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REVIEW ARTICLE

Therapeutic Potential of Mesenchymal Stem Cell-Derived Extracellular Vesicles for Tendon Regeneration: Insights into Inflammatory and Angiogenesis Modulation

Daffa Raditya Fernanda [1], Radiana Dhewayani Antarianto [2,3], Abinawanto [1,4], Retno Lestari[1,4*]

1. Department of Biology, Faculty Mathematics and Natural Sciences, Universitas Indonesia, Jl. Prof. Dr. Mahar Mardjono, Depok 16424, Indonesia
2. Department of Histology, Faculty of Medicine, Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta 10430, Indonesia
3. Stem Cell and Tissue Engineering Research Cluster (SCTE), Indonesia Medical Education and Research Institute (IMERI), Universitas Indonesia, Jl. Salemba Raya No. 6, Jakarta 10430, Indonesia
4. Cellular and Molecular Mechanisms in Biological System (CEMBIOS) Research Group, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Jl. Prof. Dr. Mahar Mardjono, Depok 16424, Indonesia

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***Corresponding**:
Retno Lestari
Address:
Cellular and Molecular Mechanisms in Biological System (CEMBIOS) Research Group, Faculty of Mathematics and Natural Sciences, Universitas Indonesia, Jl. Prof. Dr. Mahar Mardjono, Depok 16424, Indonesia.
E-mail:
retno.lestari@sci.ui.ac.id

ABSTRACT

Mesenchymal stem cell-derived extracellular vesicles (MSC-EVs) are a promising cell-free therapeutic in preclinical models of tendon healing. However, clinical translation remains to be demonstrated. MSC-EVs modulate two critical and interlinked processes in tendon repair—inflammation and angiogenesis—primarily via delivery of bioactive cargo such as microRNAs and proteins. In inflammation, MSC-EVs promote the transition of macrophages from a pro-inflammatory (M1) to an anti-inflammatory (M2) phenotype, enhancing the resolution of inflammation. In angiogenesis, MSC-EVs facilitate endothelial cell proliferation and migration, promoting early vascularization followed by vascular regression during later healing. Recent advancements in MSC-EVs engineering and delivery systems have improved their therapeutic efficacy, yet further research is essential to elucidate their molecular mechanisms and achieve precise therapeutic outcomes.

***Keywords*:** Angiogenesis, Extracellular vesicles, Inflammation, Mesenchymal stem cells, Tendon regeneration

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Introduction

Tendons are dense, highly structured connective tissues that serve as essential components of the musculoskeletal system by facilitating the attachment of muscles to bones. Histologically, tendons are composed predominantly of collagen fibers, comprise ~85% of tendon dry weight, organized into parallel collagen bundles that confer tensile strength. The primary function of tendons is to withstand biomechanical loads and facilitate efficient force transmission during movement (1). However, tendons are particularly vulnerable to injury due to excessive mechanical stress, acute trauma, or pathological conditions associated with metabolic syndrome and obesity. Furthermore, the inherent hypovascularity and hypocellularity of tendon tissue pose significant challenges in achieving effective healing and regeneration, often resulting in poor recovery outcomes and a high risk of re-injury (2).

One of the most common tendon issues is tendinopathy, a degenerative disease associated with chronic inflammation, matrix degradation, and the inability of tissue to regenerate. Tendinopathy is reported to be involved in 30-50% of all orthopedic injuries, with a notable increase over the past few decades (3, 4). The risk of tendinopathy is higher among athletes, with the highest incidence observed in basketball players (69.9 per 100 players per season) and roller hockey players (64.4 per 100 players per season). Additionally, the patellar and Achilles tendons are the most frequently affected, with incidences of 11.7 and 10.3 per 100 players per season, respectively (5).

The tendon healing process comprises three phases: inflammation, proliferation, and remodeling. The inflammatory response triggers the recruitment of pro-inflammatory cells to eliminate cellular debris from necrotic cells or damaged extracellular matrix components within the tendon. However, prolonged inflammatory responses following tendon injury have been extensively associated with the pathogenesis of tendinopathy, tendon rupture, and long-term functional deficits. Persistent inflammation disrupts the regenerative process, leading to compromised tendon healing (6, 7).

Angiogenesis, the formation of new blood vessels, increases during the proliferative phase of tendon healing to alleviate hypoxia and facilitate the delivery of essential nutrients for tendon regeneration (7). Although angiogenesis is necessary for supporting the healing process, excessive angiogenesis has been shown to be detrimental, as tendons are inherently hypovascular under normal physiological conditions. Recent findings in orthopedic research indicate that newly formed vasculature often exhibits structural deficiencies, including aberrant anastomosis and increased vascular permeability, leading to impaired tissue perfusion and regeneration (8, 9).

An optimal therapeutic approach aims to modulate the inflammatory phase to prevent pathological progression while concurrently enhancing the reparative mechanisms during the proliferative and remodeling phases. However, conventional therapeutic approaches remain limited by heterogeneous clinical outcomes, a high recurrence rate, and limited long-term efficacy. Therefore, the development of advanced therapeutic strategies to enhance tendon regeneration is of critical importance.

Recent advancements in mesenchymal stem cell (MSC)-based therapies emphasize the therapeutic potential of extracellular vehicles (EVs) as a novel cell-free strategy for tissue regeneration. Evidence suggests that MSCs mediate tissue repair predominantly through paracrine signaling pathways facilitated by extracellular vesicle-mediated communication. Extracellular vesicles are nanoscale secretome components that encapsulate diverse bioactive molecules, including proteins, peptides, and nucleic acids, particularly microRNAs. Mesenchymal stem cell-derived EVs (MSC-EVs) exhibit significant potential in promoting tendon regeneration through the modulation of macrophage polarization to facilitate inflammation resolution. Additionally, MSC-EVs regulate tendon angiogenesis to mitigate hypoxic conditions and improve the viability and proliferative capacity of stromal tendon compartments (10, 11).

Previous preclinical studies have highlighted the therapeutic potential of MSC-EVs in promoting tendon regeneration. However, the underlying mechanisms through which MSC-EVs facilitate tendon tissue repair remain inadequately understood. In particular, inflammation and angiogenesis are critical biological processes that significantly influence tendon healing (6, 11). Given that the application of MSC-EVs for tendon regeneration is an emerging and innovative strategy, a deeper understanding of their biological interactions is essential to optimize their therapeutic

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potential. Further research is needed to elucidate how MSC-EVs precisely modulate inflammation and angiogenesis to enhance tendon repair. Therefore, this review aimed to summarize current knowledge on MSC-EVs in tendon healing, with a specific focus on their regulatory roles in these two key processes.

Mesenchymal Stem Cell-Derived Extracellular Vesicles

MSC-EVs are nanoscale vesicles that facilitate intercellular communication by transporting bioactive molecules from donor MSCs to recipient cells, thereby modulating various cellular process and signaling pathways. Traditionally, EVs are categorized into exosomes (30–150 nm), microvesicles (100–1000 nm), and apoptotic bodies (500–3000 nm). However, the MISEV 2018 guidelines suggest a more practical classification based on size: small EVs (sEVs, <200 nm) and medium/large EVs (>200 nm). Among these, sEVs are the predominant vesicle type secreted by MSCs and are reported to exert therapeutic effects comparable to those of their parental cells (12). Therefore, the term "MSC-EVs" as used in this review—and frequently employed in other scientific communications—typically refers to "sEVs" or "exosomes" with a diameter of less than 200 nm.

Small extracellular vesicles (sEVs) are membrane-bound particles ranging from 30 to 200 nm. Their biogenesis begins with inward invagination of the plasma membrane, leading to the formation of early endosomes, which mature into multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs). This maturation process can proceed via two pathways: ESCRT-dependent or ESCRT-independent (13).

Biogenesis

The endosomal sorting complex required for transport (ESCRT) drive membrane scission during ILVs biogenesis. ESCRT-0 (HRS/STAM) recognizes Lys63-linked ubiquitinated cargo on PtdIns(3)P-enriched early endosomes via the HRS FYVE domain, with clathrin binding aiding microdomain organization (13, 14). Cargo is handed to ESCRT-I (TSG101 and UBAP1/MVB12), which recruits ESCRT-II (VPS36 with a GLUE domain) to seed ESCRT-III assembly (15). ESCRT-III is initiated by CHMP6, polymerizes CHMP4 (notably CHMP4B) to bend membranes, and engages CHMP1/2/3/8 to complete constriction and scission; the AAA ATPase VPS4 then disassembles ESCRT-III for recycling (15).

A recent study by Sharma et al. (2025) has demonstrated that proteins involved in the ESCRT pathway play a crucial role in MSC-EVs production, with ALIX identified as a central regulatory protein in biogenesis. Sequential siRNA-mediated knockdown of these proteins revealed their significant contributions to MSC-EVs formation and secretion. For instance, ALIX knockdown resulted in a substantial reduction in MSC-EVs yield, whereas its presence was shown to enhance MSC-EVs production by approximately 3-4 times in BM-MSCs and 5-7 times in WJ-MSCs (16).

The findings of Stuffers et al. (2009) demonstrated that ESCRT silencing in mammalian cells does not impede ILVs formation, suggesting the presence of an ESCRT-independent mechanism in MSC-EVs biogenesis (17). Tetraspanins, such as CD9 and CD63, are transmembrane proteins that regulate EV biogenesis independently of ESCRT. Furthermore, the study by Chairoungdua et al. (2010) revealed that CD9 gene knockout in mice resulted in a significant reduction in exosome production by dendritic cells compared to those derived from wild-type mice, highlighting the essential role of CD9 in the biogenesis of MSC-EVs (18). Similarly, CD63 deficiency leads to diminished MSC-EVs secretion (19). Other ESCRT-independent regulators include enzymes like neutral sphingomyelinase 2 (nSMase2), which catalyzes ceramide production to induce membrane invagination, and phospholipase D2 (PLD2), which generates phosphatidic acid (PA), a lipid promoting membrane curvature (20).

MSC-EVs biogenesis is closely regulated by interactions between endosomes and other organelles such as the endoplasmic reticulum (ER), mitochondria, and the trans-Golgi network (TGN). Micro RNAs (miRNAs), critical components of MSC-EVs cargo, are selectively incorporated into ILVs through recognition motifs and RNA-binding proteins (RBPs) including AGO2, YBX1, and MEX3C (21). ALIX, an ESCRT-associated protein, interacts with AGO2 to facilitate miRNA loading without affecting ILV quantity. YBX1, which contains a cold shock domain, initially forms protein condensates before anchoring to endosomes to assist miRNA loading. MEX3C binds to AP-2, supporting clathrin-mediated endocytosis and recruiting miRNAs either directly or via other RBPs (22, 23).

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Following maturation, MVBs either fuse with lysosomes for degradation (degradative MVBs) or with the plasma membrane to release ILVs as MSC-EVs (secretory MVBs).

Rab GTPases regulates this trafficking. Rab5, activated by Rabex-5/Rabaptin-5, initiates early endosome maturation, followed by Rab7 recruitment for lysosomal trafficking. Rab7 inactivation promotes sMVBs formation and fusion with the plasma membrane, enabling MSC-EVs release (11, 24). The fusion of MVBs with the plasma membrane is mediated by SNARE proteins, Rab GTPases, and tethering factors. SNARE proteins, characterized by a conserved motif, form trans-SNARE complexes between vesicular (v-SNARE) and target (t-SNARE) membranes, facilitating membrane fusion. Post-fusion, the cis-SNARE complex is disassembled by NSF ATPase and α-SNAP to recycle SNAREs for subsequent fusion events (25). Overall, the biogenesis of MSC-EVs is illustrated in Figure 1.

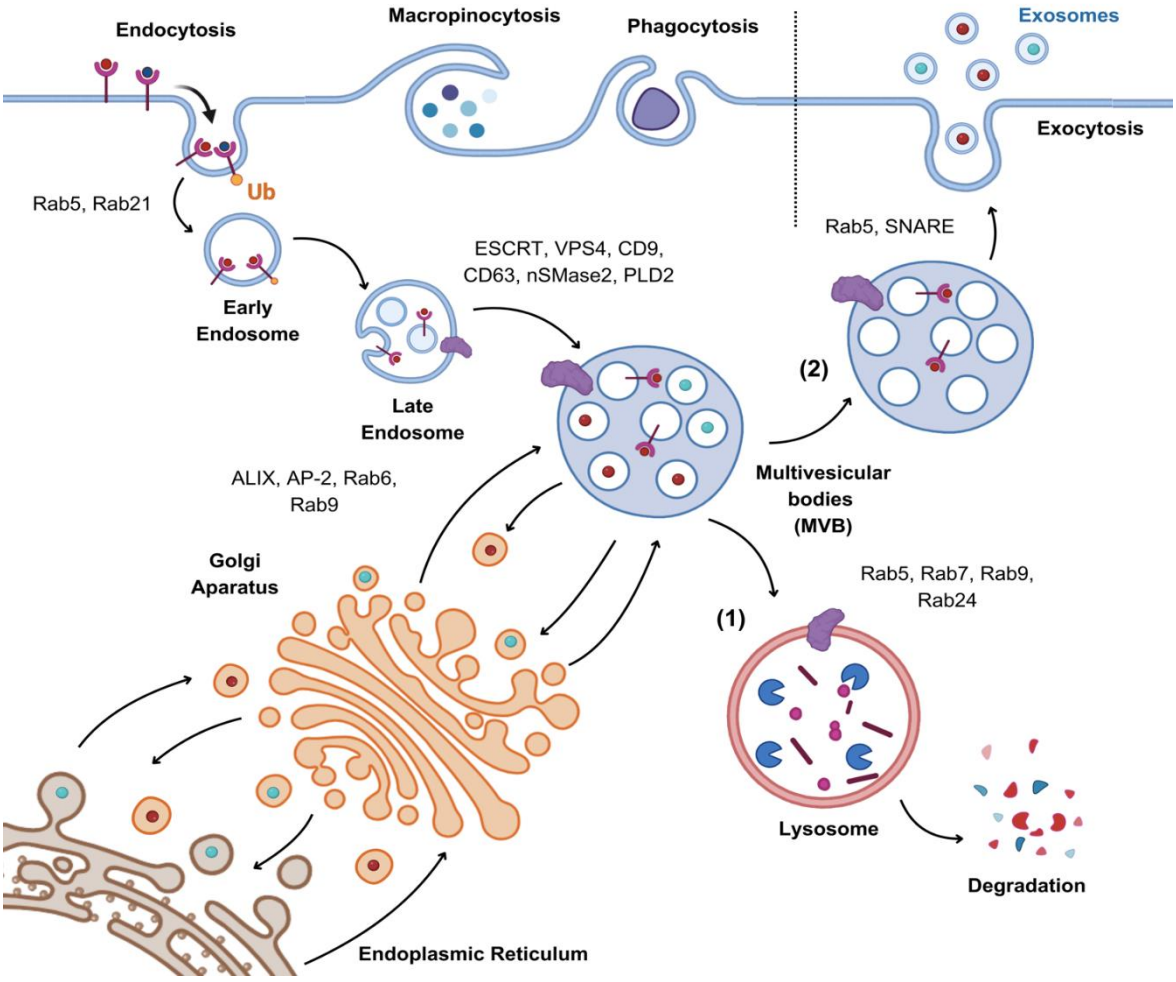

Figure 1 Overview of the biogenesis and trafficking pathways of MSC-EVs. The pathways depicted represent well-established mechanisms in mesenchymal stem cells (MSCs). A critical step in MSC-EVs biogenesis is the formation of multivesicular bodies (MVBs) proceed via both ESCRT-dependent and ESCRT-independent mechanisms. Upon maturation, MVBs can follow one of two potential fates: (1) fusion with lysosomes for degradation, or (2) fusion with the plasma membrane for secretion as exosomes (MSC-EVs). Fusion of MVBs with the plasma membrane is mediated by SNARE complexes that drive membrane docking and bilayer merger. Figure created with BioRender.

Isolation and Characterization

MSC-EVs are typically collected from cell-conditioned medium (CCM), the MSC-derived secretome. MSCs are typically cultured until they reach 70-80% confluence, after which the CCM is collected for EVs isolation. However, the composition and

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quality of CCM are highly sensitive to in vitro culture conditions. Culture media often contain exogenous EVs or vesicle-like structures that can interfere with downstream analyses. Therefore, supplements should be EV-depleted, and microbial contaminants such as *Mycoplasma* spp. must be eliminated to ensure the purity and biological relevance of isolated EVs.

MSC-EVs isolation approach includes ultracentrifugation, immunoaffinity-based methods, precipitation, microfluidics, size-based separation (filtration), and commercially available kits. The comparison of MSC-EVs isolation methods has been comprehensively discussed by Nikfarjam et al. (2020) (26). Differential ultracentrifugation (dUC) remains the gold standard for isolating MSC-EVs based on size and density via sequential centrifugation steps (27). This approach is fundamentally based on the separation of biological particles according to their size and density. In dUC, samples are subjected to stepwise increases in speed and duration, separating particles based on their sedimentation coefficients into pellets and supernatant at each stage. The method consists of three main steps: (1) initial low-speed centrifugation to remove cells and large debris; (2) intermediate-speed centrifugation to eliminate larger vesicles and apoptotic bodies; and (3) high-speed centrifugation to pellet the MSC-EVs.

An example of dUC protocol developed by Fiolin et al. (2022) is presented in Figure 2.

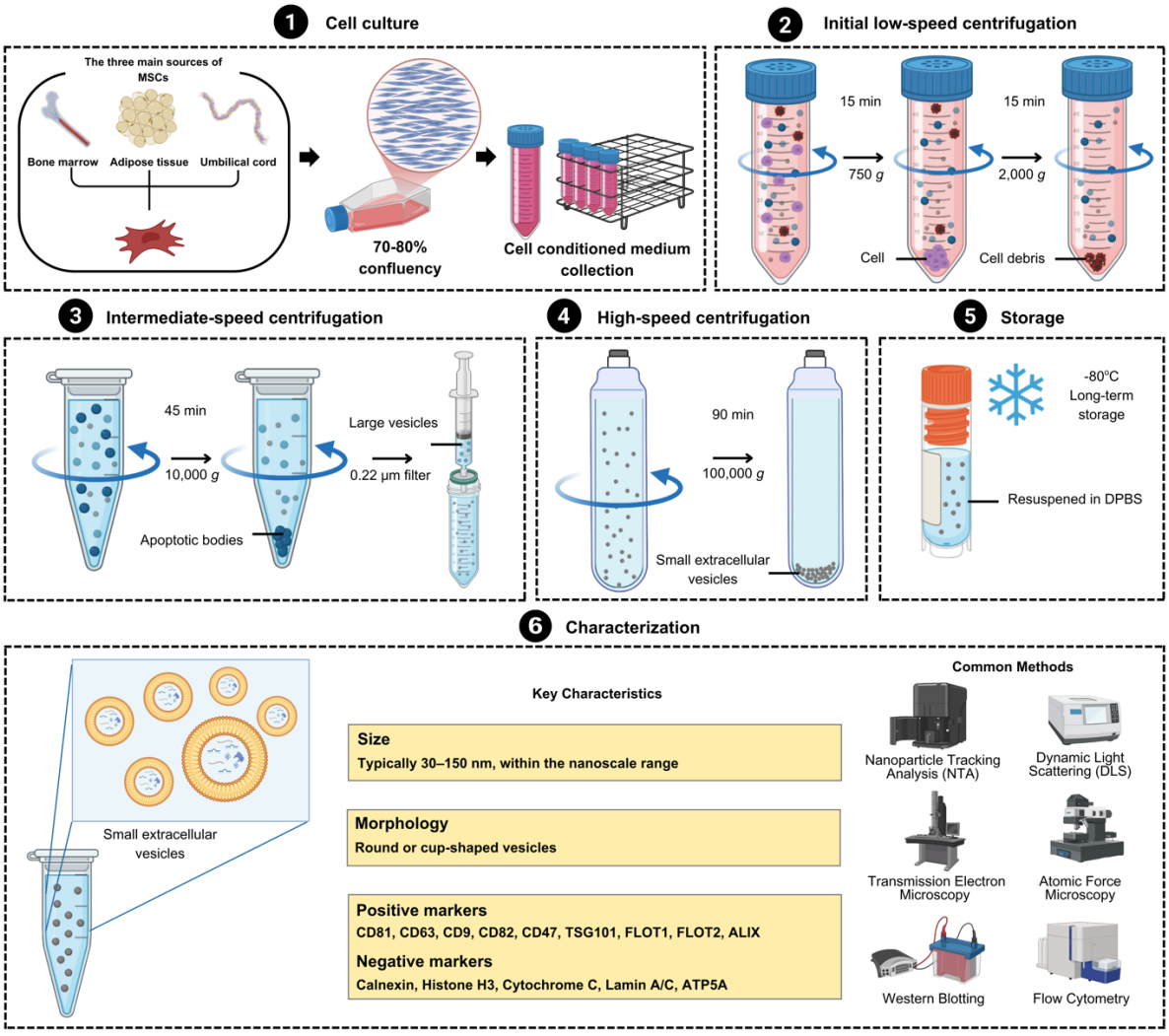

Figure 2. Workflow for Isolation, Storage, and Characterization of MSC-EVs. Conditioned medium was collected from *in vitro*—expanded MSCs at 70-80% confluence, and MSC-EVs were isolated by differential ultracentrifugation consisting of sequential low-speed spins (750-2,000 *g*), an intermediate-speed spin (10,000 *g*), and a high-speed spin (100,000 *g*). Isolates were stored at -80ºC without repeated freeze-thaw cycles and characterized in accordance with the MISEV guidelines. Figure created with BioRender.

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In Fiolin et al. (2022) (27) MSC-EVs were isolated through a multi-step density ultracentrifugation protocol. The first step involved centrifugation at 750 *g* and 2,000 *g* for 15 minutes using S-4-72 swing-bucket rotor (Eppendorf) and 50 mL tubes. The second step was performed at 10,000 *g* for 45 minutes using 24 × 1.5/2.0 mL rotor with ClickSeal™, 45° angle (Thermo Scientific), followed by filtration with a 0.22 μm filter. Finally, MSC-EVs were isolated by ultracentrifugation at 100,000 *g* for 90 minutes using T-865 fixed angle rotor, 23,5° angle (Thermo Scientific).

Despite its advantages, including simplicity, and cost-effectiveness, dUC lacks standardized protocols and often yields heterogeneous vesicle populations. The dUC approach, as reported by Ansari et al. (2024), Yields MSC-EVs with higher functionality compared to other methods such as ultrafiltration and precipitation. However, clinical-scale exosome therapy faces challenges in scalability, as it requires specialized equipment and is not easily applicable for large-scale production. The MISEV 2023 guidelines emphasize the need for precise documentation of centrifugation parameters—speed, rotor type, time, and temperature—to ensure reproducibility and data validity (28, 29).

Fiolin et al. (2022) demonstrated that exosomes isolated from adipose-derived MSCs (AD-MSCs) using dUC exhibit an average size of 88.7 nm, carry a negative zeta potential, and express CD63 and CD81 (28). According to MISEV 2018, valid EV identification requires detection of at least two positive markers, including transmembrane proteins (CD63, CD81, CD82) and cytosolic proteins (TSG101, ALIX, flotillin 1/2, HSC70, HSP84), as well as negative markers indicative of non-EV contaminants (e.g., albumin, APOA1/2). Recently, Arbade et al. (2024) have proposed additional EVs markers such as IMMT, cytochrome C, histones, and lamin A/C to improve characterization specificity (12, 30). In brief, the guidelines for MSC-EVs characterization based on MISEV 2018 and 2023 are presented in **Table 1**.

Tendon Injury and Regeneration

Tendon injury refers to structural damage or pathological alterations within a tendon resulting from acute mechanical trauma, such as tendon rupture, or from chronic degenerative conditions collectively classified as tendinopathy. The term tendinopathy is also understood as a pathological condition that represents as "failed healing response" to tendon injury. These conditions may arise due to mechanical overuse, aging, and systemic complications associated with metabolic syndrome, including obesity and type 2 diabetes mellitus (31).

Tendon pathology is characterized by changes in two major components: the cellular compartment (tenocytes) and the extracellular matrix (ECM). Approximately 85% of tendon ECM is composed of collagen fibers, with 97-98% consisting of type I collagen, which is typically aligned in a compact and parallel manner (31). During pathological conditions, this collagen fiber architecture undergoes degradation, disorganization, and disorientation, resulting in a non-parallel and separated structure, along with the replacement of collagen fibers with non-collagenous ECM components (32).

Furthermore, morphological alterations in tenocyte nuclei—from elongated and flattened to rounded shapes—serve as indicators of tendon pathology. These nuclear shape changes, along with increased cytoplasmic volume, reflect a gradual chondroid transformation and the transdifferentiation of tendon stem cells into other cell types (32). Tendon injury may also be marked by osteochondrogenesis, followed by calcification and increased deposition of ground substance (33).

Persistent tendon degeneration may ultimately lead to tendon rupture and permanent disability, which is often difficult to heal. Tendon injuries possess an inherent capacity for healing. However, the repair process is protracted, typically requiring 1 to 2 years for complete restoration. The duration and efficacy of healing are influenced by factors such as the patient's age and the severity of the injury. The healing of tendon injuries involves three overlapping phases, which parallel the structural changes observed in wound healing: inflammation, proliferation, and remodeling. Each phase is regulated by distinct cellular and molecular mediators that coordinate the histodynamic process of tendon repair (34).

In preclinical *in vivo* studies, small animals (e.g., rodents and rabbits) exhibit a rapid progression of tendon repair: the inflammatory phase lasts approximately 5 to 7 days, the proliferative phase peaks around 7 to 21 days, and remodeling begins

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shortly thereafter, continuing for several weeks. In contrast, large animal models (e.g., sheep and horses) experience a longer inflammatory phase, typically lasting 1 to 3 weeks, with the proliferative phase peaking at 2 to 6 weeks, and remodeling extending over several months to years. Regarding angiogenesis, small animals generally exhibit the onset of neovascularization within 2 weeks, whereas large animals may require several months to achieve comparable outcomes. It is important to note that each phase may overlap and is significantly influenced by the specific conditions of the study. The duration of tendon healing should be considered a critical factor in determining the timing of therapeutic interventions, which may be aimed at accelerating or resolving the corresponding phases (35-38).

Table 1. The Guidelines for MSC-EVs Characterization

Exosome Biomarkers	
General Characterization	**1. At least three positive protein markers for MSC-EVs. The positive markers used should include at least one from each of category's a-b.** Conservative Markers a. Transmembrane/lipid-bound protein CD9, CD63, CD81, CD82, CD47, MHC-I, MHC-II, ADAM10, TSAP6, TRF2 b. Cytosolic protein TSG101, ALIX, VPS4A/B, FLOT1/2, HSC70, HSP84 Optional c. Contaminant control Lipoprotein (ApoA1, ApoB), immunoglobulin, albumin, HSP90AA/B, HSPA13, LDHA/B d. Transmembrane proteins, lipids, or soluble proteins associated with intracellular compartments LMNA/C, CYC1, CANX, Grp94, BIP, GM130, ACTN1/4 e. Secretory proteins associated with the exosome corona EGF, FN1, VEGFA, FGF1/2 **2. At least one negative protein marker.** CD34, CD45, AGO2 Biomarker analysis can be performed using several methods, such as Western Blotting, ELISA, and flow cytometry.
Single Particle Analysis	
Electron microscopy	**At least one morphological analysis should be conducted using electron microscopy techniques.** For example, use: TEM, SEM, cryo-EM, AFM. Characteristics: MSC-EVs have a morphology that is typically round, cup-shaped, or elliptical, with variations in shape occurring under certain conditions.
Non-electron microscope-based	**At least one size analysis should be conducted using non-electron microscopy techniques.** For example, use: NTA, DLS. Characteristics: MSC-EVs have a size in the nanometre range, typically between 30-150 nm.

Immediately following injury, the stromal compartment of the tendon undergoes necrosis, which triggers chemotaxis and the infiltration of pro-inflammatory immune cells, such as neutrophils and macrophages.

This inflammatory response facilitates the clearance of necrotic cell debris, stimulates angiogenic factors, and promotes collagen secretion by tenocytes. The inflammatory phase typically lasts from several hours to a few days and resolves through the polarization of pro-inflammatory M1 macrophages into anti-inflammatory M2 macrophages.

Timely resolution of inflammation is essential to prevent prolonged acute inflammation, which can impair tendon regeneration. The second phase, proliferation, involves the recruitment and

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proliferation of fibroblasts, which enhance the production of type III collagen. This collagen is rapidly secreted to form a provisional matrix that temporarily replaces the damaged tissue. Concurrently, angiogenesis is upregulated to ensure adequate oxygen and nutrient supply to the repair site. The third phase, remodeling, encompasses both catabolic and anabolic activities aimed at restoring tissue homeostasis and achieving tendon maturation.

The initially deposited type III collagen, which is relatively disorganized and biomechanically inferior, is gradually degraded and replaced by type I collagen, known for its superior biomechanical strength and cross-linking capacity. The balance of collagen production and matrix remodeling is regulated by matrix metalloproteinases (MMPs), which degrade and reorganize the ECM components (34, 39).

A study by Xu et al. (2023) highlighted that inflammation and angiogenesis are two critical factors essential for the healing of tendon pathologies (7). Recent evidence further suggests that facilitating the resolution of inflammation during the early phase of tendon repair constitutes a promising therapeutic strategy to enhance healing outcomes and restore the structural integrity of the tendon. Tendon injury induces a localized hypoxic environment accompanied by a robust inflammatory response, particularly within the first 48 hours post-injury. Endothelial cells from blood vessels adjacent to the injured tendon secrete pro-inflammatory mediators that facilitate leukocyte recruitment and amplify the inflammatory cascade. Infiltrating immune cells subsequently release a variety of bioactive molecules, including pro-angiogenic factors, which initiate and sustain neovascularization. The upregulation of angiogenesis within the tendon microenvironment serves as an adaptive mechanism to mitigate hypoxia and establish a regenerative niche conducive to cellular proliferation, ECM remodeling, and overall tissue regeneration.

Tendon Inflammation

Inflammation constitutes the initial and essential biological response following tendon injury, serving a critical role in initiating tissue repair. This response is primarily driven by the release of damage-associated MSC-EVsmolecular patterns (DAMPs) from stressed tenocytes and tendon progenitor stem cells (TPSCs). Key DAMPs such as S100A8/A9, HMGB1, and heat shock proteins (HSP27 and HSP70) act as endogenous alarmins that activate pattern recognition receptors—including TLR4, TLR2, and RAGE—triggering the NF-κB signaling cascade. This results in the production of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6), chemokines (e.g., CCL2, CXCL10), and matrix-degrading enzymes (e.g., MMPs), all of which contribute to the inflammatory microenvironment (40). HMGB1 promotes macrophage recruitment and ECM remodeling through NLRP3 inflammasome activation and disrupts TPSC differentiation via the RAGE/β-catenin pathway, sustaining inflammation and fibrosis (41-43). Similarly, S100A8/A9 proteins amplify leukocyte infiltration and cytokine secretion, while extracellular HSPs modulate innate immune responses and cell survival under stress conditions (44-48).

The inflammatory milieu is further shaped by the dynamic interplay between resident immune cells—such as tenophages and mast cells—and infiltrating immune populations, particularly M1 macrophages, neutrophils, and T cells. Resident tenophages initiate immune surveillance and monocyte recruitment via NF-κB–dependent CX3CL1 expression, while mast cells contribute to neurogenic inflammation and ECM degradation through mechanosensitive interactions with tenocytes.

Persistent M1 macrophage dominance delays resolution of inflammation and transition to regenerative M2 macrophages (49-52). T cells, particularly IL-17A–producing subsets, form direct interactions with tenocytes, perpetuating a pro-inflammatory feedback loop through TRAF6/NF-κB signalling and promoting the expression of fibrosis-related genes. This chronic inflammatory state impairs the tendon's healing capacity, contributes to ECM disorganization, and underlies the pathogenesis of degenerative tendon disorders (4, 53, 54).

Several studies also reported that biomechanical parameters (e.g., Young's modulus, strain ratio, and shear wave velocity) measures correlate with inflammatory infiltration and macrophage-driven matrix remodelling, providing quantitative links between inflammation and mechanical deficit (55, 56).

Mechanistically, macrophage-mediated ECM degradation and subsequent fibrosis alter collagen content, cross-linking, hydration, and fiber architecture, thereby modulating stiffness and viscoelasticity via mechanotransduction (e.g., YAP, integrins) and enzymatic activity (e.g., MMPs), with

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changes detectable by elastography. Thus, these biomechanical readouts serve as non-invasive biomarkers of inflammatory burden and ECM remodelling.

Given that prolonged inflammation constitutes a central pathological hallmark and a key impediment to tendon healing, the identification of critical inflammatory mediators offers valuable insight into potential therapeutic strategies. The key molecular and cellular factors that have emerged as promising targets for future interventions in tendon injury are summarized in Table 2.

These mediators, particularly DAMPs, not only serve as danger signals but also play a pivotal role in regulating broader immune signalling pathways. MSC-EVs, which carry bioactive molecules, can influence inflammation either by modulating these pathways or directly interacting with DAMPs to enhance immune regulation. For example, MSC-EVs carrying miR-146a can downregulate DAMPs such as HMGB by modulating the TLR4 signalling pathway (57).

Table 2 Key Molecular and Cellular in Tendon Inflammation

Molecular/ Cellular Factor	Category	Role in Tendon Inflammation and Pathology	Ref
S100A8 & S100A9 (calgranulins)	Alarmin/DAMP	Potently induce local inflammatory responses, increase vascular permeability, promote monocyte recruitment, and amplify inflammation through the induction of chemokines and cytokines (e.g., CCL2, IL-6, IL-8).	(40)
HMGB1	Alarmin/DAMP	Release during necrosis or actively secreted by immune cells; engages TLR4 and RAGE to amplify inflammation, promote ECM remodeling, activate the NLRP3 inflammasome, and dysregulate TPSC differentiation toward osteogenic lineage.	(41, 43, 53)
HSP27 & HSP70	Alarmin/DAMP	Secreted by stressed or apoptotic tenocytes; modulate innate immune responses, promote immune cell recruitment, and prevent apoptosis, but may also sustain chronic inflammation if unresolved.	(46, 47)
NLRP3	Inflammasome	Mediates caspase-1 dependent maturation of IL-1β; contributes to persistent inflammation, ECM degradation, and impaired tissue regeneration.	(42)
NF-κB	Transcription factor	Central regulator of pro-inflammatory cytokines, chemokines, catabolic enzymes, and angiogenic factors; its dysregulation is closely associated with chronic tendon inflammation.	(44, 45, 48)
IKKβ	Enzyme	A catalytic subunit of the IKK complex; its overexpression induces severe ECM disorganization, macrophage accumulation, and tendon weakening.	(44)
Macrophages (M1 subtype)	Innate immune cell	Predominantly present during the early inflammatory phase; produce pro-inflammatory mediators and delay the transition to M2 macrophages and regulatory T cell, hindering resolution and repair.	(49, 50)
Tenophages	Innate immune cell	Hybrid cells co-expressing immune and tendon markers; mediate immune surveillance, promote monocyte recruitment via CX3CL1, and regulate inflammation, angiogenesis, and fibroblast proliferation.	(51)
CX3CL1 (fractalkine)	Chemokine	Expressed by tenocytes and tenophages; facilitates monocyte recruitment and contributes to angiogenesis and tissue remodeling via NF-κB signaling.	(51)
Neutrophils	Innate immune cell	Contribute to ECM degradation through the release of proteolytic enzymes; their prolonged presence exacerbates tendon degeneration.	(49, 50)
Mast cells	Innate immune cell	Involved in neurogenic inflammation and pain signaling; activated by tenocyte-derived substance P via MRGPRX2, promoting the release of inflammatory mediators and MMPs.	(52)

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Molecular/ Cellular Factor	Category	Role in Tendon Inflammation and Pathology	Ref
T cells	Adaptive immune cell	Actively recruited by tenocytes during inflammation; produce IL-17A, enhancing chronic inflammation, ECM disorganization, and fibrosis through TRAF6/NF-κB signaling.	(4, 53, 54)
IL-17A	Cytokine	Drives fibroblast activation and fibrotic gene expression; induces CCL20 secretion, sustaining T cell recruitment and perpetuating a pro-inflammatory microenvironment.	(4, 53, 54)

Tendon Angiogenesis

Neovascularization is the process of forming new blood vessels within tissue, which occurs through two primary mechanisms: vasculogenesis, the de novo assembly of blood vessels during embryogenesis or in pathological conditions such as ischemia and cancer (58); and angiogenesis, which typically occurs in response to tissue injury through sprouting from existing vessels (59).

In tendon pathology, neovascularization contributes to the restoration of blood supply to damaged tissue and is typically initiated alongside early inflammatory responses (60). However, unlike other connective tissue, excessive angiogenesis is not considered beneficial in tendons. Healthy tendons are characteristically hypo vascular or avascular, whereas pathological tendon often exhibit abnormal hypervascularity (8).

The regulation of neovascularization, particularly the balance between pro- and anti-angiogenic signals, is a critical consideration in conditions such as Achilles tendinopathy (59). Angiogenesis is markedly upregulated during the proliferative phase of tendon healing and gradually declines during the remodelling phase. This dynamic regulation ensures that new blood vessels from when needed for tissue repair but are restricted during the maturation of the healed tissue.

Injury-induced sprouting angiogenesis involves three main stages—hypoxia, elongation, and anastomosis—coordinated by the modulation, specification, and migration of endothelial cells from adjacent vasculature to construct new vascular branches (58).

This process is initiated by hypoxia, which triggers HIF-1α accumulation and VEGF-A upregulation, enhancing microvascular permeability and promoting the specification of tip cells (TCs) with high Dll4 and low Notch activity to guide vessel sprouting along VEGF-A gradients. TC selection is mediated by VEGFR, Notch, and Dll4 signalling, while neighbouring stalk cells (SCs) express higher Notch-1 and VEGFR1 to support directional elongation (61). VEGF-A also facilitates the recruitment of inflammatory cells and MMP production, enabling extracellular matrix degradation and endothelial migration (9).

EphrinB2–EphB4 signalling further enhances TC migration and vessel maturation via the PI3K/Akt pathway, working synergistically with VEGF to regulate sprouting and vascular remodelling (62). Angiogenic sprout elongation is primarily driven by endothelial cell proliferation regulated by VEGF and placental growth factor (PIGF). VEGF binds to three receptor tyrosine kinases—VEGFR1, VEGFR2, and VEGFR3—with VEGFR2 playing the central role in promoting angiogenesis.

Upon VEGF binding, VEGFR2 undergoes autophosphorylation at Y951 and Y1175, initiating a signalling cascade involving PLCγ, PKC, and the Ras/Raf/MEK/ERK pathway. This cascade activates transcription factors such as c-Fos and CREB, ultimately enhancing endothelial proliferation necessary for vascular elongation (63).

Anastomosis, the fusion of newly formed vessels with pre-existing vasculature, is essential for establishing functional blood circulation during angiogenesis and occurs via two main types: lumenized (type I) and non-lumenized (type II) (58, 64). Both begin with the formation of filopodial contacts between tip cells and adjacent endothelial cells, stabilized by adherens junction proteins like VE-cadherin at apical membrane initiation sites (AMISs).

Type I anastomosis involves the expansion and merging of two lumens under intraluminal pressure, while type II involves luminal fusion without blood flow, relying on apical membrane insertion and coalescence of intracellular luminal vacuoles. Pericytes, recruited by endothelial-derived PDGF-B,

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contribute to vessel stabilization through secretion of ANG-1, which activates the TIE-2 receptor to suppress VEGF-A responsiveness and promote vascular maturation (58).

This process is further regulated by TGF-β signalling via Alk1 and Alk5, where Alk1 promotes proliferation and Alk5 supports differentiation and stabilization by reducing VEGFR2 and enhancing VEGFR1 expression (9, 65).

Effects of MSC-EVs on Tendon Inflammation and Angiogenesis

Lessons Learned from Diverse *in vivo* Study Designs

The eleven studies reviewed adhered to the majority of the ARRIVE guidelines in their reporting, facilitating a comprehensive assessment of their scientific validity. These results are presented in Figure 3 and 4. Although not all studies full adhered to ARRIVE items, each study covered more than 50% of the items, with an average compliance rate of 65.6%. This suggests that all studies exhibited a low risk of bias. All studies documented receiving ethical approval from the relevant institutional animal care and use committees or regulatory bodies for the use of animal models. However, not all studies explicitly stated adherence to national or international guidelines aimed at ensuring the welfare of the animals. Despite this omission, the overall methodological rigor and ethical considerations reflected in the studies provide a solid foundation for their scientific merit.

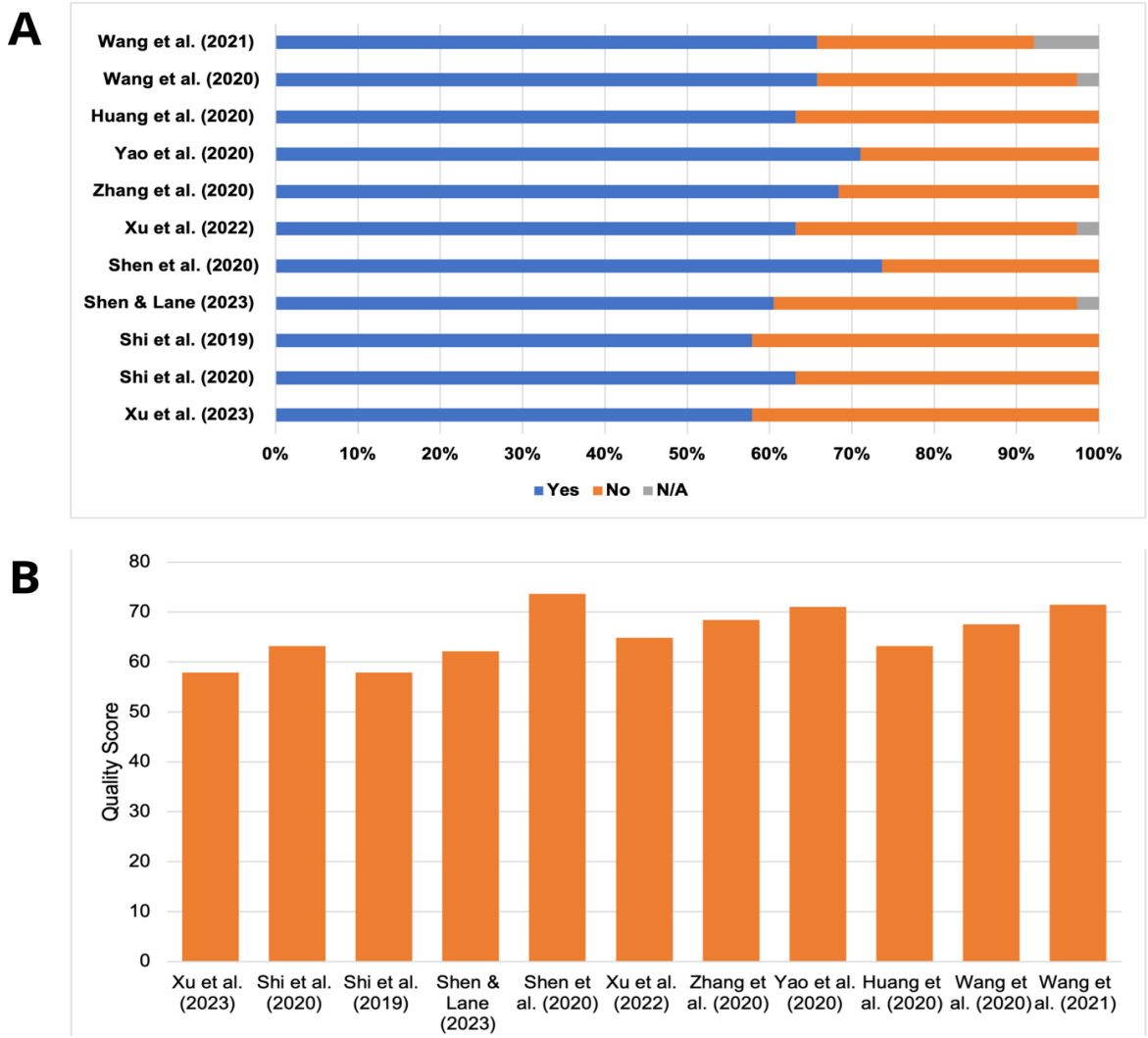

Figure 3. ARRIVE-Based Reporting Quality by Study. (A) Proportion of ARRIVE item reported (Yes), not reported (No), and not applicable (N/A) for each included study. (B) The overall quality-assessment score for

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each study (0-100%), calculated as the percentage adherence to the ARRIVE guidelines. No study achieved a perfect score or fully reported all ARRIVE items. The highest score was observed for Shen et al. (2020), at 74%, whereas the lowest was for Xu et al. (2023), at 58%. Across the 11 studies, the mean score was 65.6%.

A critical review of the 11 studies analyzed revealed several notable strengths. Fifteen items were fully reported in all studies (Figure 4), including the abstract, background, objectives, and outcomes, which were presented clearly and comprehensively. These components provided a solid foundation for interpreting the findings and understanding their scientific implications. However, the review also highlighted certain areas where information was lacking, particularly in relation to proper randomization, animal allocation, and methods for estimating sample size. These aspects are crucial for ensuring the reproducibility of preclinical research and minimizing potential biases. In light of these gaps, the studies still adhered to procedural standards appropriate of *in vivo* research, offering valuable insights that contribute meaningfully to the field.

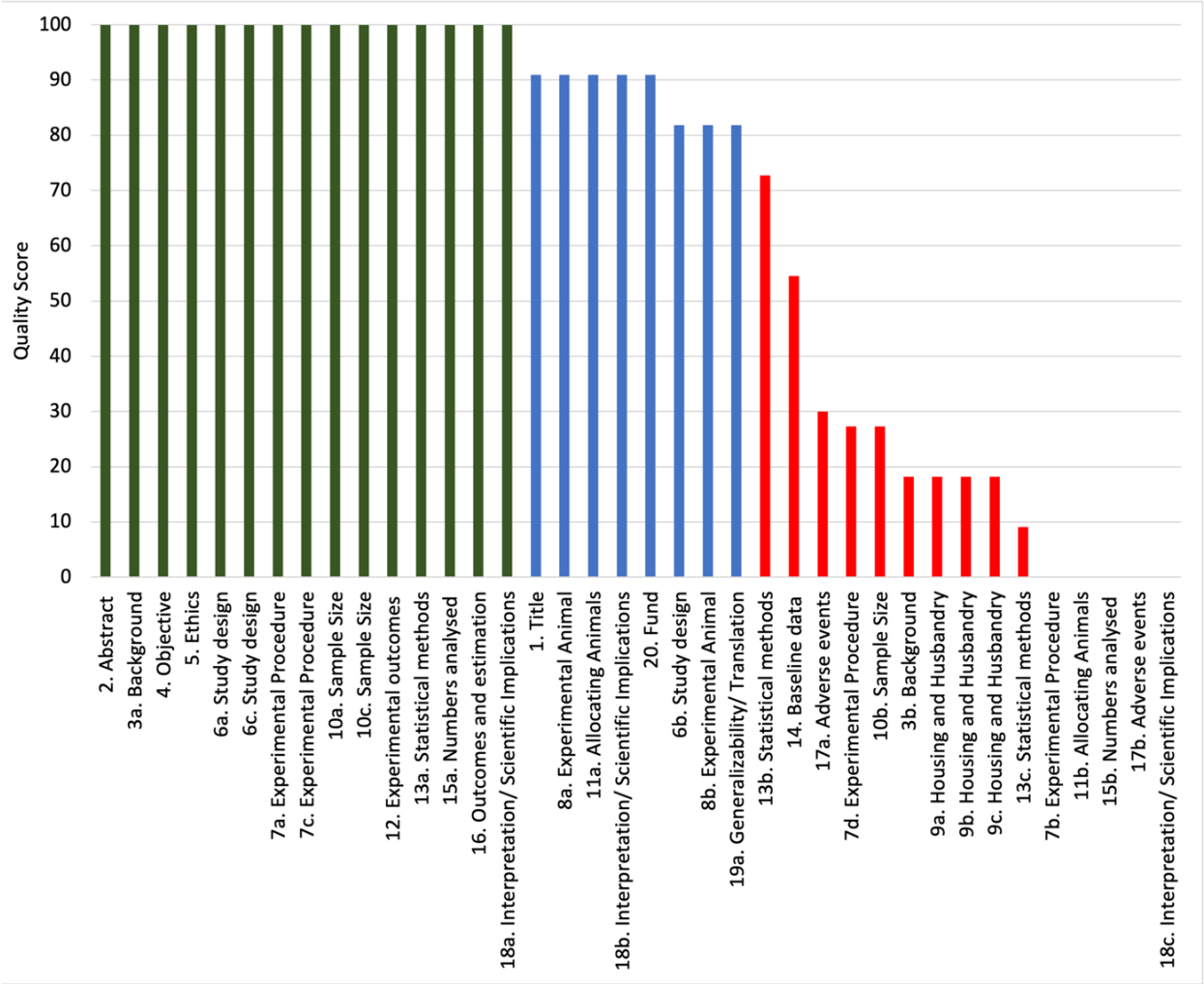

Figure 4. Item-Level Adherence to the ARRIVE Checklist Across Studies. Fifteen items exhibited perfect adherence (100%; green) across all studies. Eight items fell into the high-adherence category (80-99%; blue), and fifteen items showed moderated-to-low adherence (<80%; red). Among these lower-adherence items, five were universally unreported (score = 0): the time of day at which experiments were conducted (7b, experimental procedures); the order in which animals across experimental groups were treated and assessed (11b, allocating animals); the number of animals excluded throughout the study (15b, numbers analysed);

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protocol modifications implemented to mitigate adverse events (17b, adverse events); and a description or appraisal of how the methods relate to the 3Rs—replacement, refinement, and reduction (18c, interpretation/scientific implications).

Regarding animal model selection, only two of the 11 studies reviewed provided a clear rationale for their choice of model and its relevance to human biology. The majority did not justify the selection criteria. It is important to note that model selection may depend on institutional preferences, available resources, and the specific objectives of the study. Access to animal models is a key factor in determining the feasibility of *in vivo* research, with practical constraints often influencing the choice of species. As no single animal models can fully represents human tendon pathology, the justification for the chosen model should align explicitly with the study's objectives and be thoroughly documented. While rodents are appropriate for early-stage studies, larger animals, such as sheep and horses, are more suitable translational research due to their anatomical similarities to humans. Researchers should consider both the practical aspects of animal model access and the scientific objective when selecting the most appropriate model for their studies.

Although not all studies provided comprehensive details on housing conditions and environmental settings of the animals, it is important to recognize that ethical approval inherently ensures adherence to stringent animal welfare standards.

These standards typically encompass appropriate housing, controlled environmental conditions, and proper care practices to maintain the animal's well-being. While the absence of explicit reporting on these aspects may limit transparency, the ethical review process guarantees that the studies were conducted in accordance with the necessary animal care guidelines, as required by both national and international regulations.

In addition, even though the health status and pathogen-free conditions of the animals were not always directly reported, these criteria are also implicitly addressed during the ethical review process, which rigorously ensures that such standards are met before granting approval. Therefore, the absence of detailed reporting on these factors should not be viewed as a significant limitation, as the ethical approval process assures that all required welfare criteria are fulfilled. Future research should focus on enhancing the adequacy of reporting within study design to ensure greater clarity, reproducibility, and precision in the interpretation of results.

All studies reviewed were preclinical *in vivo* experiments evaluating MSC-EVs in tendon inflammation and angiogenesis. Given the variability in study design and reporting, we assessed study quality and risk of bias according to the ARRIVE (Animal Research: Reporting of *in vivo* Experiments) guidelines. The quality of the included studies was evaluated using the ARRIVE checklist, reformulated into 38 items by Abbas et al. (2021) (66). The full details of this quality assessment are provided in the Supplementary Material (Supplementary Table S1 and S2).

Effects of MSC-EVs on Tendon Inflammation

Recent preclinical biomedical research has aimed to explore and develop cell-free therapy strategies utilizing MSC-EVs to enhance tissue healing and regeneration. These strategies offer several advantages over conventional MSC-based therapies, including the unique nanoparticle characteristics of EVs that allow for superior penetration of biological barriers, the potential for large-scale production and cost-effective storage, reduced ethical concerns, and the potential for off-the-shelf availability. Several in vivo studies have reported the positive effects of MSC-EVs therapy on tendon healing through the regulation of inflammation, as summarized in Table 3.

Several sources of MSCs utilized in the research include bone marrow (10, 65), adipose tissue (67, 70, 71), tendon (69), and umbilical cord (68). The administration of MSC-EVs was performed across a wide range of doses, depending on the route of administration and the model employed. The injury models employed in the studies were diverse, including patellar tendon injury, Achilles tendon transection, tendon adhesion models, and bone-tendon junction injuries, demonstrating the therapeutic effects of MSC-EVs across various types of tendon injuries and anatomical locations.

The mechanism of MSC-EVs in promoting tendon injury healing has been reported to occur through the enhancement of macrophage polarization from the pro-inflammatory subtype (M1) to the anti-inflammatory

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subtype (M2). Shi et al. (2019) observed a reduction in M1 macrophages, marked by CCR7, alongside an increase in M2 macrophages, marked by CD163 (33). Similar findings were reported by Zhang et al. (2020), using the same markers; Shi et al. (2020), who used iNOS for M1 and Arg1 for M2; and Xu et al. (2023), who utilized CD86 as a marker for M1 and CD206 for M2 (7, 10, 69). These conditions led to alterations in the cytokine profile and accelerated the transition from the inflammatory phase to the proliferative phase, thereby preventing chronic inflammation that could result in tissue degeneration.

Table 3 Studies on the Effects of MSC-EVs on Tendon Inflammatory Responses

Animal Model Groups			EVs Therapeutic Group		Control Group	Follow -up	Outcomes	Ref
Animal	Injury Model	Location	Source of MSC-EVs	Dose of EVs				
Rat	Surgically tendon defects	Patellar Tendon	Bone Marrow	25 μg EVs on 10 μL of fibrin glue	1. Fibrin glue without EVs 2. Defect with no treatment	2 and 4 weeks	BM-MSC-EVs elevated CD163+ macrophage cells (M2; anti-inflammation) and reduced CCR7+ macrophage cells (M1; pro-inflammation) significantly compared to control group at 4 weeks after treatment. The administration of EVs also inhibit IL-6 secretion at week 2 and promote IL-4 and IL-10 mRNA expression at week 4 post-treatment.	(65)
Mouse	Surgically tendon defects and repair	Achilles Tendon	Adipose (naïve and IFN-γ primed)	$5\text{-}6 \times 10^9$ EVs on collagen sheet	Defect with no treatment	1, 3, and 7 days	Primed AD-MSC-EVs exhibit superior effects compared to naïve AD-MSC-EVs in suppressing early tendon inflammatory responses. The administration of EVs elevated NF-κB on the first day after repair and retained at 3 days after repair. EVs significantly suppressed NF-κB expression on day 7 post-repair compared to the control (without EVs). The administration of EVs also suppressed the	(67)

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Animal Model Groups			EVs Therapeutic Group		Control Group	Follow -up	Outcomes	Ref
Animal	**Injury Model**	**Location**	**Source of MSC-EVs**	**Dose of EVs**				
							secretion of pro-inflammatory cytokines, such as TNF and IL-1β.	
Mouse	Surgically tendon-bone junction defects and repair	Achilles tendon	Bone marrow	1×10^6 EVs dissolved in PBS and loaded with 300 μL hydrogel	1. Hydro gel without EVs 2. Defect with no treatment	7 days	BM-MSC-EVs induces the polarization of macrophage subtype 1 (iNOS+) into macrophage subtype 2 (Arg1+), reduces pro-inflammatory factors (IL-1β and IL-6), and enhances anti-inflammatory factors (IL-10 and TGF-β1) at 7 days post-EVs injection,	(10)
Rat	Surgically tendon defects	Achilles Tendon	Umbilical cord (miRNA-21-3p engineered)	200 μg EVs dissolved in 50 μL PBS	1. PBS without EVs 2. Defect with treatment	3 weeks	UC-MSC-EVs (low-abundance miR-21a-3p) inhibited the levels of inflammation-related genes p-65 and COX2.	(68)
Rat	Surgically tendon defects	Achilles Tendon	Tendon	200 μg EVs on 30 μL of gelatin methacryloyl (GelMA)	1. GelMA without EVs 2. Defect with no treatment	1 week	TSC-EVs combined with GelMA elevated CD163+ macrophage cells and reduced CCR7+ macrophage cells at 1 week post-treatment. TSC-EVs also increased anti-inflammation cytokines (IL-10) and decreased pro-inflammation cytokines (IL-6 and COX-2).	(69)
Mouse	Surgically tendon defects and repair	Achilles tendon	Adipose (IFN-γ primed)	1×10^9 EVs on collagen sheet (low dose) 5×10^9 EVs on collagen sheet (high dose)	Defect with no treatment	28 days	High dose EVs were more effective than low dose EVs in suppressing early tendon inflammatory responses. EVs injection modulates toll-like receptor 4 (TLR4)/NF-κB inflammatory signaling pathway	(70)

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Animal Model Groups			EVs Therapeutic Group		Control Group	Follow -up	Outcomes	Ref
Animal	Injury Model	Location	Source of MSC-EVs	Dose of EVs				
							through miR-147-3p.	
Rat	Surgically tendon defects and repair	Achilles tendon	Adipose (bioactive glass primed)	80 μL EVs on 20 μL of gelatin methacryloyl (GelMA)	1. Normal 2. Defect with no treatment 3. GelMA without EVs 4. GelMA with naïve EVs	3, 7, 14, and 28 days	AD-MSC-EVs induces the polarization of macrophage subtype 1 (CD86+) into macrophage subtype 2 (CD206+) earlier than other groups, beginning as early as 7 days post-injection.	(7)

Note: Dosing metrics for MSC-EVs are reported variably across studies, including particle count, total protein, or micrograms. Due to differences in measurement methods, sample preparation, and reporting standards, these metrics cannot be reliably converted into a single standardized unit. Values are presented as reported by the authors.

The therapeutic action of MSC-EVs occurs in target cells within tendon tissue, including resident and infiltrating macrophages that rapidly increase and accumulate following injury. Macrophage cellular uptake of MSC-EVs can occur through various mechanisms, as Horibe et al. (2018) reported that the cellular uptake mechanisms of EVs may vary depending on the recipient cells and the specific characteristics of the target organ (72). Since there are no specific reports on the cellular uptake mechanism of MSC-EVs by macrophages, several comparative studies have reported multiple mechanisms involved in the cellular uptake of MSC-EVs by recipient cells, including clathrin-mediated endocytosis (CME), micropinocytosis, caveolin-dependent endocytosis (CDE), and membrane fusion (73). The presence of integrin CD29 and tetraspanin CD81 is suspected to play a crucial role in EVs binding and uptake (74).

As mentioned before, MSC-EVs modulate macrophage phenotype toward an anti-inflammatory state. Representative cargo-target relationship include TGF-β1-enriched MSC-EVs engaging the miR-132/Mycbp2/TSC2 axis (75); miR-21-5p from IL-4-primed hUC-MSCs repressing PDCD4 *in vitro* and *in vivo* (76); miR-24 targeting KEAP1 to disrupting the Nrf2/HO-1 pathway (77); miR-23a-3p suppressing IRF1 (78); miR-486-5p acting through PIK3RI within the PI3K/Akt pathway (79); and miR-139-3p downregulating Stat1 (80). Collectively, these examples illustrate cargo-encoded routes by which MSC-EVs dampen pro-inflammatory signalling and support tissue repair.

The suppression of M1 macrophage populations within tendon tissue leads to a reduction in key pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-1β, while enhancing anti-inflammatory cytokines, including IL-10, IL-4, and TGF-β1. One of the signalling pathways involved in the secretion of inflammatory cytokines is the transcription factor NF-κB-mediated signalling. As reported by Shen & Lane (2023), miR-147-3p carried by MSC-EVs inhibits TLR/NF-κB signalling, thereby suppressing the expression of IL-1β and IL-6, as demonstrated *in vitro* and supported by *in vivo* findings; causal confirmation *in vivo* remains limited (70).

Additionally, NF-κB activation is also inhibited by miR-181d derived from BM-MSC-EVs, which targets KLF6 (81). Several other studies have further demonstrated the downregulation of NF-κB signalling: miR-511-3p reduces inflammation through the modulation of the TRAF6/S1P/NF-κB pathway (82), miR-146a suppresses inflammation by inhibiting the TRAF6/IRAK1 axis (83), and miR-30e initiates downregulation of NF-κB p65/Caspase-9 signalling by targeting LOX1 (84). While several miRNAs have been associated with the modulation of inflammatory signalling in tendon healing, most findings originate from single studies. Targeted *in vivo* loss-or gain-of-function experiments are warranted to validate their causal roles, and current evidence should be considered

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exploratory. As an overview, the effects of MSC-EVs on tendon inflammation are presented in Figure 5.

Based on the studies summarized in Table 3, several modifications have been implemented to enhance the therapeutic effects of MSC-EVs in tendon healing. MSC-EVs loaded with gelatine metacryloyl (GelMA) and collagen sheets demonstrated superior therapeutic effects by extending the retention time of MSC-EVs at the injury site, promoting tissue regeneration, and suppressing inflammation (7, 67, 70). The use of MSC-EVs with chitosan/β-glycerophosphate/collagen hydrogels, as reported by Yang et al. (2017) and Shi et al. (2020), significantly improved tendon regeneration compared to MSC-EVs administration without hydrogels (10, 39).

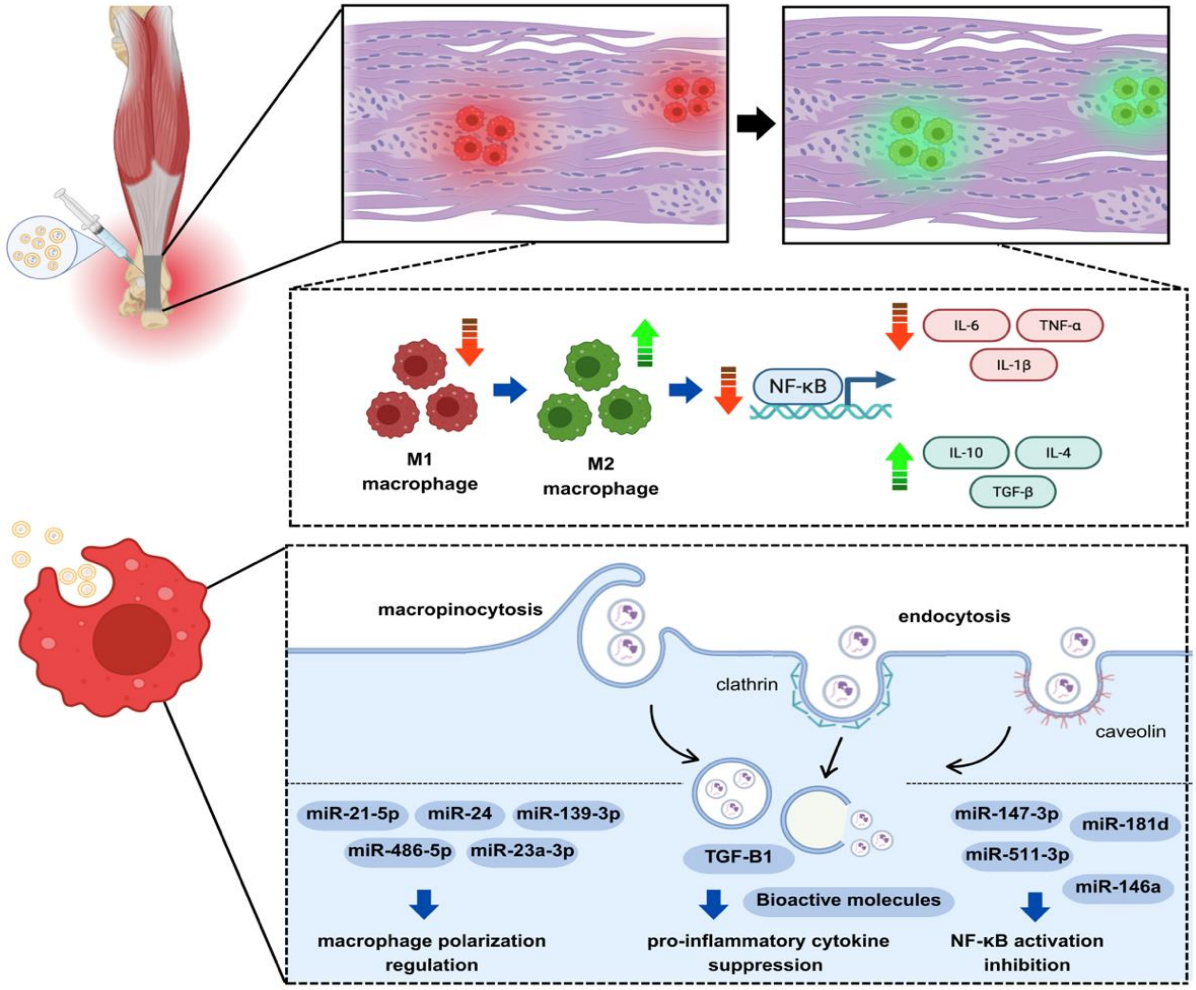

Figure 5. Effects of MSC-EVs on Tendon Inflammation. MSC-EVs act as "intercellular communication mediators" by carrying various biomolecules, particularly miRNA. MSC-EVs deliver their cargo into macrophage cells through endocytosis or other mechanisms, regulating macrophage polarization to resolve inflammation. Figure created with BioRender.

At the cellular level, pre-conditioning MSCs with inflammatory or bioactive conditions, such as IFN-γ or bioactive glass, enhanced the immunomodulatory and regenerative potential of EVs by upregulating specific miRNA expression, including miR-21a-3p and miR-21-5p, which were proven to inhibit inflammatory signalling pathways both *in vitro* and *in vivo* (11, 67, 70). However, it is important to note that priming can substantially alter MSC-EVs cargo and bioactivity compared to naïve MSC-EVs, highlighting the need for appropriate experimental control, such as unprimed EVs, conditioned medium lacking EVs, and vehicle-only treatments, to accurately delineate the effects attributable to priming. Collectively, these methods enhance healing effects and suppress tendon inflammation, providing valuable insights and

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practical approaches that can be utilized in future research to further explore and optimize MSC-EVs for tendon regeneration.

Effects of MSC-EVs on Tendon Angiogenesis

Angiogenesis is a dynamic factor that plays a critical role in determining the success of tendon healing and regeneration. Several preclinical studies investigating the effects of MSC-EVs on angiogenesis in tendon healing are presented in Table 4.

The administration of MSC-EVs to tendon has been shown to exert a modulatory effect on vascular dynamics, specifically through the regulation of angiogenesis. Huang et al. (2020) reported a significant enhancement of angiogenesis at the tendon-bone interface at 4 weeks post-implantation of MSC-EVs in a rodent model of rotator cuff defects (85). Conversely, other studies have documented a reduction in vascular density in tendons treated with MSC-EVs (64, 86, 87). Xu et al. (2023) further elucidated the temporal dynamics of angiogenesis, demonstrating an initial surge in angiogenic markers at day 7-post MSC-EVs implantation, which progressively declined at days 14 and 28 (7). These variations across studies may be attributed to differences in observation periods and study model.

Table 4. Studies on the Effects of MSC-EVs on Tendon Angiogenesis Responses

Animal Model Groups			EVs Therapeutic Group		Control Group	Follow-up	Outcomes	Ref
Animal	**Injury Model**	**Location**	**Source of MSC-EVs**	**Dose of EVs**				
Rat	Collagenase-induced tendinopathy	Achilles tendon	Adipose	1×10^{10} EVs	1. Saline 2. Ectosome	3 and 5 weeks	A significant difference in angiogenesis score was observed between the control group and the groups injected with ectosomes or exosomes. The lowest angiogenesis score was recorded in the exosome group.	(71)
Rat	Surgically rotator cuff defects	Rotator cuff tendon-bone interface	Bone marrow	200 μg of total protein	PBS without EVs	4 weeks	MSC-EVs enhance the expression of CD31 (endothelial cells) and endomucin around the tendon-bone junction and promote angiogenesis around the rotator cuff at 4 weeks post-reconstruction and implantation.	(85)
Rabbit	Surgically rotator cuff defects	Rotator cuff tendon-bone interface	Adipose	1×10^{11} EVs on 20 μL NaCl	NaCl without EVs	18 weeks	A significant difference in the number of blood vessels per low-power field was observed between	(86)

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Animal Model Groups			EVs Therapeutic Group		Control Group	Follow-up	Outcomes	Ref
Animal	**Injury Model**	**Location**	**Source of MSC-EVs**	**Dose of EVs**				
							the EVs-injected group and the control group. The EVs-injected group exhibited lower vascularization.	
Mouse	Overuse-induced tendinopathy	Rotator cuff	Adipose	1×10^{11} EVs on 10 μL NaCl	NaCl without EVs	4 weeks	A significant difference in the number of blood vessels per low-power field was observed between the EVs-injected group and the control group. The EVs-injected group exhibited lower vascularization.	(87)
Rat	Surgically tendon defects and repair	Achilles tendon	Adipose (bioactive glass primed)	80 μL EVs on 20 μL of gelatin methacryl oyl (GelMA)	1. Normal 2. Defect with no treatment 3.GelMA without EVs 4.GelMA with naïve EVs	3, 7, 14, and 28 days	The administration of MSC-EVs enhanced angiogenesis, as indicated by increased IHC expression of CD31+ and α-SMA on day 7 post-implantation, followed by a gradual decline from day 14 to day 28.	(7)

Note: Dosing metrics for MSC-EVs are reported variably across studies, including particle count, total protein, or micrograms. Due to differences in measurement methods, sample preparation, and reporting standards, these metrics cannot be reliably converted into a single standardized unit. Values are presented as reported by the authors.

Angiogenesis is observed to increase during the initial phase of tendon healing, primarily in response to acute inflammatory conditions that induce hypoxia. To resolve hypoxic conditions, an increase in blood vessel formation is required. Huang et al. (2020) utilized a model of the tendon-bone interface, a region characterized by high demand for blood supply to facilitate tissue healing (85).

The observed increase in vascularization is believed to be more critical at the tendon-bone interface than within the tendon itself, which may explain its persistence up to 4 weeks post-treatment. In contrast, within the internal tendon, Xu et al. (2023) reported a transient increase in blood supply following MSC-EVs injection, which gradually declined over time (7). This dynamic pattern reflects the natural course of vascular regression following initial tissue repair. Since degenerative tendinopathy is associated with persistent angiogenesis, effective tendon healing is characterized by vascular degradation and the downregulation of angiogenesis at later stages. This vascular reduction typically occurs between 2 to 4 weeks post-injection in murine models and up to 18 weeks in rabbit models (7, 71, 86, 87).

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Järvinen et al. (2020) stated that the characteristic of excessive vascularization in tendinopathy is associated with vascular leakage, presumably resulting from incomplete maturation and anastomosis of neovascular structures. This condition induces persistent hypoxia, leading to unresolved tissue degeneration (8). Consequently, angiogenesis in tendons is widely considered a target for suppression to achieve optimal healing. We propose a concurrent investigation of angiogenesis and tissue hypoxia across multiple time points to elucidate histodynamic processes. While CD31 is commonly used to quantify neovascularization, it alone may not distinguish between immature, leaky vessels and functional, mature vasculature. Therefore, vessel maturation and stability should also be assessed using markers such as VE-cadherin, TIE-2, and ANG-1 alongside CD31. Pericyte markers, such as NG2, PDGFR-β, and α-SMA, are also commonly employed in the analysis of angiogenesis.

In addition to selecting markers for angiogenesis evaluation, the choice of method is crucial in evaluating tendon angiogenesis. Many studies rely on basic histological techniques (e.g., Hematoxylin-Eosin staining) to quantify blood vessels, which can introduce bias as they do not differentiate whether the vessels are pre-existing vessels or newly formed (neovascular). Given that angiogenesis is the process of new blood vessel formation, simply counting vessels in HE-stained images becomes irrelevant for representing angiogenesis unless accompanied by comparative or additional strong evidence. Furthermore, some studies, such as those by Huang et al. (2020) and Xu et al. (2023), have utilized more specific methods, including immunohistochemistry or immunofluorescence for markers such as CD31, endomucin, and α-SMA (7, 85). Huang et al. (2020) also applied micro-CT analysis for three-dimensional vascular reconstruction. These methods provide superior analysis for evaluating tendon angiogenesis.

The best practices for quantifying angiogenesis in tendon *in vivo* involve integrating multimodal imaging techniques (e.g., micro-CT) with molecular assays (e.g., immunohistochemical staining for specific markers) to capture both structural and functional vascular parameters. In parallel, 3D histological image reconstruction and stereology approaches can be applied to enhance the quality of angiogenesis evaluation. Stereology offers unbiased or minimally biased estimates of three-dimensional structures from two-dimensional images, ensuring accurate and reliable data, which are critical for quantifying aspects such as volume, vessel density, and length density, thus providing a comprehensive understanding of angiogenesis (88).

The mechanism of action of MSC-EVs in modulating angiogenesis is believed to be mediated by their miRNA content. Specifically, miR-7846-3p, miR-100-5p, and miR-1246 have been reported to inhibit angiogenesis by suppressing NRP2 expression and downregulating VEGF-A. Additionally, miR-7846-3p has been shown to inactivate Hedgehog signaling, a pathway critical for endothelial cell proliferation and migration during angiogenesis. MiR-15a, miR-181b, miR-320c, miR-874, miR-199b-3p, and miR-125a-5p have also been demonstrated to induce the downregulation of genes involved in angiogenesis. These findings have been supported by preclinical *in vitro* studies using relevant cell lines, such as HUVEC, tumour endothelial cells, and fibroblast, as well as *in vivo* studies conducted on rodents and rabbits (7, 89, 90).

Overall, temporally regulated angiogenesis is essential for effective tendon healing, particularly in the context of chronic tendinopathy. In such cases, suppression of excessive vascularization is beneficial because persistent angiogenesis leads to pathological tissue remodelling, scar formation, and biomechanical deterioration. However, during the early stages of tendon repair, transient angiogenesis is critical to provide the necessary nutrients and support tissue regeneration. Properly timed angiogenic responses allow for tendon repair by balancing the formation of new blood vessels without exacerbating inflammation and matrix degradation. Thus, a therapeutic approach targeting the timing and specificity of angiogenesis modulation presents a promising strategy for tendon healing. Future research should focus on refining these strategies to optimize clinical outcomes while minimizing adverse effects, ensuring a more controlled and effective healing process (9, 91).

MSC-EVs are believed to possess a temporal regulatory mechanism for vascular dynamics, modulating angiogenesis according to the tissue healing phase and hypoxic conditions. Under this mechanism, an initial increase in angiogenesis is triggered as a compensatory response to tissue hypoxia resulting from acute inflammation. This is

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subsequently followed by vascular degradation and the inhibition of angiogenesis, driven by the resolution of hypoxia and the restoration of normoxic conditions. The modulatory effects of MSC-EVs on angiogenesis during tendon healing are summarized in Figure 6, illustrating how MSC-EVs can regulate the balance between angiogenesis and tissue repair throughout the healing process.

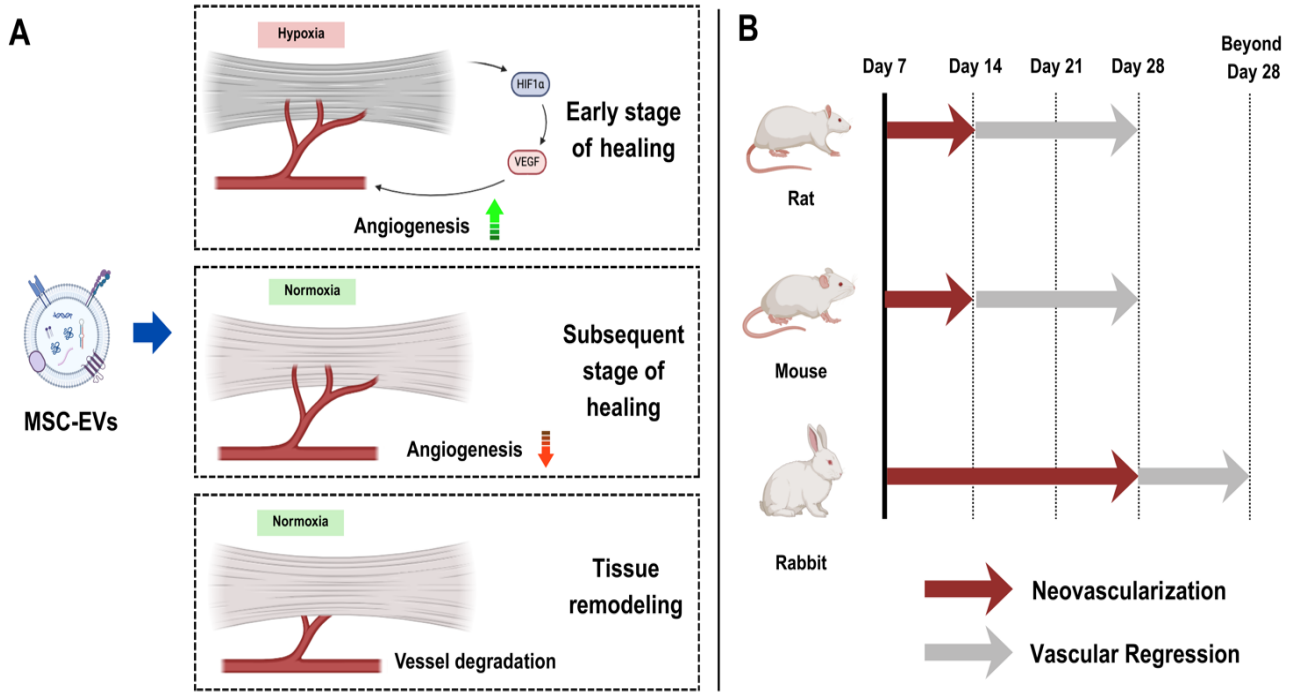

Figure 6 Effects of MSC-EVs on Tendon Angiogenesis. (A) Schematic illustration of MSC-EVs effects on tendon angiogenesis. MSC-EVs modulate tendon angiogenesis, promoting early-stage angiogenesis to resolve hypoxia, followed by suppression in later stages as tissue normoxia is restored. (B) Comparative timeline of temporal angiogenesis across three animal models. Timelines are estimated from available studies and may vary with pathology, EVs dose, and experimental design. Figure created with BioRender.

Challenges and Future Directions

MSC-EVs have emerged as a promising therapeutic modality for tendon healing, offering a cell-free alternative with substantial clinical potential. As nanovesicles, MSC-EVs possess superior characteristics that enhance healing capacity and therapeutic efficiency in terms of both production and cost. The mechanism of action MSC-EVs is primarily attributed to their ability to penetrate target cells and deliver biological molecules, such as miRNAs, which regulate tissue dynamics.

Our findings have demonstrated that MSC-EVs enhances tendon healing through the regulation of two critical mechanisms: inflammation and angiogenesis. MSC-EVs effectively modulate the cellular and molecular dynamics of immune cells, including cytokine expression, to facilitate appropriate inflammatory resolution. Moreover, MSC-EVs are capable of regulating angiogenesis and vascular reduction by adapting to tissue conditions and the ongoing phase of the healing process. These regulatory effects are both tissue- and phase-dependent, a cross-tissue comparison is warranted to delineate how MSC-EVs behaviour diverges across tendon, ligament, and cartilage. In tendon, repair is characterized by MSC-EVs-mediated-macrophage polarization, whereby a transient early shift from M1 to M2 phenotypes coincides with a brief early angiogenic burst that is dispensable at later stages. Ligament healing shares certain features (e.g., promotion of collagen alignment and inflammation resolution) but the spatiotemporal dynamics of angiogenesis is incompletely characterized. Given their dense-regular connective-tissue architecture and structural similarity to tendons, we advance the working hypothesis that ligament healing follows a broadly similar MSC-EVs-regulated histodynamic, particularly in the coordination of inflammatory and angiogenic phase (92, 93).

In addition to its advantages as a cell-free therapeutic modality capable of modulating

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inflammation and angiogenesis, MSC-EVs-based therapy is significantly limited by the heterogeneity in dosing. This lack of uniformity in dosing protocols presents considerable challenges in the accurate interpretation of dose-response relationships and in facilitating reliable comparisons across studies. To address these issues, the adoption of standardized reporting metrics is crucial. The MISEV guidelines provide a comprehensive framework for standardizing MSC-EVs research (12, 29).

These guidelines recommend specific metrics for dose reporting, including particles per millilitre (particles/mL) by nanoparticle tracking analysis (NTA), total protein content in micrograms (μg), and the particle-to-protein ratio. Using NTA to report MSC-EVs concentration ensures a consistent and quantifiable measure of MSC-EVs dose, while reporting total protein content provides a standardized metric for comparing MSC-EVs preparations across studies. The inclusion of the particle-to-protein ratio offers additional insights into the purity and composition of MSC-EVs samples, thus facilitating better cross-study comparisons.

In tendon applications, the administration of MSC-EVs is generally performed through local injection into the tendon area. For this purpose, MSC-EVs can be resuspended in PBS or DPBS (without calcium and magnesium). In addition to considering the particle dose of MSC-EVs in the solution suspension, the volume of the suspension is also an important consideration. An excessively high suspension volume may cause tendon swelling and structural damage, potentially introducing bias in the interpretation and conclusion of the study. We recommend conducting volumetric studies to determine the optimal volume that can be injected into the tendon without causing significant anatomical changes. The latest paradigm also highlights that the therapeutic role of MSC-EVs is largely mediated by miRNA. Adequate quantification of miRNA and its correlation with suspension volume could also serve as a critical consideration for future studies. By evaluating the expression of miRNAs in relation to different suspension volumes, it may be possible to optimize the therapeutic efficacy of MSC-EVs in tendon regeneration (92).

Numerous studies have reported various modifications to improve the characteristics of MSC-EVs, resulting in superior outcomes in terms of inflammation control and angiogenesis. However, further research on MSC-EVs modification strategies is essential, particularly concerning their specific mechanisms of action or target phases during the healing process. Engineered EVs, derived from the modification of native EVs, represents a forward-looking strategy to enhance the specificity and therapeutic efficacy of MSC-derived EVs (Figure 7). The development of engineered EVs can be strategically focused on: (1) improving cargo specificity and targeted delivery; (2) increasing particle stability and retention time within target tissues; and (3) optimizing additional functional attributes to promote tissue repair and regeneration. Advanced engineering strategies, such as hybrid modification of MSC-EVs with synthetic nanovesicles like liposomes or the incorporation of specific surface proteins that target cells or tissues, may be employed to enhance the precision and efficacy of MSC-EVs therapy.

Major challenges in MSC-EVs-based therapies include their stability, which is highly influenced by storage conditions. Further exploration of preservative solutions or effective lyophilization methods is warranted to maintain MSC-EVs integrity. To date, storage at -20 to -80^{o}C has been shown to preserve both structural integrity and functional activity of MSC-EVs for up to six months. Additionally, MSC-EVs face challenges in delivery and controlled release. Their rapid clearance and limited retention at target sites necessitate the development of efficient delivery systems. Engineering approaches investigated in previous studies, including hydrogels and biomaterial scaffolds such as GelMA, collagen sheets, and chitosan hydrogels, have demonstrated enhanced retention time and improved localization within tendon tissue (7, 39, 67, 69, 70). GelMA-based carriers generally provide a retention time of 7 to 14 days, which can be further extended through reinforcement strategies such as microfiber incorporation or composite formation.

Among the three carrier types, GelMA exhibits superior biocompatibility and effectively supports cell adhesion and proliferation, making it the most widely used strategy for MSC-EVs delivery. Collagen sheets and chitosan hydrogels can achieve longer retention times, ranging from 14 to 30 days or more, when modified with PEG, gelatine, or combined as composites. However, while most biomaterials are biocompatible and typically do not induce significant inflammation, some materials still carry a potential risk of inflammation. The risk depends on factors such as

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crosslinking, surface chemistry, stiffness, and topography, all of which can be optimized to minimize immune activation and enhance tissue integration (94, 95). Given that hydrogels and biomaterial scaffolds offer a promising strategy to enhance MSC-EVs retention and delivery to tendons, several challenges remain. First, optimizing the loading of MSC-EVs into biomaterials is necessary for effective results, with limited data on optimal concentrations for GelMA and chitosan hydrogels. Second, the complex interplay between scaffold design MSC-EVs requires a tailored approach for each application. The porosity, mechanical strength, and biochemical composition of the materials significantly impact loading efficiency and release kinetics. Third, the biodegradability of biomaterials also affects the release profile of MSC-EVs into the tissue. For example, GelMA undergoes approximately 43% weight loss within 14 days, whereas collagen sheets and chitosan hydrogels may vary depending on their composition and chemical bonding. Addressing these challenges will be crucial for optimizing MSC-EVs delivery and improving the therapeutic efficacy of biomaterial-based treatments for tendon regeneration (96, 97).

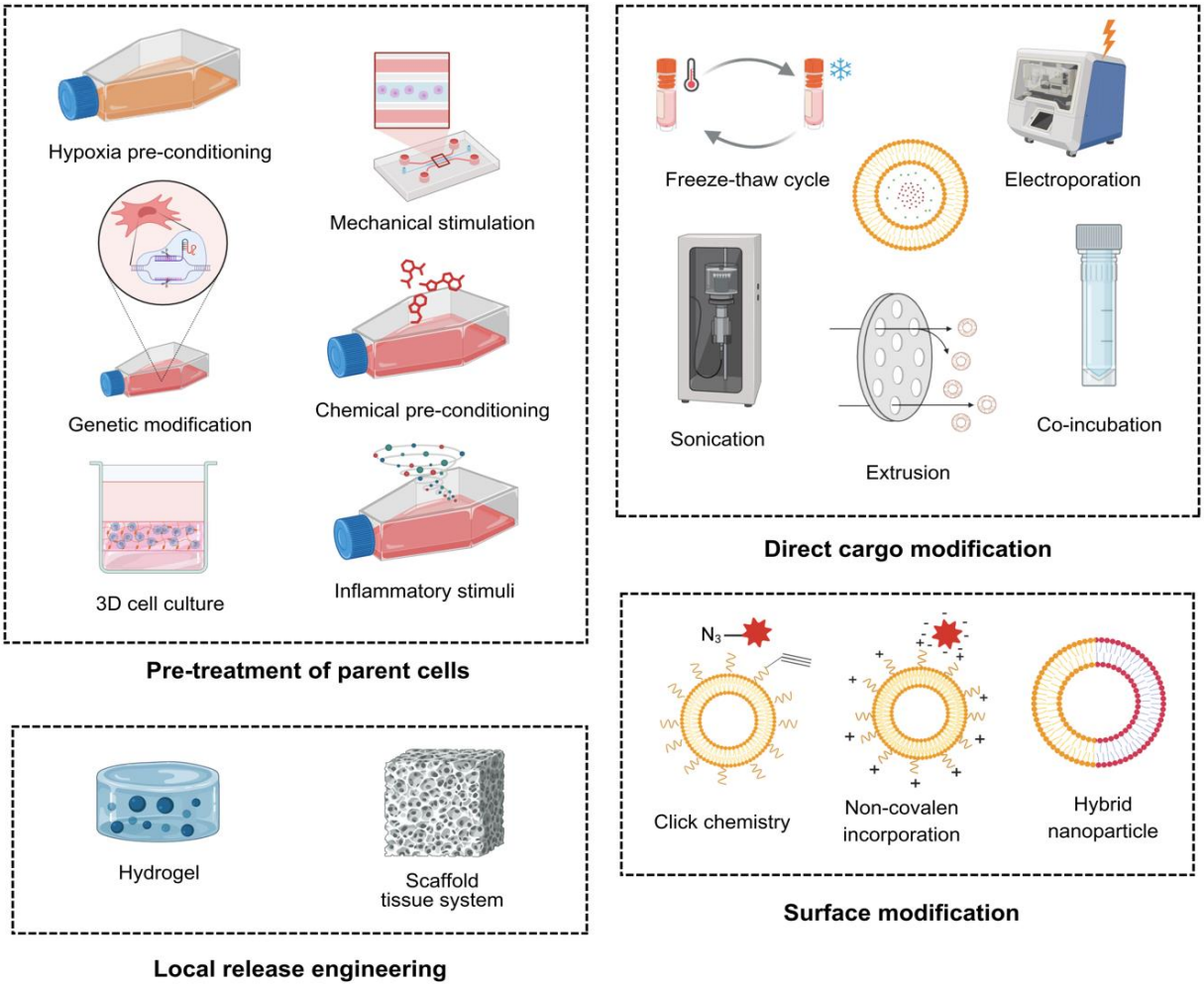

Figure 7. Engineering Strategies of MSC-EVs. Four principal approaches are commonly employed to engineer MSC-EVs: (1) pre-treatment of parent cells, (2) local release engineering, (3) direct cargo modification, and (4) surface modification. Figure created with BioRender.

Future directions in the development of MSC-EV-based therapies requires standardized protocols for EVs isolation and characterization to ensure clinical-grade quality, yield, and safety. We recommend conducting sterility testing (endotoxin, bacteria, fungi, *Mycoplasma*, adventitious virus), as MSC cultures are often susceptible to microorganisms' contamination. Furthermore, reporting culture conditions is critical, as the presence of specific contaminants may influence the characteristics of MSC-EVs and introduce bias into

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the study. Although MSC-EVs are reported to have low tumorigenic and toxicity risk, the evaluation of MSC-EVs quality and potential side effects must be thoroughly reviewed to ensure the safety of the therapy. Additional research is required to confirm their effectiveness and safety prior to their use in clinical settings (98, 99). Comprehensive cargo profiling using multi-omics approaches is essential for understanding therapeutic mechanisms. The expansion of recent omics branches, such as transcriptomics and miRNomics, has become highly relevant for evaluating the molecular therapeutic mechanisms of MSC-EVs in tendon regeneration.

Insights into specific miRNAs targeted toward tendon regeneration provide an advanced paradigm for clinical-stage therapies. Moreover, advancing toward clinical translation requires multidisciplinary collaboration, the establishment of a clear regulatory framework, and the achievement of key clinical milestones, such as demonstrating safety and efficacy in GLP-compliant animal studies, establishing biodistribution patterns, optimizing dosing protocols, and ensuring compliance with regulatory guidelines for biologics and advanced therapy medicinal products (ATMPs) (98, 99).

The development of potency assays is also crucial for evaluating the therapeutic potential of MSC-EVs in tendon repair. A potency assay for tendon repair should integrate biomechanical testing (tensile strength and load to failure), histological analysis (histomorphometry, 3D histology, or stereology analysis), molecular markers (inflammation and angiogenesis), and non-terminal longitudinal functional gait analysis. Identifying appropriate functional readouts will help correlate MSC-EVs-mediated effects with tissue repair outcomes.

In summary, MSC-EVs represent a promising cell-free strategy for promoting tendon tissue regeneration. This therapeutic potential is primarily attributed to their roles in modulating inflammation and enhancing angiogenesis. Future exploration holds significant potential for clinical translation and the rational engineering of MSC-EVs to further optimize their therapeutic efficacy.

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