stem cell research&therapy

REVIEW

Stem cells in veterinary medicine

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Abstract

The stem cell field in veterinary medicine continues to evolve rapidly both experimentally and clinically. Stem cells are most commonly used in clinical veterinary medicine in therapeutic applications for the treatment of musculoskeletal injuries in horses and dogs. New technologies of assisted reproduction are being developed to apply the properties of spermatogonial stem cells to preserve endangered animal species. The same methods can be used to generate transgenic animals for production of pharmaceuticals or for use as biomedical models. Small and large animal species serve as valuable models for preclinical evaluation of stem cell applications in human beings and in veterinary patients in areas such as spinal cord injury and myocardial infarction. However, these applications have not been implemented in the clinical treatment of veterinary patients. Reviews on the use of animal models for stem cell research have been published recently. Therefore, in this review, animal model research will be reviewed only in the context of supporting the current clinical application of stem cells in veterinary medicine.

The clinical applications of stem cells

At present, stem cell therapies in veterinary patients are not rigorously supervised by regulatory agencies in any country [1]. Unfortunately, this has led to the implementation of some therapies that have not demonstrated efficacy *in vitro* or in preclinical animal studies. In general, the therapeutic role of stem cells in regenerative medicine is not fully understood. It is unclear whether stem cells ultimately function once differentiated into a tissue-specific cell such as a tenocyte or whether they primarily improve tissue repair through secretion of immunomodulatory and bioactive trophic factors or whether a combination of the two mechanisms occurs [2]. These questions are not purely academic in nature, because if stem cells are truly immunomodulatory, then allogeneic transplantations should be possible. Safe and efficacious applications of allogeneic stem cells would imply that off-the-shelf stem cell products could be developed for increased availability and rapid implementation of stem cell therapies early in a disease course. The potential for allogeneic stem cells to be more cost-effective than autogenous stem cells is questionable. For allogeneic cells, there would be no costs associated with a tissue harvest procedure, but there would be added expenses of ensuring that the stem cell product was free of disease and of storing the stem cells until sale.

The therapeutic application of stem cell-based technologies in veterinary medicine was first used by Herthel [3] to treat equine suspensory ligament desmitis. This application involved direct injection of large volumes (20 to 60 mL) of naïve bone marrow aspirate obtained from the sternum into an injured ligament. In this report of an uncontrolled, nonrandomized case series, the technique appeared to improve return to athletic function rates over conventional therapies. However, it is unlikely that the observed results were due to stem cells, as it became known that there are very few stem cells in bone marrow aspirate. Mesenchymal stem cells (MSCs) represent a very small fraction of the total population of nucleated cells from bone marrow from humans [4] and cats [5] and are presumed to be similar in other species, including the horse. These studies indicate that 0.001% to 0.01% of mononuclear cells isolated from a Ficoll density gradient of bone marrow aspirate are MSCs. The percentage of MSCs in raw bone marrow aspirate would be less than 0.001% to 0.01% because the technique of Ficoll density gradient isolation omits several types of nucleated cells, including granulocytes and immature myeloid precursors. Any clinical effect of bone marrow aspirate might be attributed to the numerous bioactive substances in the acellular fraction such as growth factors produced by cells or platelets. For example, bone marrow aspirate that is rendered acellular through freeze-thaw has some stimulatory effects on matrix synthesis when applied *in vitro* to tendons and ligaments [6,7].

Stem cell products in clinical use

In veterinary patients, three MSC-based approaches are currently used for the treatment of tendon, ligament, or

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cartilage/joint injuries in horses or dogs. As stated previously, there are research-based but no clinical reports that document the use of stem cells to enhance fracture repair, nor are there any reports in cardiovascular, gastrointestinal, or neuroendocrine body systems. The first MSC-based method relies on a culture-expanded cell population derived from bone marrow aspirate, the second is another bone marrow aspirate-based approach using a concentrated mixed cell population derived from bone marrow aspiration, and the third method employs a mixed nucleated cell population derived from adipose tissue. Each technique has its strengths and weaknesses. Embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, and cord blood-derived cells are also beginning to be investigated in the laboratory but have not yet been applied to the clinical scenario.

Culture-expanded bone marrow-derived mesenchymal stem cells

Bone marrow-derived mesenchymal stem cells (BM-MSCs) have the advantages of being easily and relatively noninvasively obtained and have a greater capacity to differentiate into tissue types of the musculoskeletal system in comparison with other MSCs [8-10]. Furthermore, BM-MSCs have received the most scientific attention and hence are the best characterized. One disadvantage of culture-expanded BM-MSCs is the time lag of 3 to 6 weeks from bone marrow aspirate until treatment. This time lag is necessitated by the time required to grow the MSCs. Bone marrow is collected from the sternum or the tuber coxae of horses under sedation or can be collected intraoperatively if the horse is already anesthetized. The horse has seven marrow spaces in the sternum, and marrow spaces 3 to 5 are the largest (up to 5 cm in diameter). Ultrasonography can be used to isolate the marrow space but is not necessary if one is familiar with the regional anatomy. Bone marrow is typically aspirated from the proximal humerus, proximal femur, or tuber coxae in dogs.

Tendonitis

The use of culture-expanded BM-MSCs for the treatment of tendon injuries is supported by experimental investigations in horses and laboratory animals in which MSCs were implanted in surgically or collagenase-induced tendon lesions. These studies have shown favorable effects on tissue organization, composition, and mechanics of MSC-implanted tendons and ligaments [11-14]. These studies vary in experimental design with respect to the number of BM-MSCs implanted (0.5 to 10 $\times$ 10^6), vehicle for suspension (plasma, phosphate-buffered saline, bone marrow supernatant), and time post-injury to injection (up to 2 weeks). The clinical application of BM-MSCs was first reported in 2003 [15]. More recently, a small case control study (n = 11) demonstrated that, as a result of BM-MSCs, 90% of treated horses successfully returned to pre-injury athletic function and race horses suffered no re-injury of the superficial digital flexor tendon after 2 years whereas all of the horses of a control population suffered from re-injury [16]. In an unblinded, uncontrolled case series, Godwin and Smith [17] reported on 141 horses treated with cultured BM-MSCs with at least a 3-year follow-up. The authors reported a significant decrease in re-injury rate for National Hunt race horses but not flat-track Thoroughbred race horses treated with BM-MSCs when compared with conventionally treated historical controls (23% to 66%). To date, preclinical and clinical studies have focused on the ability of stem cells to enhance tissue regeneration and have not investigated the potential immunomodulatory roles of stem cells for tendon repair. This is most likely simply a matter of timing, with the concept of immunomodulation being more recent than the more traditional paradigm of stem cells differentiating and functioning as tissue-specific cells. Although the above-mentioned studies have documented stemness of the cells to varying degrees, tumor, ectopic bone, or cartilage formation has not been observed in either clinical or research investigations.

Cartilage injury/osteoarthritis

Culture-expanded BM-MSCs have been evaluated in an equine model of acute cartilage injury in which 15-mm-diameter full-thickness articular cartilage defects were created on the lateral trochlear ridge of the femur [18]. The BM-MSCs were implanted in autogenous fibrin as a scaffold in one limb, and the opposite limb was grafted with autogenous fibrin alone. At 30-day re-check arthroscopy, arthroscopy scores and biopsy assessments for the BM-MSCs lesions were significantly better than fibrin-only control grafts. However, at 8 months, no significant differences between the two groups in histologic or biochemical composition were observed. In an equine model of early osteoarthritis (OA), a direct comparison between BM-MSCs and adipose-derived stromal vascular fraction (AD-SVF) cells was made [19]. The two stem cell preparations were injected directly into affected joints 14 days after induction of OA. Joints treated with BM-MSCs showed significantly less synovial effusion and significantly lower prostaglandina E2 (PGE2) concentrations in comparison with those treated with AD-SVF cells. No differences in cartilage biochemistry or histology, synovial fluid analysis, or other clinical parameters were observed. It is interesting to note that synovial fluid PGE2 concentrations, though not directly investigated in the study, were decreased by BM-MSC treatment because PGE2 is one mechanism by which BM-MSCs modulate immune cells and exert

anti-inflammatory/immunomodulatory effects, such as suppression of lymphocyte proliferation and T-cell activation [2,20]. Several other preclinical studies in OA models using goats, sheep, rabbits, and rats have demonstrated the capacity for BM-MSCs to enhance regeneration of cartilage and even meniscus [21,22]. Combined, these studies suggest that BM-MSCs have the dual function in an articular environment to modulate the local T cell-mediated immunological response and to enhance tissue regeneration. Long-term studies using BM-MSCs in naturally occurring articular cartilage injuries in veterinary and human patients are required to demonstrate restoration of joint function, decreased articular pain, and durability of BM-MSC-based therapies.

Bone marrow concentrate

Concentrated bone marrow aspirate was designed to increase the concentration of stem cells compared with naïve bone marrow aspirate and to avoid the lag time from diagnosis to treatment when culture-expanded BM-MSCs are used. In addition to the concentration of stem cells, the concentrations of platelets and therefore anabolic growth factors are increased [23]. When combined with thrombin, the fibrinogen present in BMC is converted to fibrin and a solid scaffold forms to retain the cells and growth factors in a given location.

Tendonitis

No peer-reviewed preclinical or clinical reports on the use of BMC for tendonitis have been published. BMC is being applied clinically for ligament and tendon injuries in horses, but sufficient data are not currently available to assess its therapeutic potential.

Cartilage injury/osteoarthritis

In the equine model of acute cartilage injury discussed above (15-mm-diameter lesions), one limb was treated with BMC and microfracture and the other was treated with microfracture alone [23]. Re-check arthroscopy at 3 months demonstrated significantly improved repair tissue in BMC-grafted defects compared with microfracture tissue with increased volume and greater integration of repair tissue with surrounding host cartilage. At 8 months, all macroscopic, histologic, and magnetic resonance imaging data indicated sustained improvement in BMC-grafted repair tissue in comparison with microfracture. Like many other stem cell-based technologies, BMC is being applied in clinical veterinary and human patients, but no peer-reviewed results have been published.

Adipose-derived stromal vascular fraction cells

The currently available technique uses a mixture of cells derived from adipose tissue surgically excised from horses or dogs. The AD-SVF cells are simply isolated and injected into the patient without a cell culture step. Compared with cultured BM-MSCs, this technique has the advantage of supplying cells in a short time period (48 hours), and it should be remembered that although there are a large number of nucleated cells retrieved from the adipose digest, only a small percentage of nucleated cells are stem cells. In humans, 0.7% to 5% of nucleated cells in the stromal vascular fraction are stem cells [24].

Tendonitis

No references regarding the clinical application of AD-SVF cells in equine tendonitis are currently available. Results of a pilot study demonstrated significant improvement in histologic score in AD-SVF cell-treated tendons over phosphate buffered saline-treated control tendons [25]. Although AD-SVF cells have been available for nearly 8 years and have been used to treat several thousand horses, no reports documenting their use in clinical cases of equine tendonitis have been published. AD-SVF cells are not approved by the US Food and Drug Administration for human application at this time.

Cartilage injury/osteoarthritis

As mentioned above, AD-SVF cell application in an equine model of early OA failed to result in any detectable improvement in articular health [19]. In fact, AD-SVF cells led to an increase in synovial fluid concentration of the proinflammatory cytokine tumor necrosis factor-alpha. In dogs, two reports of improved clinical signs of OA after treatment have been published. In a double-blinded study assessing the use of AD-SVF cells in the hip joint of dogs, examining veterinarians (but not the dog owners) reported signs of clinical improvement [26]. In a second, uncontrolled study using AD-SVF cells for elbow OA, veterinarians and, to a lesser extent, owners both reported improvements in clinical signs [27]. The disparity in the clinical benefits noted by owners in these studies investigating the use of AD-SVF cells in OA is unclear but perhaps suggests that any benefit of AD-SVF cell application can be seen only in more advanced cases of OA or that changes in lameness associated with elbow OA in comparison with those of hip OA are more easily perceived by owners.

Debated hypothesis and the future of clinical stem cell therapy

Irrespective of the type of stem cell being investigated, the nature of the target tissue, or the species that is being treated, the fundamental questions underlying the clinical application of stem cells are the same and include the following: (a) What is the optimal tissue source of stem cells for each clinical application? In the current clinical applications of adult-derived stem cells, it is unlikely that a single stem cell source will be best for

regeneration of tissues from the three different embryonic germ layers (endoderm, mesoderm, and ectoderm). (b) How many stem cells are needed to effectuate regeneration? Very few dose-response studies have been performed to date, and the available data suggest that 'more is not better'. (c) What is the best means to deliver the cells? Should they be administered locally to the site of damage or intravenously? Is a scaffold necessary, and if so, which scaffold is optimal for each tissue type? (d) Is there a requirement for co-delivery of growth factors to direct the function of the implanted cells? Many of these questions are intricately linked, and carefully designed research studies will be required to answer the debated theories.

Several avenues of stem cell therapy for tendon/ligament pathologies are currently under investigation. Several types of stem cells not discussed herein, including ES cells, umbilical cord blood-derived stem cells, and iPS cells, show promise for regenerative applications. Finally, genetically modified stem cells have been investigated *in vitro* and *in vivo* and show tremendous promise for enhancing organized repair of tendons and other musculoskeletal tissues.

Clinical uses of stem cells in reproductive medicine

Currently, there are no widespread uses of stem cell-based therapies in reproductive medicine. However, the potential utility of such approaches makes them subjects of intensive research. Broadly speaking, two stem cell types are the primary topics of investigation: ES/iPS cells and spermatogonial stem cells (SSCs). Unfortunately, despite great effort, there are no completely characterized ES or iPS cells derived from species other than primates or mice [28]. For this reason, we focus here on SSCs, which are used in the techniques of testis xenografting and spermatogonial stem cell transplantation (SSCT).

Testis xenografting

The primary clinical application for testis xenografting would be as a means to preserve the breeding potential of a genetically valuable pre-pubertal male animal [29]. For example, in the captive management of threatened or endangered species, specific individuals often have high genetic value. If adult males die before contributing their genes to the population, mature sperm can be collected and cryopreserved for future use in artificial insemination or a form of *in vitro* fertilization (IVF). If neonatal or juvenile males die, testis xenografting offers a means to develop sperm from their gonocytes or SSCs, which are present from parturition. In this procedure, small pieces (1 to 2 mm^3) of donor testes are surgically grafted into immunodeficient mice. In the absence of a functioning immune system, the recipient mice nurture the foreign testis tissue, which supports spermatogenesis [30]. By means of this approach, morphologically mature sperm have been produced in xenografts from a number of species, including rabbits [31], pigs and goats [30], hamsters [32], rhesus macaques [33], sheep [34], cats [35], and dogs [36]. However, the efficiency of spermatogenesis in xenografts differs among species, with the bull [37-39], cats [35,40], and dogs [36] being less efficient. One common finding across species is that if the donor testis tissue has germ cells actively undergoing meiosis (as in puberty or adulthood), then the xenografts lose the ability to support spermatogenesis [40,41]. The fertilizing ability of graft-derived sperm has been verified by the production of viable offspring in allografted mouse [42] and xenografted rabbit [31] and pig [43]. Because there is no epididymis in this system, the functionally immature sperm can help generate offspring only through intracytoplasmic sperm injection (ICSI), a procedure in which sperm are injected directly into an oocyte. Thus, although banking of material from genetically valuable individuals of multiple species might begin now, the ultimate production of offspring is restricted until ICSI is optimized for that species.

Spermatogonial stem cell transplantation

The primary clinical uses of SSCT would be to preserve or manipulate the male germline or both [44]. Briefly, the technique involves isolation of a mixed germ cell population from a donor testis (preferably enriched in SSC if markers are known for that species). The isolated cells are then injected in a retrograde fashion into the testes of a recipient animal. To increase the SSC niches that might be open for colonization, the recipients are often treated with focal testicular irradiation [45,46] or systemic busulfan [47,48] to reduce their endogenous SSC. After time is allowed for colonization, proliferation, and spermatogenesis, semen is collected and assessed for the relative percentage that is of donor origin. Although it has been performed successfully in several species, this technique has multiple steps that are technically challenging and time- and labor-intensive. Therefore, it is likely to be used in the future primarily as a clinical tool to develop transgenic biomedical research models or for the production of transgenic farm animals that produce tissues/organs genetically engineered to be compatible across species or to produce pharmaceutical proteins [49]. Xenogeneic transplantation has been attempted with various donor and recipient species. Unless the donor and recipient are closely taxonomically related (for example, rat and mouse [50] as opposed to dog and mouse [51]), the recipient testes do not support spermatogenesis. Therefore, utilization for the conservation of threatened species would require not only the use of a suitable domestic animal recipient that would support spermatogenesis of the donor but also some method of sorting the sperm of donor origin from that of recipient origin.

Debated hypothesis and the future of stem cell technologies in clinical reproduction

Several questions need to be addressed in order to enhance the clinical utility of both testicular xenografting and SSCT approaches: Can markers that will label the SSC of various species be identified? Can cryopreservation methods for individualized SSC, pieces of testis tissue, and sperm be optimized? Can 'downstream' technologies such as classical IVF and ICSI be developed for different species? Other questions are specific to one or the other technique: Why are there differences among species in the efficiency of xenograft spermatogenesis? Why do xenografts from meiotic testes fail? Can we determine the critical parameters that define the taxonomic gulf between SSC donor species and the species that might be able to function as recipients?

Conclusions

The clinical use of stem cells in veterinary medicine is clearly in its early stages. Applications for BM-MSC and AD-SVF cells in the treatment of musculoskeletal pathologies are currently in use in several species, although the differential efficacies of various approaches are still being investigated. Optimization of these stem cell-based therapies will focus on cellular origin, isolation, enrichment, and processing as well as on the timing, route of administration, formulation, and dosing of those therapies. Development of confirmed ES or iPS cells in domestic species would greatly facilitate the development of a wider range of clinical applications. Use of stem cell-based approaches in attempts to preserve the germ plasm of threatened species could begin on an opportunistic basis in the form of xenografting of testis tissue obtained quickly after the death of pre-pubertal individuals. However, this must still be considered a research endeavor given the largely unknown causes of species differences in the success of spermatogenesis as well as the need to perform subsequent techniques of assisted reproduction which have themselves not yet been determined for most species.

Abbreviations

AD-SVF, adipose-derived stromal vascular fraction; BM-MSC, bone marrow-derived mesenchymal stem cell; ES, embryonic stem; ICSI, intra-cytoplasmic sperm injection; iPS, induced pluripotent stem; IVF, *in vitro* fertilization; MSC, mesenchymal stem cell; OA, osteoarthritis; PGE2, prostaglandina E2; SSC, spermatogonial stem cell; SSCT, spermatogonial stem cell transplantation.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LAF generated the review on the clinical use of stem cells. AJT provided information regarding the use of stem cells in reproductive medicine. Both authors read and approved the final manuscript.

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