Introduction to Equine Biologic and Regenerative Therapies

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KEYWORDS

• Equine • Regenerative medicine • Orthobiologics • Autologous conditioned serum • Platelet-rich plasma • Platelet lysate • Autologous protein solution • Stem cell • Mesenchymal stem cell

KEY POINTS

- Regenerative medicine therapeutic use is growing in equine practice and it is important that practitioners be familiar with and have a basic understanding of available products.
- Autologous conditioned serum, platelet-rich plasma, platelet lysate, and autologous protein solution are all minimally processed orthobiologics obtained from blood.
- Bone marrow aspirate, bone marrow aspirate concentrate, and adipose-derived stromal vascular fraction are obtained after tissue is harvested and processed, releasing a concentrated cell population with minimal processing.
- Culture-expanded stem cells are manipulated in culture, establishing a desired stem cell phenotype. Stem cells exert their effects at the site through paracrine signaling.

INTRODUCTION TO EQUINE BIOLOGIC AND REGENERATIVE THERAPIES

Introduction

Regenerative medicine is defined as the "process of replacing or regenerating cells, tissues, or organs to restore or establish normal function."[1] This broad field has gained significant momentum in equine veterinary medicine specifically in the treatment of damaged musculoskeletal tissues with a limited capacity for intrinsic healing such as cartilage, tendons, ligaments, and meniscus. The term "orthobiologics" has become more commonly, and more appropriately used to encompass the biologics used to assist in musculoskeletal recovery. According to the National Football League Physician Society Orthobiologics Consensus Statement, "orthobiologics" is not

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narrowly limited to a specific product but is defined as techniques and procedures designed to manufacture and deliver platelet-rich plasma (PRP) or stem cells derived from bone marrow, amnios, or adipose tissue.[2] Additionally, in equine orthopedics, the orthobiologics landscape includes other preparations such as bone marrow aspirate (BMA), bone marrow aspirate concentrate (BMAC), autologous conditioned serum (ACS), and autologous protein solution (APS). Despite this categorization, the exact formulation of each orthobiologic, the possible conditions for that they are useful, and the setting of their optimal application share *uncertainty* as an unifying commonality.[3] Considering that the effects of these products are multifactorial and not entirely understood and that the veterinary community is still lacking appropriate clinical information, orthobiologics should continue to be meticulously assessed with carefully designed clinical trials aimed at assessing their clinical value in the context of the accepted standard of care.

MINIMALLY PROCESSED BLOOD-DERIVED ORTHOBIOLOGICS

Blood-derived orthobiologics are manufactured after collecting blood from the eventual recipient of the final product. With few exceptions, these preparations are obtained with minimal processing outside of the recipient and are intended for autologous use. As a rule, blood is either collected in the presence of an anticoagulant to achieve a plasma product such as PRP and APS or is allowed to clot and then separate serum as a final product, as in the case of ACS. The collected blood is further processed to separate the liquid from the solid components of blood. This is achieved by centrifugation, gravity filtration, or a combination of both. During this processing the desired blood components of the orthobiologic are captured for therapeutic use. Equipment used for processing are meant to be simple and straight forward, requiring little to no technial expertise from the practioner, allowing these producuts to be manufactured rapidly and conveniently, stall-side. Unfortunately, there are significant differences in the equipment that is used to manipulate blood and achieve the final product. The type of equipment, the starting blood volume, any blood activation mechanism, the specifics regarding time and speed of centrifugation, and the type of filtration system used play a major role in determining the characteristics of the final product. These factors result in major challenges in our collective ability to provide a uniform and consistent assessment of the benefits and detriments of using blood-derived orthobiologics in our equine patients.

Autologous Conditioned Serum

ACS has gained widespread popularity in the management of synovial inflammation in horses. Veterinarians have become accustomed to this biological product as a treatment option for arthritis and tenosynovitis both in the management of horses with performance limitations and in the postoperative management of horses undergoing arthroscopy and tenoscopy. The premise for this treatment is that acute joint inflammation driven primarily by IL-1 and TNF-α contributes to the progressive downfall of the articular cartilage and over time joint degradation and osteoarthritis. Over the last few decades, method refinements have led to the development of specific blood collection techniques aimed at stimulating leukocytes, especially monocytes, to produce disease-modifying cytokines such as IL-1 receptor antagonist (IL-1ra) and others, including IL-10 and anabolic growth factors such as transforming growth factor-beta (TGFβ) and insulin growth factor 1 (IGF-1). Even though the exact mechanism behind this treatment modality has not been completely clarified, these anti-inflammatory cytokines benefit the synovial environment by counteracting inflammatory processes

especially those driven by IL-1. Once manufactured, ACS is conveniently stored in a household freezer and maintains its cytokine profile for a long time allowing veterinarians to perform repeated treatments as needed. Even though injection protocols may be highly variable depending on the target and the veterinarian's experience, most of the time, practitioners will perform a series of injections 1 week apart for 3 to 5 treatments.

Platelet-rich Plasma

PRP has a long history in the medical field and has been applied in maxillofacial and periodontal surgery since the 1980s. Platelets, also called thrombocytes, develop from the bone marrow. Platelets are nucleated, discoid cells with different sizes and a density of approximately 2 μm in diameter, the smallest density of all blood cells. The physiological count of platelets circulating in equine blood ranges from 100,000 to 200,000 platelets per microliter. Platelets contain several secretory granules that are crucial to platelet function. There are 3 types of granules: dense granules, α-granules, and lysosomes. Platelets are primarily responsible for the aggregation process contributing to homeostasis through the processes of adhesion, activation, and aggregation. During a vascular lesion, platelets are activated, and their granules release factors that promote coagulation.[4]

Platelets were thought to have only hemostatic activity, although, in recent years, scientific research and technology have provided a new perspective on platelets and their functions. Studies suggest that platelets contain an abundance of growth factors and cytokines that can affect inflammation, angiogenesis, stem cell migration, and cell proliferation.[5] These features of PRP are somewhat captured in other terms used to define this orthobiologic such as platelet-rich growth factors, platelet-rich fibrin matrix, and platelet concentrate. This nomenclature gives us an indication of the medical rationale for its use in clinical practice. At the simplest level, the process involves collecting platelets and concentrating them to subsequently deliver the concentrate to the injured tissue thereby introducing a milieu of growth factors and proteins capable of guiding the healing response. The use of PRP in human sports medicine has attracted widespread attention in the media and has been extensively used in this field to include the treatment of tendonitis and desmitis in the equine athlete.

PRP is a natural source of signaling molecules, and on activation of platelets in PRP, the P-granules are degranulated and release growth factors and cytokines that will modify the pericellular microenvironment. Some of the most important factors released by platelets in PRP include vascular endothelial growth factor, fibroblast growth factor, platelet-derived growth factor, epidermal growth factor, hepatocyte growth factor, insulin-like growth factor 1, 2 (IGF-1, IGF-2), matrix metalloproteinases 2, 9, and interleukin 8.[6,7]

PRP plays a role in the treatment of osteoarthritis, in fact, a specific manufacturing process formed the basis for the development of APS as another autologous blood-derived product used for the treatment of osteoarthritis in horses. Similarities exist between ACS and APS because both include the concentration of IL-1ra as an important feature. Additionally, however, the APS manufacturing process includes a separator that sequesters white blood cells and platelets that are then transferred to an APS concentrator. The APS concentrator filters the product through polyacrylamide beads and desiccates it, resulting in a concentrated solution of WBCs, platelets, and plasma proteins.[8]

Despite an early publication regarding the use of APS in horses and sharp contrast with the widespread clinical use of this product, no additional follow-up clinical studies have occurred to further refine the use of APS for the treatment of equine joint disease.

Platelet Lysate

Platelet lysate (PL), otherwise known as platelet releasate, is the product of processing a platelet concentrate to obtain a final preparation that contains all the platelet injury modifying factors outlined above but that is free of cellular debris. The absence of platelets may allow the use of PL from donors in recipients of the same species and, although scientific discovery is still in its infancy, PL seems to not elicit an immune response and has been shown to exert a powerful inhibition of cell-mediated inflammatory responses.[9] Specifically, TNF-α produced by LPS-stimulated monocytes decreased over a thousand-fold in the presence of PL compared with those incubated in the presence of serum.[9] Even though PL has not been extensively studied as an orthobiologic, it is likely to contain the elements that have distinguished PRP in the treatment of soft tissue injuries and other forms of orthopedic trauma. Therefore, it would not be surprising if PL garnered a similar level of attention from veterinary professionals.

Finally, PL has recently captured the interest of the veterinary scientific community because of its reported antimicrobial effects. In addition to their well-known role as regulators of thrombosis and inflammation, there is mounting evidence to suggest that platelets play a central role in the host's response to infection.[10] Platelets kill bacteria by producing antimicrobial oxygen metabolites such as superoxide, hydrogen peroxide, and hydroxyl free radicals.[11] Moreover, platelets participate in antibody-dependent cell cytotoxicity against microbial pathogens. These effects are thought to be mediated either by a direct interaction between platelets and bacteria or, perhaps more interestingly, via peptides released by activated platelets.[12] In fact, a broad-spectrum antibacterial effect has been attributed to a select group of peptides including platelet factor 4, beta-defensin 1, and connective tissue activating peptide 3.[13,14]

TISSUE-DERIVED ORTHOBIOLOGICS: MINIMALLY MANIPULATED AND CULTURE EXPANDED

Tissue-derived orthobiologics are delivered following the collection of donor tissue. The donor tissue is processed to concentrate the cells from the collected tissue which are then either delivered to the recipient as a concentrated cellular product or the cellular population is placed in culture for expansion. The population of cells that is concentrated following tissue processing is diverse, containing several different types of cells. However, stem cells are the desired cell in this population for therapy, yet stem cells represent only a small portion of the concentrated cells that are delivered. BMAC and adipose-derived stromal vascular fraction (ADSVF) are the 2 concentrated cellular products most commonly used by equine practitioners. These products are attractive to equine practitioners because the tissues can be easily harvested, processed, and re-implanted stall-side with little-to-no delay in treatment. When the harvested cellular population obtained from the tissues is placed in culture, stem cells become established because media and growth conditions are chosen to favor stem cell growth in culture over time. The stem cells are manipulated and grown in culture to obtain the desired number of cells with the desired phenotype. This process can take several weeks following tissue harvest. When ready, the cells are harvested from the culture and then delivered to the recipient. Culture expansion allows the delivery of a greater number of stem cells than concentrated cellular products, usually 10 million stem cells or more constitute a stem cell dose. Culture expansion also allows the delivery of a more homogenous population of stem cells than concentration alone, though some diversity in cellular

phenotype following culture remains.[15] Ideally the delivered cells would undergo testing (characterization) to ensure that the cells are actually stem cells before therapy, this should be the case in experimental studies, but is rarely the case in commercially grown stem cells if used in an autologous manner. The minimum criteria for characterization of MSCs include demonstration of plastic adherence, expression and lack of expression of certain surface markers, and demonstration of trilineage differentiation.[16] It is important to understand that differences in tissue source; donor age, donor health status, donor relation to the recipient, and culture methods including isolation and expansion as well as harvest and delivery techniques result in stem cells with differing therapeutic potential.[17] In addition, these stem cells are being used therapeutically to treat a variety of conditions with differing levels of severity. These factors make it challenging for practitioners to understand exactly what these therapies are doing for their patients and how they are meant to use them.

STEM CELL THERAPY: CELLULAR CONCENTRATES

Bone Marrow Aspirate and Bone Marrow Aspirate Concentrate

BMA contains a rich milieu of platelets, red blood cells, white blood cells, hematopoietic and non-hematopoietic precursor cells that include stem cells, as well as several important anabolic growth factors, cytokines, and chemokines important for tissue repair. The aspirate can be further concentrated via centrifugation using either density gradient media or a gravitational separation device. Concentration of BMA yields a product with a higher number of nucleated cells including progenitor and stem cells, growth factors, cytokines, and platelets.[18] This product is referred to as BMAC. It is important to recognize that although concentration results in a greater percentage of mesenchymal stem cells (MSCs), the number of cells within BMAC remains low, approximately 0.001% to 0.01% of the mononuclear cell population of BMAC are MSCs.[18] Despite the low MSC population, BMAC has been shown to have reparative, anabolic effects experimentally on musculoskeletal tissues.[18,19] Most equine research has centered on the use of BMAC for the treatment of suspensory ligament desmopathy while clinically, treatment of naturally occurring acute and chronic suspensory desmopathy with BMAC has proved promising.[20,21]

Adipose-derived Stromal Vascular Fraction

ADSVF is a cellular product obtained from adipose tissue. Adipose is harvested stall-side from the patient via either lipoaspiration or lipectomy. The adipose aspirate or tissue is then subjected to enzymatic digestion or mechanical agitation followed by centrifugation resulting in an aqueous fluid with a concentrated cellular pellet, termed the stromal vascular fraction.[22] The cellular pellet contains a heterogenous population of cells that includes endothelial cells, monocytes, lymphocytes, myeloid cells, pericytes, and hematopoietic stem cells.[22] Adipose tissue has a greater number of MSCs within tissue, therefore the MSC yield is greater than BMAC.[23] Currently, this product requires shipment of the adipose tissue to an approved commercial laboratory for processing. Cells are typically shipped back within 48 hours for treatment. There are currently no commercially available medical devices to produce ADSVF stall-side for the equine patient which differs from BMAC.

STEM CELL THERAPY: CULTURE EXPANSION

Stem cells are immature, unspecialized cells with the capacity for self-renewal and differentiation. Differentiation is the process by which the cell changes to a more mature,

specialized cell. Stem cells are found in limited quantities in all body tissues and are responsible for replacing injured, diseased, or aged stromal cells.

Stem cell therapy was first introduced because of the stem cells self-renewal and differentiation capacity. MSCs were injected into the site of injury and thought to integrate and differentiate into the desired tissue. However, studies have shown that MSC engraftment, retention, and direct differentiation are limited.[15] Despite this, improvement in clinical signs of the patient and quality of repair tissue have been reported. Therefore, our current understanding is that the injected MSCs interact with native cells within the injured environment via cell–cell interactions and secreted factors (paracrine signaling). These interactions modulate the wound environment to a more immunotolerant that aids rather than hinders tissue repair by native cells.[15]

Stem cells are categorized based on various criteria including their differentiation capacity (potency), tissue of origin, and relationship to the recipient of cellular therapy (autologous, allogenic, and xenogeneic). Stem cells can be obtained from various stages of organismal development that determines their potency. Cells can be totipotent, pluripotent, multipotent, oligopotent, bipotent, or unipotent.[24] Totipotent cells can form any tissue in the body and are derived from the fertilized ovum whereas pluripotent cells are obtained from the embryo and can emerge into cells of all 3 germ layers.[24] Pluripotent stem cells can be obtained either from the embryo termed embryonic stem cells (ESCs) or cells can be obtained from somatic tissues and re-programed to an embryonic state, termed induced pluripotent stem cells (iPSCs). Multipotent cells only differentiate into cells of the germ layer from which the cell was obtained. These are the most common type of cells used for therapy in veterinary medicine.[24] Multipotent stem cells are commonly obtained from connective (mesenchymal) tissue and can differentiate into another type of connective tissue and are termed MSCs.[24] Differentiation of MSCs into the 3 tissues: bone, fat, and cartilage is termed trilineage differentiation and this is one of the criteria used to characterize cultured cells as MSCs. Oligopotent, bipotent, and unipotent cells are obtained from the target tissue and can only produce cells of the specific tissue from which they were obtained. As the cells lose potency, they also lose the capacity for self-renewal, meaning that totipotent or pluripotent stem cells have a greater capacity for replication than multipotent stem cells. Although ESC and iPSCs have been successfully obtained and cultured from horses, MSCs from fetal or adult tissue remain the most common type of stem cell therapy used in equine practice.[25]

MSCs can be obtained from either fetal or adult tissues. Fetal tissues used in practice may include placental tissue, umbilical cord blood, or umbilical cord tissue. These tissues are obtained from the fetus at the time of birth, processed, and then banked for future use. More commonly, MSCs are obtained from adult tissues such as peripheral blood MSCs, adipose SCs, or bone marrow (BMSCs). In equine practice, adipose tissue and bone marrow are the most commonly harvested tissues for concentration or culture expansion of MSCs.[25,26] Bone marrow is harvested either from the sternum or the tuber coxae based on clinician preference and comfort. Adipose is harvested via an open technique or through a small stab incision with insertion and redirection of a lipoaspiration needle just lateral to the tailhead. Of these 2 tissues, BMSCs are the most commonly researched MSC in veterinary medicine[24] and the most commonly used MSC therapy in equine practice.[26]

Another consideration for MSC therapy is the tissue donor. Tissue can be obtained from the patient (autologous), a donor different from the patient but of the same species (allogenic), or a donor from a different species (xenogenic). Autologous tissue is commonly used in equine practice, however, when growing cells in culture there can

be significant delays in treatment (~4 weeks) when using autologous cells. In addition, the age and disease status of the patient can change the self-renewal and differentiation capacity of the cells. Allogenic cells are an attractive alternative because cells can be harvested from young, healthy donors, expanded in culture, and characterized for phenotype and function. These culture expanded characterized cells are stored for rapid use with little-to-no delay of treatment. Allogenic use in the horse has been investigated because MSCs are considered hypoimmunogenic due to their low expression of major histocompatibility complex II.[15] Despite this, both cellular and humoral immune responses to administered allogeneic stem cells have been observed.[15]

As previously stated, of the factors secreted from MSCs play a major role in tissue repair. These secreted factors, termed the secretome, are gaining interest as an additional avenue of stem cell therapy. The secretome consists of cytokines, chemokines, growth factors, extracellular vesicles, lectins, prostaglandins, and enzymes from MSCs.[17] Extracellular vesicles are membrane-bound vesicles important in cellular signaling that contain several bioactive factors including mRNAs, microRNAs, cytokines, and other proteins.[17] MSCs can be manipulated in culture via serum starvation, hypoxia, and induced inflammation (priming) to produce different secreted factors in the culture media that can be harvested and used for therapy.[27]

SUMMARY

The use of regenerative medicine in equine practice is increasing. Yet there is no clear understanding of each product's specific mechanism of action, treatment efficacy, or best treatment practices. To address some of these challenges, standards defining each therapeutic and its components continue to be refined. These standards need to be measured and reported within the scientific literature to understand the similarities and differences of each therapy. Finally, to understand treatment efficacy, more well-designed, properly powered, placebo-controlled studies are needed. Regenerative medicine holds great promise for our equine patients.

DISCLOSURE

The Authors have nothing to disclose.

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