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Review

Stem Cells in Veterinary Medicine

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ABSTRACT

Today, with the advancement of technology, medical science has shifted its focus to regeneration. Regenerative medicine aims to restore irreparable tissues and organs to their normal function by stimulating the body's repair mechanisms using different approaches. Regenerative medicine is currently one of the important research areas in both human and veterinary medicine. This field of medicine provides diversity in veterinary medicine due to the abundance of animal species and anatomical and physiological differences, but the abundance of variables requires more meticulous work. Due to the difficulty in translating clinical results from one species to another, the scope of the work is quite limited. Today, stem cell applications are widely used in veterinary medicine, particularly in horses and dogs with muscle and skeletal system diseases. This chapter evaluates mesenchymal stem cells, their production, the use of mesenchymal stem cell therapies in horses and dogs with muscle-skeletal system diseases, and the use of mammary stem cells and stem cells in reproductive medicine.

Keywords: Intervertebral disk degeneration, mesenchymal stem cells, osteoarthritis, stem cell, tendon ligament injury, veterinary medicine.

Mesenchymal stem cells (MSCs), also known as adult stem cells, were first discovered in 1976 by Friedenstein[1] using a fetal bovine serum to culture bone marrow cells and observing that they could differentiate into fat and bone cells.

Later studies revealed that these cells were non-hematopoietic pluripotent cells capable of differentiating into various cell types, such as fat, cartilage, neuron, muscle, and bone cells.

Mesenchymal stem cells are characterized by their ability to adhere to plastic surfaces, undergo multipotent differentiation, and express stromal surface antigens. They can be isolated and expanded from bone marrow, dental pulp, adipose tissue, liver, placenta, amniotic fluid, peripheral blood, and synovial fluid. However, MSCs are present in very small numbers in their native tissues, and therefore, they are usually expanded by passaging. However, this process may alter the properties of the cells and has some disadvantages. Mesenchymal stem cells form spindle-shaped clusters when observed in culture. They are used in cases of severe damage, organ failure, and bone fractures, and can be obtained from peripheral blood.[2]

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It is important for us to know where MSCs are obtained from because their biological properties differ. Mesenchymal stem cells can be isolated from almost any tissue. One of the tissues with the highest number of MSCs in the body is adipose tissue. Extensive research has been conducted on MSCs derived from adipose tissue. Adipose tissue covers most of the human body and is classified as white and brown adipose tissue according to its function. Cells obtained from these tissues are called adipose tissue-derived mesenchymal stem cells (AD-MSCs) derived from adipose tissue and have the most extensive research due to their safe tissue samples and easy accessibility by quick retrieval.[3]

Adipose tissue is obtained by elective, laparoscopic, or liposuction methods. Adipocytes, or fat cells, are removed from the collected adipose tissue through processes. As a result, smooth muscle cells, cells rich in

growth factors, adipocyte precursors, macrophages, endothelial cells, pericytes, and mesenchymal stem cells remain, and the fluid containing them is called the stromal vascular fraction. Isolating results from the stromal vascular fraction is easier.[4] The stromal vascular fraction can only be used for autologous treatments, meaning it can only be taken from and used in the same individual because it contains different cell types that cause an immune reaction. In dogs and cats, abdominal visceral fat can be used as a source of MSCs during a simple surgical procedure.[5]

In horses, fibroblast-like cells derived from peripheral blood and MSCs derived from bone marrow can differentiate into different mesenchymal lineages with less potential than human MSCs. Both cell types also have a more limited capacity to produce lipid droplets than human MSCs. In addition, the MSC yield decreases with increasing donor age.[6]

MESENCHYMAL STEM CELL ISOLATION

Mesenchymal stem cells are obtained from peripheral blood or adipose tissue. Firstly, the isolation process is performed with a gradient density centrifugation protocol. Then, an enzymatic process is required. Afterward, cells are seeded onto plates for adhesion and the culture medium is replaced with a new one.[7] These procedures take approximately one to ten days. Then, adherent cells are formed and slowly cover the bottom of the culture plate with a layer. After this stage, cell layers are separated by trypsinization and re-seeded to allow them to proliferate upon reaching confluency. Mesenchymal stem cells are not passaged more than four times for clinical applications because their ability to maintain their stemness decreases with each passage and their morphology changes.[4]

MESENCHYMAL STEM CELL CULTURING, CRYOPRESERVATION, AND APPLICATION METHODS

Basal medium eagle, Dulbecco's Modified Eagle's Medium, and Minimum Essential Media are primarily used as culture media for mesenchymal stem cells. Fetal bovine serum is important for growth factors, and the use of antibiotics (mostly penicillin) is important to prevent bacterial contamination.[8] The pH value ranges between 7.2-7.4. Additionally, it is usually incubated at 5% CO2 to maintain pH, and NaHCO3 can be used to buffer the culture. After all of these processes, cryopreservation is performed. This method involves freezing biological material using liquid nitrogen at -196 degrees Celsius or using carbon dioxide at -80 degrees Celsius to store and preserve the material without deterioration. Freezing with liquid nitrogen allows cells to remain in a state of complete dormancy for years, but it is believed that this process changes the structure and function of the cells. Many parameters are used to protect the cells in this process. A mixture of fetal bovine serum and dimethyl sulfoxide is used as the standard cryoprotectant in veterinary medicine.[9]

The typical clinical use of mesenchymal stem cells involves administering the cells using needles. For pathological lesions in the joints, MSCs are directly administered into the joint. In tendon lesions, MSCs are also applied directly to the lesion.[10]

CELL TRANSPLANTATION

There are two types of transplantation in stem cell therapy: autologous and allogeneic cell transplantation. Using the patient's own stem cells is called autologous transplantation, while transplantation of stem cells obtained from a donor is called allogeneic transplantation. Autologous transplantation is better for the patient's safety, but it is a time-consuming process to multiply the right amount of cells for implantation, and it usually involves surgical procedures and is also dependent on age, gender, and the disease.[11]

There are many studies on the use and safety of allogeneic MSCs. Allogeneic MSCs can be stored in banks for a long time, saving time. In Australia, allogeneic dog adipose-derived mesenchymal cells have been commercially available since 2010. In a study, pain relief, improvement, and increased quality of life were observed in 203 dogs diagnosed with degenerative arthritis after treatment.[12] In most studies, it was observed that there was no systematic response in the applied patient, and the recovery was high.

THE USE OF MESENCHYMAL STEM CELLS IN VETERINARY MEDICINE

Mesenchymal stem cells are defined as multipotent progenitor cells that can differentiate into other cells, replicate themselves, and regenerate damaged tissues. Due to their ability to secrete factors that support the repair of damaged tissues, their targeting abilities to migrate to areas of injury and inflammation along the endothelium, and their immunospecific activities, among many other features, they are widely used in veterinary medicine. Some reasons for the

widespread use of MSCs include their applicability, easy isolation process, lack of ethical debate, and lack of immunogenicity that allows for the use of cells from a single donor.[4] Many applications related to tissue sampling and MSC culture in veterinary medicine are similar to those used in human medicine.[13]

Musculoskeletal disorders that are associated with both acute and chronic pain are common pathologies in veterinary clinical practice. These disorders usually arise from excessive use of the musculoskeletal system, muscle fatigue, and inflammation of tendon structures. Treatment of musculoskeletal disorders involves the use of systemic or intra-articular anti-inflammatory drugs, hyaluronic acid (HA), platelet-rich plasma (PRP), as well as autologous and allogeneic stem cell transplantation.[14]

Examples of musculoskeletal disorders, osteoarthritis (OA), tendon ligament injury, and intervertebral disk degeneration (IVDD) can be given.

OSTEOARTHRITIS

Osteoarthritis is the most common form of joint disease that affects humans and horses, resulting from progressive degeneration of joint cartilage, ligaments, and underlying bone. It is characterized by a reduction in cartilage and loss of function in the affected joint. It is difficult to reverse and leads to inflammation.[15]

Mesenchymal stem cells are used as a therapy for osteoarthritis in dogs and horses. In these cases, MSCs are applied by intra-articular injection into the joint. Adipose tissue is generally used as the cell source for dogs. When mesenchymal stem cells are applied together with HA and PRP, positive results have been achieved in reducing lameness, improving joint functionality, and reducing pain.[16] Additionally, dogs treated with AD-MSCs containing plasma rich in growth factors have shown the release of growth factors.[17]

Degenerative joint diseases in horses are the reason why horses affected by this disease retire early from their equestrian and performance careers. In a study by Broeckx et al.[18], 75 horses affected by joint osteoarthritis were examined. Fifty horses were given an intra-articular injection of allogeneic plasma and allogeneic chondrogenically induced mesenchymal stem cells, while 25 horses were injected with saline solution to form the control group. They reported that the treated horses achieved better results in short and long-term lameness and flexion testing. Adipose tissue or bone marrow-derived cells, as well as MSCs produced from blood to change their combinations with allogeneic plasma, were used in this study. It was also reported that there was no immunological effect after the use of allogeneic MSCs.

TENDON-LIGAMENT INJURY

Tendons transmit forces generated by muscles to move the joints they pass through. The tendon-ligament injury affects a large proportion of the horse population, and achieving positive results with conventional treatment is difficult due to the limited capacity for tendon tissue regeneration in horses.[19,20] Therefore, in recent years, attention has shifted towards regenerative medicine for treating damaged tendons. This typically involves visualizing the tendon by ultrasound and directly injecting a simple cell suspension into the damaged tissue.[21]

There are many studies showing that allogenic cells can be safely used for the treatment of naturally occurring tendon injuries in horses.[22,23] In fact, these treatments are compared to traditional treatments by comparing the rate of return to activity after rehabilitation. This comparison provides information about the animal's ability to return to previous activities. Cell therapy has resulted in significantly lower rates of re-injury compared to traditional treatments. Following traditional treatments such as hyaluronan, beta aminopropionitrile fumarate, or polysulfated glycosaminoglycans, the rate of re-injury varies between approximately 23-80%, while with MSC treatment, this rate is between 13-36%.[18,24,25]

Canine tendinopathy has been less studied compared to horses. Supraspinatus tendinopathy (ST) is a major cause of forelimb lameness in dogs. Caanapp et al.[26] applied adipose-derived MSCs together with PRP in the treatment of 55 dogs with ST and observed positive results of MSC application for ST. In another study, the use of PRP with combined bone marrow aspirate concentrate resulted in a 13.8% improvement in tendon size, fiber pattern, and echogenicity, with positive sonographic results. The treated dogs returned to a completely normal fiber pattern 90 days after treatment.[27]

Semitendinosus myopathy is not a tendinitis, but a fibrotic musculoskeletal disease commonly seen in active dogs (especially police dogs). It affects the muscle that is a part of the Achilles tendon. In a study aiming to treat semitendinosus myopathy in eleven police dogs using AD-MSCs in a single application, a comparison of six-month and one-year follow-ups showed that all patients returned to work

at six months, and an improvement in walking was observed at one-year follow-up.[28]

INTERVERTEBRAL DISC DEGENERATION

Intervertebral disk degeneration is the primary cause of back pain in humans and currently has no cure. Therefore, treatment focuses on pain management. Efforts in this area are based on strategies that can at least partially restore or repair the functioning of the intervertebral disc structure. One of these strategies involves the intradiscal injection of MSCs. Similar medical and surgical approaches to humans are used in dogs with intervertebral disk degeneration.[16] Therefore, dogs are considered a disease model for ineffective therapeutic research for human IVDD. Steffen et al.[29] conducted studies on six dogs suffering from naturally occurring degenerative disc disease. These six dogs were given autologous bone marrow-derived MSCs and no side effects were observed. However, when examined by magnetic resonance imaging (MRI), they did not show any significant regenerative effect of the treatment. This study has been a precursor for naturally occurring IVDD. Therefore, there may be negative results due to differences between injury-related and naturally occurring IVDD. In another study, autologous MSCs were mixed with collagen microcarriers with or without a cross-linked distribution system of transforming growth factor beta (TGF-β1) in 20 dogs with spontaneous lumbosacral IVDD and back pain, as detected by MRI. After decompressive surgery, the dogs were divided into three groups, each receiving a different intradiscal injection: MSC-microcarriers, MSC-TGF-β1 microcarriers, and only microcarriers. *In vivo*, injection was successful in all dogs, and their clinical functionality was regained. However, unwanted side effects of schmorl nodes were observed in 45% of dogs, which decreased by half when injection volume was reduced. Although Schmorl nodes were observed in all groups, clinical improvement was seen in all groups, but microcarriers and MSCs did not rebuild the structure of the IVDD.[30] In a study of 34 dogs with IVDD without deep pain, surgical treatment was combined with either alone or allogenic AD-MSC transplantation into the spinal cord. Neurological improvement was observed, and the success rate for the AD-MSC group was significantly higher than for surgery alone. This demonstrated the potential therapeutic efficacy of MSCs.[31]

Although MSCs appear to be a viable approach for IVDD treatment in veterinary medicine, it should be noted that damaged tissue may not always be treated by MSCs using the therapeutic protocols proposed so far.[32]

MAMMARY STEM CELLS

Breast Gland

The breast gland, which is a structure that distinguishes mammals from other living creatures, is composed of milk-producing glands located inside the breast. It serves the function of producing milk necessary for breastfeeding after childbirth. These glands are controlled by hormones and grow during pregnancy due to increased levels of progesterone and estrogen hormones.

Breast glands undergo changes in size, structure, and activity throughout the life of a mammal and functionally go through cycles. Moreover, these changes that the breast undergoes are affected by many external factors such as environment, climate, and diet in addition to being a result of physiological changes.[33,34]

Breast gland development is a process that involves partially stromal tissue and epithelium.[35-37] The stromal region is composed of a structure consisting of fibronectin, collagen, and proteoglycans.[35] The most effective of these stromal components is the adipose tissue pad. The architecture of these pads varies among animal species. For example, in mice, this breast pad consists mostly of adipose tissue after birth, while in cows and humans, there is a greater amount of interlobular fibroblastic adipose tissue in the breast pad and only a limited number of adipose cells.[38-41] The other epithelial compartment contains the glandular network of the breast gland. There are two types of epithelial cells here: luminal and basal. Luminal cells form the luminal epithelial channels and secretory alveoli.[42-44] Basal epithelium contains myoepithelial cells. It is believed that all differentiated cells with the potential for these different morphologies actually originate from mammary gland stem cells.[45-47]

Mammary Stem Cells

Evidence for the existence of mammary stem cells has been gathered from mice and humans in recent times. The first study to demonstrate the presence of self-renewing and multipotent adult stem cells in mice was conducted by DeOme and Daniel.[48-50] Furthermore, in 1998, Kordon and Smith[34] showed that transplantation of a tissue fragment into mammary fat pads could lead to degeneration of the entire murine mammary gland with the lineage

of a single cell. In mice, a progenitor stem cell that commits to either luminal or myoepithelial cells is generally defined to explain mammary epithelial hierarchy.[51]

Why Should We Work with Mammary Stem Cells?

The cleared fat pad model in mice is frequently used to understand the functional role of mammary stem cells in the development of a normal mammary gland. The transplantation and growth of mammary stem cells are done in conjunction with the normal anatomic region and physiological environment.[51-53]

Mice are indispensable experimental animals in breast cancer research today. This is because these rodents are preferred in human breast cancer studies, in conjunction with transgenic mice expressing oncogenes, due to the fat pad transplantation system that lacks mammary glands. Breast cancer is most commonly seen in humans and carnivores.[54]

Horses, cows, pigs, and old-world primates such as macaques rarely develop mammary tumors.[55] The reason for this difference has not been fully explained. However, according to Borena and colleagues[56], this difference may arise from differences in the functional behavior and regulation of mammary stem cells, which are the driving force behind mammogenesis. Therefore, they argue that studying mammary gland stem cells in different species is crucial.

Mammary Stem Cell Production

Isolating stem cells from breast tissue is quite challenging, and the number of stem cells is very low. Nevertheless, the presence of stem cell-like cells in breast tissue has been identified, and research continues on isolating and expanding these cells.

Isolating stem cells from breast tissue is typically an invasive procedure that requires procedures such as breast biopsy. During a biopsy, a small sample of breast tissue is taken and sent to the laboratory. Here, the cells in the sample are separated, and attempts are made to identify stem cells.

Stem cells isolated from breast tissue can be expanded and differentiated into specific cell types. However, the number and potential of stem cells in breast tissue are quite limited.

Dogs and Cats Also Have Mammary Stem Cells

The incidence of breast cancer is high in women today, and studies have shown the presence of cancer stem cells or tumor-initiating cells in these cancerous breast tissues.[57] Breast cancer, commonly seen in humans and carnivores, is the most common cancer in female dogs and the third most common in female cats.[58,59] Mammary gland tumors in cats and dogs have similar histological appearance, genetics, and biology, making them valuable animal models for studying breast cancer.[60,61] Therefore, research on canine and feline stem cells has mainly focused on the cancer stem cell theory. This theory suggests that stem cells are the only type of cell in an adult organ that undergoes enough mutations to transform into cancer cells and can survive for a long time, thus showing that cancer is actually a stem cell disease.[62] However, it is still unclear whether cancer stem cells are mutated normal tissue stem cells or differentiated cells that have acquired primitive and stem cell-like properties due to mutations.[63] Currently, knowledge about feline and canine mammary stem cells and cancer stem cells is limited.

In 2009, Cocola and colleagues[64] were the first to isolate mammary stem cells and cancer stem cells from dog mammary gland tissues. These cells were expanded in cultures as mammospheres and tumor clusters for three to five passages. They were observed to have self-renewal ability and pluripotent differentiation potential and were able to form tubular structures *in vitro* and tumors *in vivo* in NOD scid gamma mice. Western blot analysis showed that the dog cancer stem cells were clearly positive for integrin alpha 6, prominin-1, cyclin-dependent kinase inhibitor 1, and keratin 14, and moderately positive for integrin beta 1 and CD44. However, there was no data available for these markers in normal mammary stem cells. Therefore, it is unfortunately still unknown what is present in normal mammary stem cells in this model and whether there are any differences between normal mammary stem cells and cancer stem cells.

In a study conducted by Barbieri and colleagues[65] in 2012, cancer stem cells derived from feline mammary cancer showed self-renewal, proliferation, and *in vivo*, tumorigenicity under *in vitro* conditions, and immunofluorescence staining showed that these feline cancer stem cells were positive for CD44, estrogen receptor alpha, and epidermal growth factor receptor.

As a result, mammary stem cells are necessary for the regeneration, growth, and differentiation of mammary tissue. These cells are important for the restructuring, growth, and differentiation of mammary tissue, and they could potentially be used for the treatment of breast cancer and other breast diseases. Therefore, studies on mammary stem cells

are important as a potential therapeutic approach for breast cancer treatment, mammary tissue regeneration, and other breast diseases.

THE USE OF STEM CELLS IN REPRODUCTIVE MEDICINE

Currently, stem cells are used for many different purposes in reproductive medicine. For example, stem cells are used for infertility treatment, egg production, fertility preservation, and prevention of genetic diseases. New assisted reproductive technologies are being developed to apply the properties of spermatogonial stem cells (SSCs) to protect endangered animal species. Generally, two types of stem cells have been focused on: induced pluripotent stem cells (iPSCs), which are obtained by reversing differentiation, and SSCs. Unfortunately, completely characterized ES or iPSCs cells from species other than primates or mice do not exist at present. Therefore, research has been focused on SSCs used in testis xenografting and SSC transplantation techniques.[66]

Testis Xenografting

Testis xenografting is the transfer of testicular tissue or cells from one animal species to another (usually from mice to humans). This procedure is being investigated as an option for treating testicular damage that causes infertility and other reproductive problems in humans. Testis xenografting is performed by transplanting testicular tissue from one animal species to another, allowing the transplanted testicular tissue cells to survive and functionally operate. This process can also preserve the sperm cells within the transplanted testicular tissue, potentially allowing for the continuation of threatened and endangered species. If adult males die without passing on their genes to the population, mature sperm can be collected and frozen for future use in artificial insemination or *in vitro* fertilization.[66] If newborns or young males die, sperm can be generated from gonocytes or SSCs present during birth using this technique. In this process, donor testis fragments of 1-2 mm^3 are transferred to immunodeficient mice via surgical methods.[67] These mice, which do not have an immune system, nourish the foreign testicular tissue and support spermatogenesis. Using this method, morphologically mature sperm has been produced in xenografts from many species including rabbits, monkeys, sheep, cats, pigs, and hamsters.[67-71] However, the efficiency of spermatogenesis in xenografts is less effective in bulls, cats, and dogs.[72-77]

Xenotransplantation is a challenging procedure due to the incompatibility of immune systems between different species. The recipient's body tends to reject foreign tissues or cells and uses the immune system to eliminate them. Therefore, during xenotransplantation, immunosuppressive drugs can be used to prevent the donated tissue or cells from being rejected by the recipient. The xenotransplantation process still carries many risks, and studies are investigating how safe the use of this method is and its long-term outcomes.

Spermatogonial Stem Cell Transplantation

Spermatogonial stem cell transplantation is a method used for the treatment of infertility in men. In this method, SSCs from the patient's testis are replaced with stem cells obtained from another area. Spermatogonial stem cells are the cells necessary for sperm production in men. In men experiencing infertility, SSCs may have been damaged or lost their function. In this case, SSC transplantation aims to renew the SSCs and restore healthy sperm production. Spermatogonial stem cell transplantation involves the isolation of a mixed germ cell population from a donor testis. The isolated cells are then retrogradely injected into the recipient animal's testes, usually under local anesthesia. Time is allowed for colonization, proliferation, and spermatogenesis to occur, and then semen is collected. The relative percentage of donor-derived semen is evaluated after these procedures. This technique involves many steps that require challenging time and intense labor. In the future, this method could be used as a clinical tool for producing genetically engineered tissues/organs compatible across species or for producing transgenic farm animals to produce pharmaceutical proteins.[77]

Xenogeneic transplantation has been attempted in various donor and recipient species. Unless the donor and recipient are taxonomically closely related, the recipient's testes do not support spermatogenesis. Therefore, in endangered species, this procedure would require not only the use of an appropriate domestic animal recipient that would support donor spermatogenesis but also a method to separate donor-derived sperm from recipient-derived sperm.[66]

In conclusion, MSCs are defined as self-renewing and differentiating multipotent progenitor cells that can differentiate into different cell types. In veterinary medicine, many studies have been conducted on

isolation processes, culture conditions, cell tissue sources, cryopreservation, dosage, administration route, and frequency to determine the most effective procedures in MSC-based therapies. In recent years, with the studies conducted, MSCs have emerged as a promising therapeutic tool in the treatment of musculoskeletal system pathologies in veterinary medicine. The selection of animal species is also important in these studies. Using laboratory animals in studies creates an induced model that is far from the clinical reality of human medicine. Therefore, using naturally occurring animal pathologies is seen as a better strategy. Referring to spontaneous diseases that occur in domestic animals (primarily dogs, cats, and horses) allows for the use of disease models that are more similar to humans in terms of pathogenesis, evolution, and healing mechanisms. Additionally, since cats and dogs share the same living environment as humans, they also share the factors and underlying pathologies that affect the pathological onset, evolution, and healing processes. The long life expectancy expected from companion animals makes them a suitable model for long-term studies. Cell therapies based on the use of MSCs in musculoskeletal disorders aim to regenerate damaged tissue. The complexity of the healing processes induced by MSCs necessitates the use of natural pathologies to understand the cells' true therapeutic potential. This also enables current therapeutic opportunities and long-term follow-up of companion animals. From studies conducted so far, animals affected by naturally occurring diseases have shown more reliable results than laboratory animals, and have shown the potential for treatments that can also be used in human medicine. Dogs and horses have a high prevalence of musculoskeletal disorders that are quite similar to those seen in humans. Veterinary medicine provides useful therapeutic protocols for human medicine for osteoarthritis, tendinitis, and intervertebral disc diseases. In addition, experimental animals also provide a potential model for breast cancer, a commonly seen cancer in women today. Through research on experimental animals, it is possible to contribute to the development of treatments that will be used in human medicine. Many animal species today are endangered. Conservation of animal populations and the use of SSC transplantation and testis xenografting for male infertility treatment in human medicine can be beneficial.

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