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Exosomes Derived from Mesenchymal Stem Cells in the Treatment of Animal Tendon Injuries: A Review on Their Isolation and Application

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Abstract

Tendon injuries are a major part of musculoskeletal injuries in animals, particularly in horses. So far, no complete cure has been found for this disease, and most treatments focus on pain control. The advantages of using exosomes over cell-based therapies and the effects of mesenchymal stem cells (MSCs) on tissue repair suggest exosomes derived from MSCs as an appropriate treatment option in repairing tendon injuries. This paper aimed to review various protocols for exosome isolation and the role of MSCs- derived exosomes on tendon tissue repair of animals, especially in horses. In the treatment of tendon disorders, exosomes are more stable than cells, have a lower risk of immune rejection after allogeneic administration, and can be used as an appropriate alternative therapy. Exosomes derived from MSCs of different sources stimulate the proliferation and migration of tenocytes and fibroblasts, modulate collagen fiber arrangement, macrophage functions, and inflammatory responses, inhibit adhesion, and generally repair damaged tendons. Exosomes are involved in cell-cell communication due to the exchange of proteins and genetic materials. The use of MSCs-derived exosomes is considered a treatment option due to easier maintenance and reduction of the risk of rejection by the immune system, reducing the possibility of aneuploidy compared to cell-based methods.

KEYWORDS: Equine, Extracellular vesicles, Techniques, Tissue healing, Treatment strategies

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Introduction

One of the main reasons for the wastage of the horse industry is a musculoskeletal disease, and it is economically important for the welfare of animals (Weeren & Back, 2016). Tendons and ligaments are the most important musculoskeletal structures; damages in these structures cause serious complications and early retirement of horses in all sports (Clegg, 2012). Exercise and other strenuous activities often lead to tendon injuries. Naturally, the healed tendon often has less mechanical properties and is prone to re-injury, and patients often suffer from prolonged pain, discomfort, and even disability (Gaspar *et al*., 2015).

Tendon injuries require a longer recovery period, and tendon healing is accompanied by the formation of scar tissue that lacks the elasticity and strength of the main tissue and provides the basis for re-injury (Dakin, 2017). Therefore, new therapeutic aproaches for tendon repair are important to ensure animal welfare and reduce economic loss. The application of mesenchymal stem cells (MSCs)-derived exosomes is considered as an optimum treatment option due to easier maintenance and reduction of the risk of rejection by the immune system, reducing the possibility of aneuploidy compared to cell-based methods.

The use of MSCs-derived exosomes in myocardial infarction, stroke, and limb ischemia, as well as in kidney, bone marrow, and perinatal hypoxic-ischemic brain injuries, has recently been identified as a new therapeutic strategy in tissue regeneration (Dakin, 2017; He *et al*., 2012). Here, we are going to review isolation protocols and the effects of exosomes derived from MSCs on tendon repair, particularly in animals.

Extracellular Vesicles

Extracellular vesicles (EVs) are membrane vesicles identified during reticulocyte maturation in 1983 (Konala *et al*., 2016). The term EV includes exosomes, prostasomes, ectosomes, microvesicles (MVs), microparticles, tolerosomes, apoptotic bodies, and nanovesicles (Théry *et al*., 2018). EVs are found in physiological fluids, such as urine, blood, bronchial lavage fluid, breast milk, saliva, cerebrospinal fluid, amniotic fluid, synovial fluid, and malignant ascites (Konala *et al*., 2016; Raposo & Stoorvogel, 2013). EV is secreted by a variety of cells, including mast cells, epithelial cells, endothelial cells, nerve cells, cancer cells, oligodendrocytes, Schwann cells, embryonic cells, T cells, B cells, dendritic cells, platelets, and MSCs (Raposo & Stoorvogel, 2013).

It has been shown that in cell-cell communication, EVs play a significant role. EVs are involved in important processes, such as immune responses, maintenance of homeostasis, coagulation, inflammation, development of cancer, angiogenesis, and presentation of antigens. Thus, EVs take part in both physiological and pathological conditions (Konala *et al*., 2016; Raposo & Stoorvogel, 2013).

Mesenchymal/stromal stem cells are one of the most common cells for the treatment of diseases, as they affect tissue regeneration by their paracrine mechanisms and/or exosome/MV transfer (Spees *et al*., 2016). EVs are generally divided into three subgroups as follows: (a) exosomes with a diameter of 40–150 nm, (b) MVs with a diameter of 150–1000 nm, and (c) apoptotic bodies with a diameter of 50–2000 nm (Andaloussi *et al*., 2013). In the past decade, exosomes have received more attention as an important type of EV (Ni *et al*., 2020).

Microvesicles

MVs originate from the surface of the membrane through the outer membrane of the plasma membrane (Revenfeld *et al*., 2014). MVs are hetero-geneous populations with irregular shapes and different sizes and densities (Badimon *et al*., 2016; Revenfeld *et al*., 2014), characterized by phosphatidylserine (PS) in the outer membrane. MVs can be isolated from different biological samples by the size and composition of specific cell markers (Giusti *et al*., 2013). The most common MV markers include CD40 ligand, adenosine diphosphate ribosylation factor 6, and several integrins and selectins (Pugholm *et al*., 2015).

Apoptotic Bodies

The apoptotic bodies can be the largest, with a diameter ranging from 800 to 5000 nm and a density between 1.16–1.28 g/mL (Revenfeld *et al*., 2014), characterized by the presence of fragmented nuclei

and DNA fragments, histones, and proteins (Martinez & Freyssinet, 2001). Apoptotic bodies are released by apoptotic cells and phagocytosed by macrophages during normal growth (Erwig & Henson, 2008). This process is performed by transferring PS to the outer leaflet of the fat layer. The binding of PS to annexin V is also detected by phagocytes (Martinez & Freyssinet, 2001). The oxidation of surface molecules also causes membrane changes. These changes create sites for the binding of thrombospondin or complementary C3b protein. These two, in turn, are recognized by phagocyte receptors (Erwig & Henson, 2008). Thus, annexin V, thrombospondin, and C3b act as markers of apoptotic bodies (Friedl *et al*., 2002).

Exosomes

The term exosome for understanding the biological process of transformation from a reticulocyte to a mature erythrocyte was invented by Dr. Rose Johnstone (Johnstone *et al*., 1987). Exosomes are surroundded by a lipid bilayer membrane. They are 30–150 nm in diameter, have a density of 1.10–1.18 g/mL, and secreted by various healthy cells (Abels & Breakefield, 2016).

Exosome Biogenesis

Small vesicles are produced by the inward budding of the plasma membrane. The fusion of these vesicles together forms the primary endosome. In the end, endosomes accumulate and form multivesicular bodies (MVBs) that are released into the extracellular environment as "exosomes." During endosome formation, proteins, lipids, RNAs, and other substances are trapped in the lumen (Kalra *et al*., 2016). The presence of membrane transfer and fusion proteins (GTPases, annexins, and flotillin), tetraspanins (CD9, CD63, CD81, and CD82), heat shock proteins (Hsc70, Hsp90, Hsp60, and Hsp20), proteins involved in the biogenesis of multicellular bodies (Alix, TSG101), lipid-related proteins, and phospholipases in exosomes is due to their endosomal origin. Also, exosomes are specific in enriching cholesterol, ceramide or other sphingolipids, and phosphoglycerides with long and saturated fatty-acyl chains (Robbins & Morelli, 2014; Zhang *et al*., 2012).

Due to the exchange of proteins and genetic materials (messenger RNA [mRNA], microRNA [miRNA], pre-miRNA, and other noncoding RNA), exosomes are involved in physiological processes, such as cell growth, immune system regulation, angiogenesis, neural communication, and cell migration (Fauré *et al*., 2006), as well as in the pathogenesis of various diseases (Fevrier *et al*., 2004).

In order to study exosomes and their practical use, they must first be separated and purified from other vesicles and cell compounds. For this purpose, various techniques have been developed.

Exosome Isolation

Techniques that isolate exosomes in addition to the high efficiency should be capable of separating exosomes from various sample matrices (Khatun *et al*., 2016). Different methods (including ultracentrifugation, size-based, immunoaffinity purification, precipitation, and microfluidics-based isolation techniques) for exosome separation have been devised based on the size, shape, density, and surface proteins of exosomes (Gurunathan *et al*., 2019; Li *et al*., 2017; Yang *et al*., 2020; Zarovni *et al*., 2015).

Ultracentrifugation: Ultracentrifugation is the most frequent method used to separate different biological components, such as viruses, bacteria, subcellular organelles, and EVs, in which the constituents of the particles of a heterogeneous mixture are precipitated by centrifugal force according to their density, size, and shape. Ultracentrifugation is to generate high centrifugal forces up to 1 000 000*g* (Gurunathan *et al*., 2019).

Analytical and preparative ultracentrifugation processes are two types of ultracentrifugation. Analytical ultracentrifugation is used to investigate the physicochemical properties of particulate materials and molecular interactions of polymeric materials. Preparative ultracentrifugation plays an important role in exosome isolation because it is used to fractionate small bioparticles, such as viruses, bacteria, subcellular organelles, and EVs (Zarovni *et al*., 2015). There are two types of preparative ultracentrifugation, i.e., differential ultracentrifugation and density gradient ultracentrifugation.

Differential ultracentrifugation usually consists of a series of centrifugation cycles of different centrifugal forces for the isolation of exosomes. Duration to isolate exosomes is based on their density and size differences from other components in a sample

(Rechavi *et al*., 2009). The density gradient ultracentrifugation consists of two types of isopycnic ultracentrifugation and moving-zone ultracentrifugation. The isolation of EVs (such as exosomes) has become popular using density gradient ultracentrifugation. The separation of exosomes through density gradient ultracentrifugation is done based on their size, mass, and density.

In isopycnic ultracentrifugation, the separation of exosomes is dependent on their density difference from all other solutes, but in moving-zone ultracentrifugation, exosomes in the sample are separated based on their size and mass instead of density (Miranda *et al*., 2014). The requiring advanced supercentrifugation and consuming extensive time are the shortcomings of ultracentrifugation. In addition, the structures of the exosomes may be affected by ultracentrifugation, which would prevent downstream analysis (Ni *et al*., 2020).

Size-Based Technique: Ultrafiltration is one of the popular size-based exosome isolation techniques used to harvest exos-omes from urine, serum, cerebrospinal fluid, and cell culture medium by particle size or molecular weight (Li *et al*., 2017).

This method uses membranes with specified pore diameters to isolate the particles of a pre-determined size range (Konoshenko *et al*., 2018). First, larger particles are excreted by using filters with pore diameters of 0.8 and 0.45 µm, leaving an exosome-rich filtrate. Then, smaller vesicles are dropped from the filtrate using membranes with pores smaller than the desired exosomes (0.22 and 0.1 µm) to move into a waste eluate. The exosomes are collected via the first and last pore filtration membranes by maximum and minimum size ranges.

To confirm the isolated exosomes, the Western blot method is used to identify the biomarkers of the exosomes, and an electron microscope is used to examine the typical features of the exosomes (McNamara *et al*., 2018). This protocol can be used as a stand-alone technique or a complement to ultracentrifugation to separate large MVs and exosomes. The recovery of exosomes is dependent on the type of filter because of various membrane types and pore sizes. This exosome isolation method is simple and does not need complex equipment. However, this method has some challenges (Li *et al*., 2017; Gurunathan *et al*., 2019).

Immunoaffinity Purification: The presence of plenty of proteins and receptors in the membrane of exosomes offers an excellent opportunity to develop highly specific techniques for the isolation of exosomes by trapping on immunoaffinity interactions between those proteins (antig-ens) and their antibodies, as well as on specific interactions between the receptors and ligands (Li *et al*., 2017). This method uses antibodies against specific exosome surface markers, especially tetraspanins, i.e., CD9, CD63, and CD81. Exosome isolation by immuneaffinity capture can be accomplished by incubating the sample with magnetic beads (Koliha *et al*., 2016) or nanocubes of gold-loaded ferric oxide (Boriachek *et al*., 2019), coating with surface protein antibodies.

Parent cell markers, such as chondroitin sulfate peptidoglycan 4 (Sharma *et al*., 2018), epithelial cell adhesion molecule (EPCAM; Zhou *et al*., 2016), or exosome binding molecules, such as heat shock proteins (Ghosh *et al*., 2014) and heparin (Balaj *et al*., 2015), have been used in other affinity methods. To improve the purity of isolated exosomes, immunoaffinity is used as an extra step combined with differential ultracentrifugation. The primary downside of this approach is that the consumer selects a subset of marker-specific vesicles that may not represent all exosomes. Although it decreases the exosomal yield, since only the exosomes recognized by the antibody are captured, the extracted exosomes would be of greater purity. Further, unless the antibodies can be removed from the post-precipitation vesicles, the integrity of exosomes may be impaired (Reiner *et al*., 2017).

Another problem that restricts the use of this technique is the specificity and quality of the antibody, as most antibodies available for immunoprecipitation are non-specific. The capture of immunoaffinity is one of the most expensive methods for exosome isolation from a large sample volume, as high quantities of antibody-conjugated beads are needed, which may restrict its use. Thus, it may be enough only for studies requiring a limited sample size, which indicates a limit to any possible therapeutic application (Sidhom *et al*., 2020).

Precipitation: By changing the solubility or dispersibility, exosomes can be settled out of biological fluids. Water-excluding polymers, such as polyethylene glycol (PEG), are involved for this reason (Zeringer *et al*., 2015). PEG precipitation is used to separate viruses and tiny particles (Gurunathan *et al*., 2019). Polymers can bind to water molecules, reducing the solubility of exosomes, and this effect can be used to isolate exosomes from conditioned media, serum, or urine (Batrakova & Kim, 2015). It has been shown that urinary exosome precipitation with those kits achieves the highest yield compared to differential ultracentrifugation and nanomembrane concentrators; also, for their next profiling analysis, the highest concentrations of miRNAs and mRNAs are extracted (Alvarez *et al*., 2012). The use of exosome separation precipitation is convenient and requires no special equipment. Also, the concentration of isolated exosomes is high. However, there are still many issues with this process, such as poor recovery and high impurities (Ni *et al*., 2020).

Microfluidics-Based Isolation Techniques: This is an alternative form of isolation focused on physical and biochemical characteristics, such as scale, density, and immune affinity (Lee *et al*., 2015; Wang *et al*., 2013). This technique is a high-throughput approach used to separate exosomes via microfluidic devices based on several concepts, including immunoaffinity, size, and density (Chen *et al*., 2010). Furthermore, it is a new method of sorting involving acoustic, electrophoretic, and electromag-netic procedures (Lee *et al*., 2015; Wang *et al*., 2013). The steps of this method are immunoaffinity, sieving, and exosome trapping on porous structures (Liga *et al*., 2015).

The immuno-microfluidic procedure, which is similar to the method of immunoaffinity capture isolation, is the most used technique. Exosomes are separated by the special binding of immobilized antibodies to exosome markers on microfluidic instruments, also known as chips. ExoChip with the CD63 antibody is a popular microfluidic system that has been used to isolate exosomes (Chen *et al*., 2010). Gold electrodes with the CD9 antibody (Vaidyanathan *et al*., 2014), graphene oxide/polydopamine (GO/PDA), nanointerface with the CD81 antibody (Zhang *et al*., 2016), and a herringbone groove with the CD9 antibody are other microfluidic instruments (Hisey *et al*., 2018). Small quantities of sample volume, reagents, and separation time are needed by this process (Wunsch *et al*., 2016). Also, microfluidics-based isolation in conjunction with other exosome separation methods could enrich exosomes and enhance purity (Gurunathan *et al*., 2019). However, this method requires specialized equipment that may limit its large-scale application (Ni *et al*., 2020).

Exosome Identification

In order to recognize the cellular combination of exosomes, a wide range of methods have been used, including trypsin digestion and mass spectrometry, Western blotting, and fluorescence-activated cell sorting (FACS) analyzes in different cell types (Théry *et al*., 2002). The identification of exosomes is primarily dependent on morphological properties, particle size, and signature proteins (Lässer *et al*., 2012). There are various approaches to assess the properties of exosomes (Gurunathan *et al*., 2019).

First, to classify exosomes specifically, scanning electron microscopy (SEM) or transmission electron microscopy (TEM) may be used. The exosome surface microstructure is detected by SEM, while TEM has a maximum resolution of 0.2 nm and may expose the internal structure and morphology of exosomes (Wu *et al*., 2015). Second, the particle size and exosome concentration can be studied by nanoparticle tracking analysis (NTA). The NAT-based detection method is relatively easy, and the outcome can be better quantified. Third, Western blot technology in estimating specific marker proteins in exosomes, such as CD63, CD8, TSG101, flotillin-1, ALIX, CD9, CD81, and CD82, plays an important role. Fourth, flow cytometry (FCM) through labeling targeted exosomes with specific antibodies or fluorescent dyes can be used to analyze the size of exosomes. FCM has several benefits for exosomal analysis, including high-throughput screening and data quantification. Also, FCM can be used to identify various exosomal subpopulations. In addition to the mentioned techniques, atomic force microscopy, tunable resistive pulse sensing, and dynamic light scattering (DLS) can also be used for the detection of exosomes (Gurunathan *et al*., 2019).

Anatomy of the Healthy Tendon

The tendon is a string that is flexible but inelastic and interposed between muscles or muscles and bones. These anatomical structures transfer the force produced by the muscle to the bone and allow joint mobility (Kannus, 2000; Rowson *et al*., 2016). Each muscle typically consists of two ends of the tendon, proximal and distal. The place that attaches the tendon to the muscle is called the myotendinous junction (MTJ), and where it connects to the bone is called the osteotendinous junction (OTJ). Healthy tendons are microscopically bright white and have a glistening appearance. A healthy mature tendon is a collection of collagen structures that join tenocytes and are placed inside an extracellular matrix (ECM). The paratenon is known to be the outermost layer of the tendon and consists of elastic fibrils of collagen types I and III and allows the tendon to move freely toward the surrounding tissue (Killian *et al*., 2012).

A tendon consists of bundles of fascicles on a macroscopic scale (1 mm to 10 mm), which are covered by connective tissues, epitenon and endotenon. The neurovascular structure supplying the tendon is located inside these connective tissues. The next surface of the tendon structure is composed of parallel collagen fibrils (50 nm to 500 nm). In the following, there are microfibrils and tropocollagen molecules about 1.5 nm in diameter (Rio *et al*., 2014). Further, 65% to 80% of tendon dry mass is collagen type I that forms the main structure of tendons. Tropocollagen is a triple helix polypeptide consisting of two α1 and one α2 chains, which are secreted in ECM (James *et al*., 2008; Sharma & Maffulli, 2008).

In tendons, collagen and tendon cells are surrounded by ECM containing 1% to 5% proteoglycans and glycoproteins, 2% elastin, and 0.2% inorganic molecules, including copper, manganese, and calcium (Lin *et al*., 2004). Tendon ECM proteoglycans and glycoproteins include tenascin-C, cartilage oligomeric matrix protein (COMP), decorin, fibromodulin, biglycan, lumican, and tenomodulin (Tnmd; Jones & Jones, 2000; Pajala *et al*., 2009). Proteoglycans attach to collagen fibrils through their glycosaminoglycan (GAG) side chains to interconnect the fibrils in a parallel alignment and ensure the gliding of collagen fibrils throughout locomotion. They also allow for rapid diffusion and cell migration of water-soluble molecules.

Dermatan and chondroitin sulphates are the two main GAG components of the tendon (Sharma & Maffulli, 2008). The glycoprotein tenascin-C is a member of the tenascin gene family. It is abundantly found to interact with fibronectin in the ECM of developing vertebrate embryos and bind to integrins and components of ECM, such as collagens (Jones & Jones, 2000; Pajala *et al*., 2009). The thrombospondin family of extracellular calcium-binding proteins contains the pentameric, noncollagenous ECM protein (COMP). It consists of a five-stranded coiled-coil containing five similar subunits of glycoprotein, and its 3D structure is stabilized by disulfide bonds. COMP binds to collagen and plays a catalytic role in the creation of tendon ECM (Rock *et al*., 2010).

The proteoglycans of decorin, fibromodulin, biglycan, and lumican are composed of a protein nucleus containing leucine repeats with different GAG chains. Decorin's GAG chains consist of either dermatan or sulfates of chondroitin. There are four keratin sulphates in fibromodulin. Biglycan's GAG chains are made up of chondroitin or dermatan sulphates, whereas lumican's GAG chains are made up of keratin sulphates (Rees *et al*., 2009). Decorin binds directly to type I fibrils of collagen and has been implicated in the lateral fibrillogenesis of collagen (Yoon & Halper, 2005). Fibromodulin, biglycan, and lumican, like decorin, bind to collagen I fibrils and take part in the collagen fibrillogenesis of the lateral tendon (Rees *et al*., 2009). Tnmd is a type II transmembrane glycoprotein with a C-terminal anti-angiogenic domain that is expressed in tendon tissue and is thought to be one of the primary factors in tendon maturation (Shukunami *et al*., 2006).

In different tissues and organs, elastin plays an essential function. Elastin cooperates with collagen as an essential component in ECM, mainly functioning in elastic stretch and recoil, especially for tensile resistance, and regulates the interactions between cells and ECM (Wu *et al*., 2017). Tendons and ligaments have excellent resistance to mechanical loads due to their special arrangement of collagen fibers. The orientation of longitudinally, transversely, and horizontally of collagen fibers offers strong buffer capacity and rotational forces during movement. Under a light microscope, by applying tensile forces at rest, the fibrils' wavy pattern disappears and reaches its initial

configuration by removing the force. The wavy configuration is not maintained by increasing stretch forces and excessive stretching (Kannus, 2000).

Regarding the cellular content of the tendon, it was initially considered an inactive tissue for a long time because no cells were found in tendon tissue. Today, various cells, such as non-committed cells and more mature cells, have been discovered. In addition to tenocytes, a type of stem cell has been found in the tendon. Also, there are small amounts of fat, nerve cells, and endothelial cells in the tendon (Dex *et al*., 2016). Although the structure of the tendon and its molecular composition are well described, the exact protein signature that distinguishes it from other musculoskeletal tissues, ligaments, or sub-variants of tendons is still questionable (Wu *et al*., 2017).

Tendon Injuries

Tendon injuries, which predominantly affect the superficial digital flexor tendon (SDFT), are one of the most common orthopedic disorders in sport horses and the leading cause of early retirement or wastage (Shojaee & Parham, 2019). Tendon injuries are divided into two categories: tendinopathy and tendon rupture. Hyperpronation, excessive loading, and microtrauma can all lead to tendon injuries (Nigg, 2001). Tendinopathy is a general term that applies to a number of tendon pathologies that arise from overuse and unnecessary mechanical loading, causing the tissue to become unable to sustain additional stress (Lui, 2013; Sharma & Maffulli, 2005; Wang, 2006).

Inflammation of the sheath and the subsequent destruction of the tendon body are pathophysiological responses to tendon damage. Several theories have discussed this process. Robi *et al*. (2013) described four theories on the mechanism of tendon degeneration: (1) mechanical, (2) vascular, (3) neural, and (4) alternative theories. The mechanical theory focuses on the effect of overload on the tendon, which initiates the pathologic process, while the vascular theory blames the tendon's hypovascularity for the poor healing response. According to the neural theory, substance P produced by neutrally mediated mast cell degranulation, in combination with inflammatory cascades localized around tendon vessels, may be responsible for the disease. Finally, an alternative hypothesis proposed that exercise-induced localized hyperthermia could threaten tenocyte survival (Robi *et al*., 2013).

The amorphous, grey-brown appearance and loss of the glistening-white of the tendon are histological features of tendinopathies and ruptures. Also, collagen degeneration, fiber disorientation and thinning, hypercellularity, rounded tenocytes nuclei, increased vascular in-growth, and a change toward fibrocartilaginous composition are visible (Rees *et al*., 2006; Riley, 2008). Fibrin deposits, calcifications, and lipid accumulations are sometimes observed in tendon degeneration (Milz *et al*., 2004). The tendon degeneration can be occurred due to an imbalance between matrix decomposition and synthesis, which is caused by a variety of stresses and mechanical loads (Sharma & Maffulli, 2008). The imbalance of metalloproteinases and tissue inhibitors of metalloproteinases (TIMPs), which are endogenous inhibitors of metalloproteinases, plays a key role in degeneration (Jones & Jones, 2000).

The upregulation of types I and III collagen mRNA, change in the ratio of type III collagen higher than type I collagen in ECM, and increase in the levels of fibronectin, tenascin-C, GAGs, aggrcan, and biglycan are major structural and molecular changes in the degenerative process (Riley, 2008). In addition, an increase in inflammatory mediators, such as prostaglandin E2 and interleukin-1, enhanced expression of cyclooxygenase-2, growth factors, including transforming growth factor β (TGF-β) and platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), and neurotransmitters, such as glutamate and substance B, are also seen in tendinopathy (Riley, 2008; Sharma & Maffulli, 2008).

Tissue formed after tendon injury has a different structure and composition. Degenerative tendinopathy, along with a decrease in type I collagen, leads to a decrease in tensile strength and ability to withstand the mechanical load, which indicates a weak tendon (Subramanian & Schilling, 2015). Also, the expression of type III collagen in damaged tendons does increase. In comparison to collagen type I, collagen type III has fewer crosslinks (Jo *et al*., 2012; Keller *et al*., 2011), and fibers are smaller and thinner with a lower resistive ability (Eriksen *et al*., 2002).

Tendon tissue produced after healing is much less organized, which is because of a loss of structure and a decreased mechanical strength (Wang, 2006). It is believed that after a tendon injury, increased production of type III collagen compared to type I creates adhesion sites, which leads to a decrease in lubrication between the tendon and adjacent tissues and causes friction and pain (Kuo *et al*., 2014); these prevent the tendon from slipping and reducing its mobility (Longo *et al*., 2011). Also, decreased mechanical strength, risk of rupture, and scar tissue formation occur with increased type III collagen (Jo *et al*., 2012).

Unlike apoptosis of tenocytes, which reduces cellulite (Wang *et al*., 2013), tendinopathy can also exhibit hypercellularity, particularly in tendinosis and chronic tendon pain (Andersson *et al*., 2011). After the injury, cells increase their proliferation in response to intrinsic and external signals. The morphology of these cells varies compared to healthy tenocytes (Rolf *et al*., 2001). These include abnormalities in the structure of the matrix and arteries, changes in protein composition and content, increased tenascin-C and fibronectin, and decreased decorin expression (Keller *et al*., 2011).

Exosomes in Tendon Repair

Musculoskeletal injuries are very common in racehorses and often manifest as a multifactor problem with a high economic effect on the equine industry (De Schauwer *et al*., 2013; Hillmann *et al*., 2016). Tendon repair involves three stages of inflammation, proliferation, and regeneration. Initially, severe inflammatory responses occur, leading to scar formation in the later stages of tendon repair (Thankam *et al*., 2018). Thus, to improve the quality of treatment, controlling inflammation is very important. After scar healing, changes in the histology, biochemistry, and biomechanical properties of the tendon occur, as a result of which the tendon is unble to return to its original strength and natural tract-ion and may break again after injury (Cui *et al*., 2011).

Current treatments for tendon injury are often associated with incomplete repair or healing and increase susceptibility to re-injury (Shojaee & Parham, 2019). None of the treatments available has been effective in completely repairing tendon tissue. However, one of the newest and fastest choices for musculoskeletal injuries in horses and other animals is tissue engineering (Lui & Chan, 2011). The ability of MSCs to differentiate into tenocyte cells *in vivo* and *in vitro* suggests them as a suitable treatment for tendon rupture, tendon growth, and improving tendon repair (Javanshir *et al*., 2020; Liu *et al*., 2017).

It has been shown that exosomes derived from MSCs have roles like MSCs, such as repairing tissue damage, suppressing inflammatory responses, and modulating the immune system. Exosomes are more stable and reservable than cells, have no chance of aneuploidy, have a lower risk of immune rejection after *in vivo* allogeneic administration, and may do use as an alternative therapy for a variety of diseases (Yu *et al*., 2014). Many studies have shown that the primary inflammatory response to tendon injury could be inhibited by exosomes derived from MSCs and improve tissue healing (Ding *et al*., 2019; He *et al*., 2019; Li *et al*., 2016).

Cell reproduction and migration are also essential processes to repair tendon damage in the next stage (Chen *et al*., 2018). According to Shen *et al.'s* findings (2020), a collagen sheet system containing exosomes from adipose-derived stem cells enhanced tendon regeneration by regulating macrophage inflammatory response. It has been reported that bone marrow-MSCs (BMSCs) stimulate proliferation and migration of tendon stem/progenitor cells (TSPCs) through paracrine exosomes and improve the tenogenic differentiation of TSPCs as well. They also observed that BMSCs-exosomes embedded in fibrin glue represented a practical method that allowed the sustained release of BMSCs-exosomes and internalization of BMSCs-exosomes by TSPCs.

In addition, BMSCs-exosomes embedded in fibrin glue significantly improved tendon regeneration and expanded proliferation of TSPCs *in vivo* (Yu *et al*., 2020). Yao *et al*. (2020) studied the effect of human umbilical cord MSC-derived exosomes (HUMSC-exosomes) on tendon adhesion—as a common complication of tendon injury—and found that HUMSC-exosomes reduced rat fibroblast proliferation and inhibited tendon adhesion and the expression of fibrosis genes, including collagen type III and α-smooth muscle actin (α-SMA) *in vitro*.

As it was mentioned, the primary inflammatory response plays an important role in the process of

tendon damage and directly affects the healing of tendon tissue (Dagher *et al*., 2009). Inflammatory macrophages (M1) can cause ECM degradation, inflammation, and cell apoptosis in many types of tissue damage, including tendon injury (Dai *et al*., 2018; Laplante *et al*., 2017), while anti-inflammatory macrophages (M2) regulate ECM balance and tissue repair (Chamberlain *et al*., 2019). Manning *et al*. (2015) showed that transfer from pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages could improve tendon injuries. Numerous studies have shown that exosomes derived from MSCs cause cell proliferation, survival, and migration (Hu L *et al*., 2016; Zhang *et al*., 2015).

Zhang *et al*. (2020) also found that tendon stem cell-derived exosomes (TSC- exosomes) enhanced the proliferation and migration of tenocytes in a dose-dependent manner. They also observed that TSC-exosomes enhanced anti-inflammatory and tendon injury repair by modulating macrophages and related cytokines (Zhang *et al*., 2020). In the mouse Achilles tendon-bone reconstruction model, mouse BMSC-derived exosomes in the hydrogel increased fibrocartilage in the exosome group compared to other groups. Also, the biomechanical properties of the tendon-bone junction significantly improved in the exosome group (Shi *et al*., 2020).

Discussion

Tendon injuries, as one of the most common musculoskeletal diseases, still have no definitive cure. Hence, it can cause great economic loss, especially in the horse industry. Accordingly, the advent of tissue engineering as one of the fastest and newest therapies can be promising. In recent years, the use of exosomes instead of cells has been considered due to their role in intercellular communication in many physiological and pathological processes. Exosomes and mother cells have similar functions, with anti-inflammatory, anti-apoptotic, tissue repair, and immune regulatory functions. In addition, using exosomes as a non-cellular, non-surgical treatment option is easier and less time-consuming than cell therapy.

Compared to the whole-cell therapy of MSCs, the cell-free therapy of MSC-derived exosomes is well tolerated and has low immunogenicity and greater potential in the clinical field. However, this method has its own limitations, including separation, purification, and small amount. As noted in this review, experimental data from the repair of damaged tendons with exosomes derived from MSCs are so promising and support them as biological repair agents for tendon injuries. However, due to the proteomic and genomic complexities of exosomes, this treatment is still in preclinical research and optimization phase. In order to reach their full potential and become a suitable treatment option, their possible mechanisms and exact combinations need to be further investigated.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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اگزوزوم‌های سلول‌های بنیادی مزانشیمی در درمان آسیب‌های تاندونی حیوانات: مروری بر جداسازی و کاربرد آنها

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چکیده

آسیب‌های تاندونی بخش عمده ای از آسیب‌های اسکلتی عضلانی در حیوانات، به ویژه در اسب را شامل می‌شود. تاکنون، هیچ درمان جامعی برای این عارضه معرفی نشده است و بیشتر درمان‌ها بر کنترل درد تمرکز دارند. با توجه به مزایایی که اگزوزوم‌ها در مقایسه با روش‌های مبتنی بر سلول درمانی و اثرات خود سلول‌های بنیادی مزانشیمی بر روی ترمیم بافتی دارند، اگزوزوم‌های سلول‌های بنیادی مزانشیمی به‌عنوان یک گزینه درمانی مناسب در ترمیم آسیب‌های تاندونی مطرح است. هدف این مقاله مرور پروتکل‌های مختلف جداسازی اگزوزوم‌ها از سلول‌های بنیادی مزانشیمی و نقش آنها در ترمیم ضایعات تاندونی حیوانات، به ویژه در اسب است. در درمان اختلالات تاندونی، اگزوزوم‌ها نسبت به سلول‌ها پایدارتر هستند، پس از تجویز آلوژنیک خطر دفع ایمنی کمتری دارند و می‌توانند به عنوان یک درمان جایگزین مناسب استفاده شوند. اگزوزوم‌های مشتق از سلول‌های بنیادی مزانشیمی منابع مختلف باعث تحریک تکثیر و مهاجرت تنوسیت‌ها و فیبروبلاست‌ها می‌شوند؛ موجب تنظیم آرایش فیبرهای کلاژن، تعدیل پاسخ‌های التهابی و عملکرد ماکروفاژها می‌شوند؛ از چسبندگی جلوگیری می‌کنند و به طور کلی تاندون‌های آسیب دیده را ترمیم می‌کنند. در درمان اختلالات تاندونی، اگزوزوم‌ها نسبت به سلول‌ها پایدارتر هستند، پس از تجویز آلوژنیک خطر دفع ایمنی کمتری دارند و می‌توانند به عنوان یک درمان جایگزین مناسب استفاده شوند. اگزوزوم‌های مشتق از سلول‌های بنیادی مزانشیمی منابع مختلف باعث تحریک تکثیر و مهاجرت تنوسیت‌ها و فیبروبلاست‌ها می‌شوند؛ موجب تنظیم آرایش فیبرهای کلاژن، تعدیل پاسخ‌های التهابی و عملکرد ماکروفاژها می‌شوند؛ از چسبندگی جلوگیری می‌کنند و به طور کلی تاندون‌های آسیب دیده را ترمیم می‌کنند. اگزوزوم‌ها به دلیل دارا بودن پروتئین‌ها و مواد ژنتیکی و حمل آنها، در ارتباط سلول‌ها با یکدیگر دخیل هستند. استفاده از اگزوزوم‌های سلول‌های بنیادی مزانشیمی به دلیل نگهداری آسان تر و کاهش خطر پس زدن توسط سیستم ایمنی بدن و کاهش احتمال بروز آنوپلوئیدی در مقایسه با روش‌های مبتنی بر سلول، به عنوان یک گزینه درمانی مطلوب مطرح هستند.

واژه‌های کلیدی: وزیکول‌های خارج سلولی، تکنیک‌ها، ترمیم بافتی، اسب، راهبردهای درمانی

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