EVs and their cargo warrants further investigation [100]. Moreover, studies have found that miRNA profiles in follicular fluid vary with age in women, influencing sperm characteristics after treatment [153]. This field is susceptible to more applied research.

Epigenetic effects

Research has indicated that cryopreservation induces genetic and epigenetic changes in zebrafish genital ridges and roosters [41, 42]. Epigenetic alterations are a crucial factor in the decline of sperm motility and fertilization capacity [43]. In recent years, it has been demonstrated that epididymal EV-mediated miRNAs can induce epigenetic modifications [154]. Small EVs (sEVs) in semen carry a significant population of small non-coding RNAs (sncRNAs, 20 to 100 nucleotides) that, through various epigenetic and post-transcriptional mechanisms, play a major role in gene expression regulation [155]. Previous studies have shown that modifications in sperm RNA, triggered by external factors, can alter paternally inherited traits in offspring, such as insulin sensitivity [156, 157]. The potential epigenetic role of EVs in mitigating cryoinjury-induced epigenetic damage in sperm requires further investigation.

Antioxidant properties

Oxidative stress is recognized as a key factor contributing to sperm cryodamage [3, 4]. Research has demonstrated that MSC-EVs are rich in antioxidant enzymes such as glutathione peroxidase, superoxide dismutase 1 (SOD1), SOD2, CAT and others [158]. Additionally, small EVs in semen exhibit antioxidant properties that help protect sperm from oxidative stress [159]. Moreover, various antioxidants can be loaded into EVs and delivered to sperm, offering potential prevention or treatment for oxidative stress-related damage.

Extracellular vesicles (EV) engineering to improve viability and fertility of cryopreserved sperm

The natural role of EVs in spermatogenesis and sperm capacitation for fertilization has been well established [18] as well as their positive impact on reducing sperm cryopreservation injuries [122, 124, 145]. Given these findings, along with the potential of EV engineering [17], there is growing interest in leveraging EV engineering to address sperm cryopreservation-related damage. EV engineering is commonly used to introduce or enhance specific biomolecules to improve therapeutic outcomes [17]. It has been suggested that SP-EVs can be extracted and engineered in vitro by loading specific molecules, then standardized for use in artificial reproductive technology (ART) applications [121]. This approach could also involve harvesting EVs from other beneficial cell

Table 2 Important direct EV engineering methods that are used for loading molecule types in EVs

Direct EV engineering methods	Loading types	Used for loading of molecule types			Reference(s)
		Proteins	RNA types	Small molecules	
Electroporation	Active	✓	miRNA or siRNA	✓	[171, 218–220]
Sonication	Active	✓	miRNA or siRNA	-	[166, 169]
Incubation	Passive	✓	miRNA	✓	[167, 169, 221, 222]
Freeze/thawing	Active	✓	miRNA	-	[169]
Saponin permeabilization	Active	✓	miRNA	✓	[169, 171, 223]
Extrusion	Active	✓	-	✓	[169, 171]
$CaCl^{2+}$heat shock	Active	-	miRNA	-	[167]

sources within the male or female reproductive tracts for treating sperm cryoinjury. Overall, EV engineering strategies can be divided into two main approaches: direct and indirect.

It is important to note that EVs required for treating cryopreserved sperm can be sourced either from secretions of specific cells in the human body or from secretions during the cultivation of these cells. The benefit of obtaining EVs from body fluids is that the secreting cells remain in their natural environment, though this method typically yields a smaller quantity of EVs. In contrast, EVs derived from cell culture are usually produced in larger amounts but the cells are no longer in their natural niche. Therefore, it is essential to compare the quality-to-quantity ratio between these two methods and evaluate their respective effectiveness in addressing the complications of cryopreserved sperm.

EV targeting can be enhanced through engineering to improve uptake by recipient cells. For instance, increasing the presence of various integrins, CD63, the tetraspanin 8 (TSPAN8)-integrin α4 complex and glycans on the EV surface can boost EV targeting efficiency [17, 160]. Modifying the EV surface with these molecules can improve their targeting capabilities and retention in sperm after thawing.

Extracellular vesicle (EV) engineering before the production (indirect EV engineering)

Indirect EV engineering can be done by engineering parental cells that secrete EVs. This EV engineering strategy could lead to the customized, effective and rich production of EVs. Genetic manipulation is one of the most important tools in this strategy [161]. For example it has been shown that over expression of neutral sphingomyelinase II (NSMase2) enhanced the miRNA production in EVs [162]. Furthermore, incubation of parental cells with selected molecules such as miRNAs and proteins is another procedure of this strategy. In this procedure, parental cells could be incubated with therapeutic molecules in a sub-lethal concentration for a certain period. The incubated molecules will be internalized by the cells and their secreted EVs will contain a certain fraction of incubated molecules [163]. Because the effects of miR-222 [150] and miR-22 [164], respectively on sperm viability maintaining, apoptosis decreasing and preserving ATP levels in injured cardiomyocytes have been observed. Their over expressing in the parental cell could be reflected in their EV and could be useful for sperm cryopreservation treatment. It seems that further studies in this new field can also be very helpful for the improvement of sperm cryopreservation treatment, with considering their other bioactive molecules (Tables 1 and 2**and** Fig. 1). In recent years, Muñoz et al. (2022) have well-reviewed EVs in mammalian reproduction including their very different EV cargoes from various cell sources, that could inspire EV engineering for sperm cryoinjury treatment.

Extracellular vesicle (EV) engineering after the production (direct EV engineering)

Extracted EVs can be directly engineered after secretion from their cell sources using various techniques, including electroporation, sonication, incubation, saponin permeabilization, extrusion, CaCl2-heat shock, and freeze/thawing [161]. In electroporation, a high-voltage electrical pulse disrupts the EV membrane, creating temporary pores that allow cargo molecules to enter. This is the most commonly used method for loading miRNAs into EVs [165]. Sonication uses sound wave energy to temporarily disrupt the cell membrane, enabling molecules to enter EVs [166]. Incubation, the simplest technique, involves incubating molecules like miRNAs with EVs at room temperature for a set period, allowing gradual entry of the cargo into the EVs [167]. Saponin is a detergent-like molecule that forms temporary pores in membranes by interacting with cholesterol [168]. In another method, EV cell sources and cargo molecules are mixed in a syringe and forced through a porous membrane, mechanically disrupting the EV membrane to trap molecules inside [169]. This approach can also generate artificial EVs while simultaneously loading cargo molecules. A novel method involves incubating EVs in CaCl2 followed by heat shock to directly load molecules into exosomes [167]. In freeze-thawing, cargo molecules are first incubated with exosomes at room temperature, then quickly frozen in liquid nitrogen and thawed again at room temperature [169]. However, the drug-loading capacity of freeze-thawing is generally lower than sonication or extrusion methods [170]. Electroporation has been proven superior for EV

loading compared to sonication, incubation, extrusion and saponin permeabilization [171]. Various molecules, such as miRNAs and proteins can be loaded into EVs based on therapeutic needs (Table 2) [161, 163]. Notably, miR-222 [150]and miR-22 [164] are promising candidates for EV loading to treat sperm cryopreservation complications due to their anti-apoptotic effects. Moreover, it has been observed that epididymal EV-mediated miRNAs can induce epigenetic modifications during sperm development [154]. Studying these miRNAs and their specific roles could pave the way for their incorporation into EVs for delivery to sperm, potentially aiding in the treatment of epigenetic damage in cryopreserved sperm.

Since cryopreservation causes damage to the sperm plasma membrane [24], EVs have great potential for engineering the delivery of membrane-associated proteins after sperm cryopreservation [143]. As previously noted, treating spermatozoa with cholesterol-loaded cyclodextrins can help preserve motility-related proteins during the cryopreservation process [62]. Additionally, engineered EVs functioning as bilayer membranous vesicles can transport surface cargoes such as proteins, lipids, and phospholipids to cryodamaged sperm. These vesicles can also deliver bioactive molecules including antioxidants and proteins as internal cargo to support the recovery of cryoinjured sperm (Fig. 2). Consistent with this, it has been shown that pig SP contains all TGF-β isoforms (TGF-β1, 2, and 3), with a significant portion being associated with SP-EVs, which can modulate the immune environment in the female reproductive tract [172].

The enhancement of the antioxidative effect can be achieved through EV engineering. EVs can directly deliver antioxidant substances such as superoxide dismutases (SOD), CAT, peroxiredoxin (PRDX), GPX, glutathione S-transferase (GST) and thioredoxin (TRX) to recipient cells. Additionally, EVs can indirectly supply either a single regulatory agent or multiple regulators to recipient cells [173]. This approach could be beneficial for preventing and treating oxidative stress during sperm cryopreservation.

As noted earlier, enhancing ATP production in sperm can aid in repairing and improving motility. Therefore, it is recommended to deliver mitochondria via engineered m/lEVs to sperm to enhance quality after freezing [139]. Additionally, mitovesicles could enhance ATP synthesis, serving as an engineering method to restore ATP-deficient mitochondria [141].

Proper cell sources for extracellular vesicles (EV) engineering for sperm cryopreservation treatment

EVs released from various cell types can exhibit distinct therapeutic effects [132, 133]. Therefore, to address complications related to sperm cryopreservation, it is preferable to utilize cell sources whose EVs can naturally treat these issues or whose EVs can be engineered to enhance their effectiveness in mitigating such complications. Among the first group, male cell sources like Sertoli cells and epithelial cells from the epididymis and prostate are noteworthy for their EVs' natural role in sperm differentiation. Additionally, female cell sources such as oviductosomes have been shown to interact with sperm [86], and improve sperm function after thawing [2]. Other female-derived EVs including uterosomes, vaginosomes, endometriosome, and oocytosomes have also been observed to interact with sperm, making them suitable sources for EV harvesting and engineering. From the second group, mesenchymal stem cells (MSCs) stand out as the best option due to their inherent healing properties and widespread use in EV engineering [174, 175] (Fig. 1). Besides bone marrow MSCs and adipose-derived MSCs and other MSCs, particularly those derived from urine, have gained attention for EV harvesting due to the non-invasive extraction method. Recent findings indicate that producing native or engineered EVs from urine-derived MSCs represents a powerful platform [176]. It has also been reported that MSC-EVs can modulate mitochondrial content in microglia [177], potentially increasing ATP levels in the cells. Additionally, recent studies have found that EVs derived from aloe vera cells exhibit antioxidant activity and are effectively taken up by skin cells during the wound healing process [178], which could be beneficial for treating cryoinjured sperm.

Choosing autologous EV sources is preferred over allogeneic ones, as the potential side effects associated with using allogeneic EVs in post-thaw sperm treatment require further exploration. This aspect could be advantageous in addressing issues related to cryopreserved sperm. However, it's essential to recognize that research in this field is still in its infancy and more studies are necessary to investigate the effects of EVs from different cell sources on complications arising from sperm cryopreservation.

Restrictions of EV application

Although the therapeutic effects of EVs are increasingly recognized, some limitations also must be regarded in their application, for example in sperm cryoinjury treatment. The reproducibility (replicability), purity and recovery rate of EVs must be considered for perfect EV therapeutic applications [179]. It should be noted that heterogeneity can affect EV reproducibility. Reducing heterogeneity appears to be beneficial for reproducibility and also makes EV quantification easier. The heterogeneity of the EV main population and their various subpopulations can be attributed to differences in their biogenesis [180]. Heterogeneity is often recognized between EVs harvested from different cell types, it is also seen within EVs released from a single cell type [181]. Furthermore,

other factors such as extraction technique [182] and storage conditions [183] can also result in variations in the EV's physical properties and function that change EV heterogeneity. So far, various methods for extracting EVs have been employed, the most common of which are ultracentrifugation, size exclusion and sedimentation techniques using polymers such as polyethylene glycol (PEG) [184, 185]. Among these methods, centrifugation is the most commonly used. Current insights indicate that the choice of extraction method can influence both the uptake of EVs [186] and their therapeutic properties. Different methods are used to isolate EVs, and not only do their results differ, but even within one method such as centrifugation, the effect of rotor type and centrifugation time on the yield and purity of EVs is observed [187]. Veerman et al. have suggested that no method is ideal for all studies, rather, different methods are suitable relying on sample type and desired EV subtype, sample volume and budget [182]. Hence, it seems that in a nascent research field like EV use for frozen sperm treatment, different cell sources, cultivation and extraction methods should be compared and the most appropriate method should be selected for EV extraction. Consequently, more research is needed in this field. It has been shown that EV storage destabilizes their surface characteristics, morphological features and protein content which could decrease their therapeutic effects [188, 189]. To increase accuracy and reproducibility, EV-TRACK was created as a crowd sourcing knowledge base that allows authors to deposit their EV extraction and characterization protocols and obtain advice on potential weaknesses in experimental design [190].

In addition to improving therapeutic effects, large-scale EV production is also a significant challenge. Recent studies have indicated that using three-dimensional (3D) cell culture methods can significantly enhance EV production [191–193]. Increasing EV yield not only reduces costs but also makes production more economical for clinical applications. Additionally, different 3D culture methods and cell sources have been shown to impact exosome production [194]. For example, culturing MSCs in 3D has been found to yield EVs with enhanced production and greater therapeutic efficacy [192]. Large-scale cultivation methods, such as bioreactor systems that use microcarriers and hollow-fiber bioreactors, can produce substantial amounts of EVs. Moreover, employing co-culture techniques with various cell types can help recreate the natural cellular environment, further enriching EV production [195]. The advancement of techniques for producing, isolating and yielding EVs is hoped to reduce production costs, increase yields and provide better circumstances for EV research and clinical trials especially in reproduction research.

Presently, various methods from production to engineering, and administration are used for improving therapeutic features and large-scale production of EVs, which need to be standardized and defined. In this regard, Minimal Information for Studies of EVs (MISEV) guidelines that have been released and updated by ISEV that is an important step for the standardization of EV research and clinical applications [13]. In addition, Welsh et al. published a paper that defines reference materials for measuring extracellular vesicle refractive index, epitope abundance, size and concentration [196]. It seems that special comprehensive guidelines and standardization protocols of EV engineering for clinical application are also needed [17], especially in the field of reproduction, there is a need for greater ethical sensitivity because the consequences can potentially affect future generations.

Ethical aspects considering

Compliance with Good Manufacturing Process-like conditions (GMP) standards in the production of EVs is essential for clinical safety [197], including the treatment of human sperm for use in fertilization. Furthermore, EV cargo is transferred to gametes during the cryoinjury treatment, which potentially could lead to changes in the offspring. For instance, miRNAs and sncRNAs can induce epigenetic modifications, making it essential to consider the ethical aspects in this context.

For sperm cryopreservation complications treatment, the primary priority is for EV harvesting from the same sperm-producing parent [135]. The secondary priority can be on using EVs from the ovum-producing parent [125]. Additionally, it is important to ensure that the cell sources used for EV harvesting are obtained through non-invasive methods whenever possible. For example, prioritizing isolated EVs from semen or MSCs derived from urine is advisable [176]. Today, with the progress of stem cell technologies, about induced pluripotent stem cells (iPSCs), could produce personalized iPSC-derived EVs at high efficiency. These cells are easy to get through the direct reprogramming of a patient's somatic cells, in addition, iPSCs can secrete many more EVs than human MSCs, which will significantly decrease probable immunogenicity besides avoiding potential ethical issues [198]. It should be noted that the use of EVs derived from immortalized cell lines or their co-culturing with other cells does not seem permissible for sperm treatment due to possible germ line modification.

Overall, it is crucial to compare the effectiveness of EVs extracted from various cell sources and determine the most suitable source, highlighting the need for further research in this area. In addition, although EV therapy is considered an approach with low immunogenicity, factors such as origin, size, surface composition, internal cargo, production/storage methods, dosage and the

biomolecular corona could affect EV immunogenicity [199], more detailed research is needed, especially about engineered EVs.

Conclusion and future perspective

Cryopreservation of sperm for artificial insemination has been practiced for decades, yet it often leads to various types of cryodamage, including harm to the plasma membrane, acrosome, nucleus, mitochondria and essential molecules like proteins, lipids and nucleic acids. As a result, a range of conventional and modern methods have been employed to mitigate the effects of cryodamage but these solutions have yielded unsatisfactory outcomes. Consequently, researchers are exploring innovative strategies to address the challenges of sperm cryopreservation. It has been revealed that EVs secreted from male sources such as Sertoli cells, the prostate and the epididymis as well as from female sources including the oviduct, uterus, vagina, endometrium and oocytes can interact with sperm and perform critical functions. These EVs contain a variety of cargoes such as proteins, lipids and nucleic acids that may aid in sperm restoration. Given their roles during spermatogenesis and the influence they have on sperm after ejaculation within the female reproductive system, using EVs from both sexes could be beneficial for treating cryopreserved sperm. In males, EVs from Sertoli cells, the prostate and the epididymis have been shown to interact with sperm, while in females, EVs from the oviduct, uterus, vagina, endometrium, and oocytes fulfill similar functions. Although extracting EVs from SP is one of the best sources for addressing sperm freezing complications, access to this resource can be limited. To overcome the scarcity of in vivo resources, abundant and enriched EVs can be obtained through in vitro culture of suitable cells using 3D culture methods or co-culture techniques. The engineering potential of EVs whether direct or indirect allows for the customization of their content, particularly through the inclusion of miRs and proteins, tailored for treating frozen sperm. The combination of EVs from different cell sources may enhance the efficacy of treatment for cryopreserved sperm, a hypothesis that warrants further investigation. Ultimately, EV engineering strategies can complement both traditional and novel approaches to improve sperm cryopreservation outcomes. This innovative use of EVs offers new possibilities for addressing post-thaw complications in sperm and could play a significant role in reducing infertility rates. Furthermore, these strategies may also benefit other areas of infertility treatment, including the prevention or mitigation of complications associated with oocyte freezing. Finally, although the research results are promising, we still need more research to comprehensively understand the EV therapeutic properties, their bioactive molecules and their engineering potential in sperm cryopreservation.

Abbreviations

3D	Three-dimensional
ADH5	Alcohol dehydrogenase 5
AE	Acrosome exocytosis
AI	Artificial insemination
ART	Artificial reproductive technology
CAT	Catalase
CeO_2	Cerium oxide
CLC	Cholesterol-cyclodextrin complexes
CPAs	Cryoprotective agents
cSrc	C-terminal tyrosine-protein Src
DLS	Dynamic light scattering
ELSPBP1	Epididymal sperm-binding protein 1
EVs	Extracellular vesicles
FF	Follicular fluid
FT	Freeze-thawing
GFRA1	GDNF family receptor α1
GliPr1L1	Glioma pathogenesis-related 1-like protein 1
GMP	Good Manufacturing Process-like conditions
GPI	Glucose-6-phosphate isomerase
GPX	Glutathione peroxidase
GPX5	Glutathione peroxidase 5
GST	Glutathione S-transferase
iPSCs	Induced pluripotent stem cells
ISEV	International Society for Extracellular Vesicles
LDHB	Lactate dehydrogenase B
MDA	Malondialdehyde
Me2SO	Glycerol or dimethyl sulfoxide
miRs	MicroRNAs
MISEV	Minimal Information for Studies of EVs
MSCs	Mesenchymal stem cells
MVH	Mouse vasa homologue
MVs	Microvesicles
NPs	Nanoparticles
NSMase2	Neutral sphingomyelinase II
PCs	Principal cells
PEG	Polyethylene glycol
PGAM1	Phosphoglycerate mutase 1
PLZF	Promyelocyticleukaemia zinc finger protein
PRDX	Peroxiredoxin
PSCA	Prostate stem cell antigen
PUFA	Polyunsaturated fatty acid
SC-EXO	Sertoli-derived exosomes
SeNPs	Selenium nanoparticles
sEVs	Small EVs
SLC	Single-layer centrifugation
sncRNAs	Small non-coding RNAs
SOD	Superoxide dismutase
SOD1	Superoxide dismutase 1
SP	Seminal plasma
SPAM1	Sperm adhesion molecule 1
SP-EVs	Sperm-derived extracellular vesicles
SSCs	Spermatogonial stem cells
STRA8	Stimulated by retinoic acid 8
TAC	Antioxidant capacity
TEM	Transmission electron microscope
TMPRSS2	Type 2 transmembrane serine protease
TRX	Thioredoxin
TSPAN8	Tetraspanin 8

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